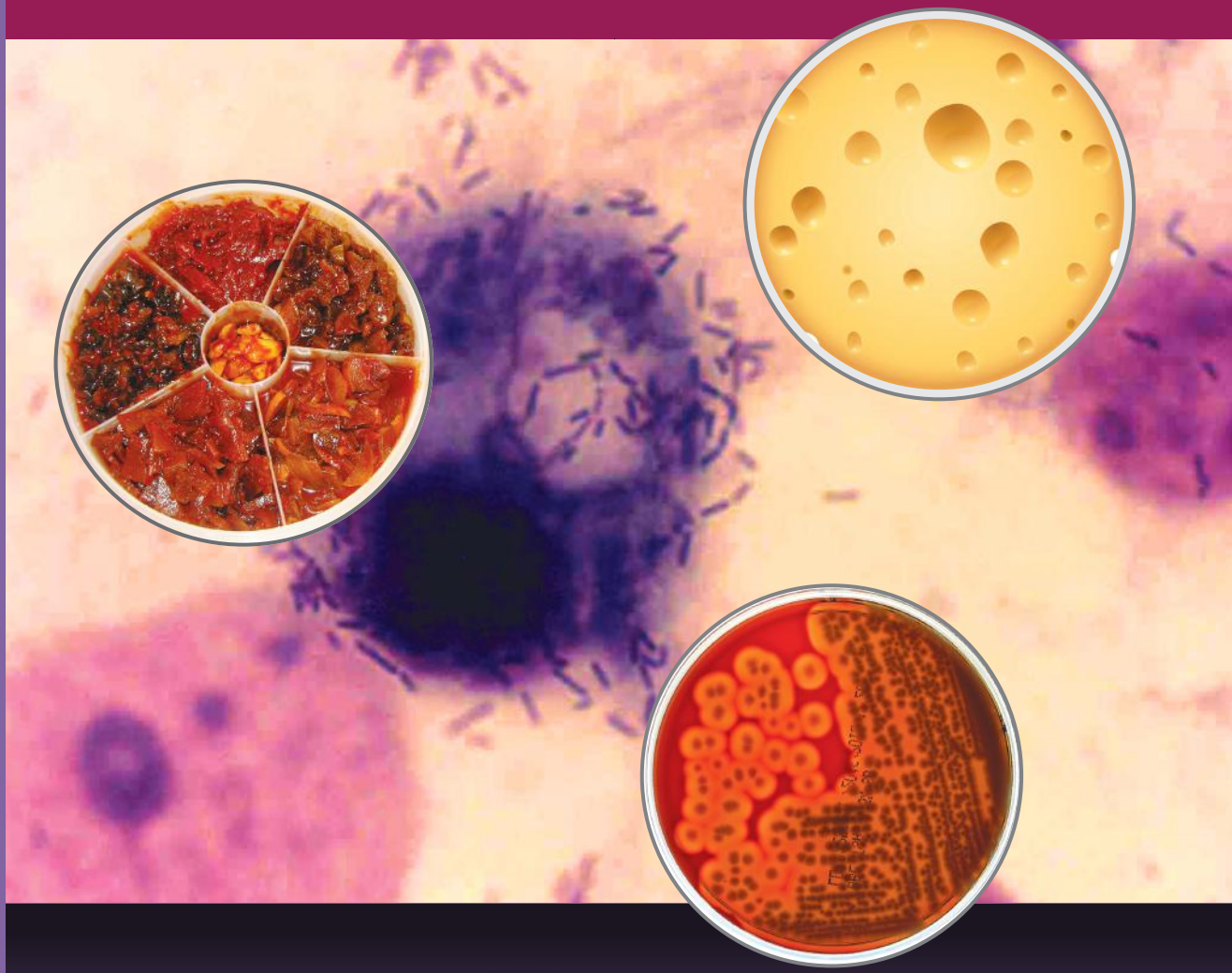
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FIFTH EDITION

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# Preface to the Fifth Edition

The golden era of food microbiology has begun. All three areas of food microbiology, beneficial, spoilage, and pathogenic microbiology, are expanding and progressing at an incredible pace; thus, it is difficult for one to stay abreast of new developments. A genuine attempt has been made to capture those developments in this new edition. Spoilage-causing microorganisms are a major concern. To feed the growing population in the world, microbial food spoilage must be reduced, and improved intervention technologies must be developed. To achieve that goal, it is necessary to understand the problems; that would help to develop effective control methods for different types of foods, especially using the hurdle concept. In the past decade, foodborne pathogens have broadened their association with varieties of foods that were unexpected, such as *Salmonella* in peanut butter, fish, and spices; *Listeria monocytogenes* in cantaloupe and celery; and a new strain of Shiga-toxin producing *Escherichia coli* (O104:H4) with sprouts. To have a greater understanding of pathogen association in preharvest plants and meat animals, the concerted efforts of plant biologists, animal scientists, soil chemists, environmental scientists, agriculture and biological engineers, social scientists, and microbiologists are needed to address food safety and quality issues. In the past decade, we have also witnessed enormous developments in intervention technologies, such as application of bacteriophages and novel antimicrobials to control pathogens and spoilage microbes, and novel processing technologies, such as high hydrostatic pressure, pulsed electric fields, low temperature plasma, infrared, X-rays, ultrasound, and many more. Microbial ecology (microbial community) in food animals, soils, plants, and raw and processed foods are receiving greater attention to understand pathogen behavior in the complex environment. Subsequent application of natural microorganisms as biocontrol and probiotics are receiving renewed interest in controlling pathogens in meat and poultry production or controlling diseases in humans. In this edition, several new topics have been added, such as microbial biofilm formation; quorum sensing; gut microbiota and their role in chronic diseases, including diabetes; obesity; application of food nanotechnology in food preservation; bacteriophages as intervention and detection tools; and emerging pathogens (*Cronobacter sakazaki*, *Clostridium difficile*, *Escherichia coli* O104:H4, and *Nipah virus*). All chapters in this fifth edition have been thoroughly revised and updated with recent reference articles when possible.

I am indebted to my wife, Banashri Bhunia, for her assistance with the collection of literature materials, inspiration, and for her patience while I work late hours, weekends, and holidays for several months on this book; to my colleague, Bruce Applegate, for research photographs, insightful discussions, and his love of microbiology; to my postdoc, Atul Singh, for generating photographs of several microorganisms; and to the students for their continued support.



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Personally, my sincere gratitude goes to Prof. Bibek Ray for his trust in me to carry on this project and to update the content with the most recent developments in the field of food microbiology.

It is gratifying to convey that the fourth edition of *Fundamental Food Microbiology* has been translated into the Spanish and Korean languages as was the third edition into Chinese. We thank the CRC group for this endeavor. We sincerely hope that the fifth edition will continue to generate interest as a trusted source of knowledge and information to students and professionals around the globe.

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# Preface to the Fourth Edition

Food microbiology is a rapidly growing and highly dynamic field of study because of continued incidents of foodborne outbreaks, the threat of intentional contamination of the food supply, and, of course, the renewed interest in the health-beneficial effects of lactic acid bacteria. Therefore, to keep up with the advancements in the field, a new edition was warranted. In this fourth edition, all chapters have been revised and updated, especially chapters covering foodborne diseases. Brief description of the mechanisms of pathogenesis with easy-to-follow illustrations has been included for several common foodborne pathogens. A new chapter on detection has been included. In fact, Appendix E from the third edition, which contained a brief description on various detection methods, has been updated with more advanced rapid techniques, such as those of biosensor- and nanotechnology-based methods. In this edition, attempts were also made to provide easy-to-follow illustrations and diagrams as appropriate. As before, this book is written primarily for undergraduate students; however, with updated revisions in key chapters, this book is an essential reference for graduate students and professionals working in different areas of food microbiology or food safety. I take this opportunity to thank my graduate and postdoctoral students in collecting literature materials for this edition.



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# Preface to the Third Edition

In the third edition, substantial changes have been made in most of the chapters and in their logical arrangement. In addition, one new chapter has been added. The chapter on microbial stress has been written to include various manifestations of bacteria under stress and their importance in food microbiology. As before, this book is written primarily for students taking undergraduate food microbiology courses. However, it can be used as a reference in other related courses in many disciplines as well as by professionals engaged directly and indirectly in food-related areas. I thank Elizabeth Smith for her excellent typing and editing in the preparation of the manuscript. Finally, I thank my students for their helpful suggestions, especially for the new materials included in this edition.



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# Preface to the Second Edition

It is gratifying to find that CRC Press showed interest in a second edition within three years of the initial publication of *Fundamental Food Microbiology*. As indicated previously, this book was written primarily as a text for undergraduate food microbiology courses. The main objective was to provide basic and applied information in as many areas as possible in about 500 pages. In the second edition, the materials were carefully edited and new information was included to keep it up to date. As before, the second edition will be important not only to undergraduate students in a food microbiology course, but also as a valuable reference book to graduate students in the food science area and to individuals associated with the science, application, production, and regulation of foods as related to microorganisms in academic institutions, research institutions, and food-testing laboratories. In addition, short-course organizers, food consultants, food industries, food regulatory agencies, and food science professionals will find this book valuable to understand and solve problems associated with microbiological aspects of food. I thank Deb Rogers for her excellent typing and editing in the preparation of the manuscript and the students in the food microbiology class for their helpful suggestions, including the new material in the second edition.



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# Preface to the First Edition

Between the time I first studied food microbiology as an undergraduate student and now, the discipline has undergone a radical change. This change is well expressed by Dr. David Mossel of the Netherlands in his letter published in *ASM News* (59, 493, 1993): from "no challenge in plate count and coliform scouting" to "linkage of molecular biology to food safety (also food bioprocessing and food stability) strategies—proclaim a new era in food microbiology." This transition was necessary to meet the changes that occurred in the food industry, especially in the United States and other developed countries. The necessary knowledge, techniques, and expertise for this transition were available. This book reflects this transition from the traditional approach to an approach to meet the needs of those who are directly or indirectly interested in food microbiology.

Introductory food microbiology is a required course for undergraduates majoring in food science. In some form it is also taught in several other programs, such as microbiology, public health, nutrition and dietetics, and veterinary science. For the majority of food scientists, except those majoring in food microbiology, this single course forms the basis of the study of microorganisms and their interactions to food. Similarly, for the latter group, food microbiology is probably the only course that provides information on the interaction of food and microorganisms. This book was written with the major objective of relating the interaction of microorganisms and food in relation to food bioprocessing, food spoilage, and foodborne diseases. Thus, it will be useful as a text in the introductory food microbiology courses taught under various programs and disciplines. In addition, it will be a valuable reference for those directly and indirectly involved in food and microbiology, including individuals in academic institutions; research institutions; federal, state, and local government agencies; food industries; food consultants; and even food lobbyists.

The subject matter is divided into seven sections. For undergraduate teaching, the first six sections can be taught as a semester course; Section VII (appendices) can be used as advanced information for an undergraduate course, which contains materials that are either taught in other courses, such as advanced food microbiology, or food safety courses and laboratory courses. Section I describes the history of food microbiology, characteristics of microorganisms important in foods, their sources, and significance. Section II deals with microbial growth and metabolism of food, and the significance of microbial sublethal injury and bacterial sporulation in foods. Section III explains the different beneficial uses of microorganisms, which include starter cultures, bioprocessing, biopreservation, and probiotics. Section IV deals with spoilage of foods by microorganisms and their enzymes and methods used to determine food spoilage. In addition, there is a chapter on problems and solutions of some emerging spoilage bacteria in refrigerated foods. Section V deals with foodborne pathogens associated with intoxication, infections, and toxicoinfections, and those considered to be opportunistic pathogens, as well as pathogenic parasites and



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algae. In addition, a chapter has been included on emerging pathogens and a chapter on indicators of pathogens. Section VI discusses different methods used to control undesirable microorganisms for the safety and stability of food. A chapter on new nonthermal methods and a chapter on the hurdle concept in food preservation are included.

The materials in each chapter are arranged in logical, systematic, and concise sequences. Tables, figures, and data have been used only when they are necessary for better understanding. At the end of each chapter, a limited list of selected references and suggested questions have been included. To reduce confusion, especially for those not familiar with the constant changes in microbial genera, three first letters have been used to identify the genus of a species. The index has been prepared carefully so that the materials in the text can be easily found.

I thank Deb Rogers for her excellent performance in typing the manuscript. Finally, I thank my students, who, over a period of the past 20 years, have suggested what they would like to have in a food microbiology course. Their suggestions have been followed while writing this text.

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# Authors

**Bibek Ray, PhD,** currently professor emeritus, was a professor of food microbiology in the Department of Animal Science at the University of Wyoming, Laramie. Professor Ray earned BS and MS degrees in veterinary science from the University of Calcutta and University of Madras in India, respectively. He received his PhD in food science from the University of Minnesota in 1970 and joined the faculty in the Department of Food Science, North Carolina State University, and then the Department of Biology at Shaw University, both at Raleigh. He joined the University of Wyoming in 1981. There, he expanded his research to intestinal beneficial bacteria, bacteriocins of Gram-positive bacteria, and high hydrostatic-pressure preservation of food along with his previous research activities in the area of microbial sublethal injury. He also taught courses in food microbiology, food fermentation, food safety, and a course titled "Safety of Our Food" to nonscience undergraduates. His laboratory was involved in extensive and thorough studies in both basic and applied areas of the bacteriocin pediocin AcH from *Pediococcus acidilactici* H. In addition, his group studied various aspects of bacteriocins produced by *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* as well as *Bacillus* and *Staphylococcus* spp. He received research funding from the National Science Foundation, American Public Health Association, National Live Stock and Meat Board, United States Department of Agriculture, United States Army Research, North Atlantic Treaty Organization (with Turkey) and Binational Agriculture Research Development Agency (with Israel), Wyoming Development Fund, and the industry. Before retirement, he was studying the combined effect of bacteriocins, ultrahigh hydrostatic pressure, and pulse field electricity and sublethal injury on the destruction of microbial cells and spores and its application in food preservation. In addition, Dr. Ray established collaborative research programs with research institutes and universities in Turkey, Israel, India, Indonesia, and France. He also participated in symposiums, offered short courses, presented research activities, and visited Research Laboratories in Japan, Indonesia, Thailand, Australia, New Zealand, India, Turkey, France, Spain, Germany, Norway, Belgium, England, Denmark, Netherlands, Hungary, Austria, Greece, Italy, Mexico, Canada, and Argentina.

Professor Ray has published over 100 research articles, reviews, book chapters, proceedings articles, and popular articles on food microbiology. He has also edited four books: *Injured Index and Pathogenic Bacteria* (1989) and *Food Biopreservatives of Microbial Origin* (1992, with Dr. M.A. Daeschel), both published by CRC Press, Boca Raton, FL; *Lactic Acid Bacteria: Current Advances in Metabolism, Genetics, and Applications* (1996, with Dr. Faruk Bozoglu), Springer, New York; and *Novel Processing and Control Technologies in the Food Industry* (1999, with Dr. Faruk Bozoglu and Tibor Deak), IOS Press, Washington, DC. He was a member of the American Society for Microbiology and the Institute of Food Technologists and a fellow of the American Academy of



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Microbiology. He also served on the editorial boards of the *Journal of Food Protection, Applied and Environmental Microbiology*, and the *Indian Journal of Microbiology*. In 1994, Professor Ray was awarded the University of Wyoming Presidential Achievement Award in recognition of his excellence in academic performance. He retired from the University of Wyoming in September 2002. Since then, he and his wife, Purnima, have built a center (Google "Bibek Ray" or "Gohaldanga Sevakendra") and together are engaged in improving the health, education, and economic conditions of underprivileged poor people in deep rural villages, about 100 miles from Calcutta, India, and spends a great deal of time with Purnima in the village of his birth every year.

**Arun K. Bhunia, BVSc, PhD,** is a professor of molecular food microbiology in the Department of Food Science and also in the Department of Veterinary Comparative Pathobiology (Courtesy) at Purdue University, West Lafayette, Indiana. Professor Bhunia received his bachelor of veterinary medicine degree (1984) from Bidhan Chandra Krishi Viswa Vidyalaya (currently West Bengal University of Fisheries and Animal Sciences), West Bengal, India. He received his PhD (1989) from the University of Wyoming (USA) under the mentorship of Professor Bibek Ray and postdoctoral training (1995) from the University of Arkansas, Fayetteville, under the mentorship of Prof. Michael G. Johnson and then joined Alabama A&M University (Normal, Alabama) as an assistant professor in 1995. In 1998, he joined the Department of Food Science at Purdue University. He teaches three graduate level courses: Microbial Foodborne Pathogens (major topics: Mechanism of pathogenesis of foodborne pathogens and toxins and the host-parasite interactions); Microbial Techniques for Food Pathogen (a laboratory course that incorporates rapid methods employing immunoassays and genetic and biosensor tools), and codirects a graduate level journal club, Intestinal Microbiology and Immunology. In addition, he routinely lectures in courses such as Principles of Public Health (both undergraduate and graduate levels) on foodborne diseases, and Veterinary Microbiology and Mycology on several foodborne pathogens. Professor Bhunia's laboratory maintains a balanced research program (basic and applied) to address problems related to foodborne pathogens in the following areas: (a) pathogen and toxin detection; (b) understanding the mechanism of pathogenesis of enteric pathogens, including *Listeria monocytogenes*; and (c) application of bioengineered probiotics and natural antimicrobial peptides coupled with nanotechnology to control pathogens. His research has been funded by grants from the U.S. Department of Agriculture, National Science Foundation, National Cattleman's Beef Association, National Institute of Health, and Center for Food Safety Engineering.

To date, Professor Bhunia has published 132 refereed and 33 proceedings articles, 137 research abstracts, written 15 book chapters, and delivered over 90 invited presentations in national and international meetings or institutions. He has also written a graduate level textbook (*Microbial Foodborne Pathogens: Mechanisms and Pathogenesis*. First Edition, 2008, Springer, New York), coedited two books (*Foodborne Pathogens: Microbiology and Molecular Biology*, 2005; Caister Academic Press, and *The Science of Homeland Security*, 2006, Purdue University Press).

He is a member of the American Society for Microbiology (ASM), Institute of Food Technologists (IFT), and International Association for Food Protection (IAFP) and was a past member of the Society for Photo Optical Engineers and American Association for the Advancement of Science, and he currently serves as a member of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF: 2013–2015); editor-in-chief of *Open Journal of Medical Microbiology* (SR Publishing), and *Open Journal of Applied Biosensor* (SR Publishing); editor for *Gut Pathogens* (2010–present); *Journal of Nutrition and Food Science* (2010–present); *Conference Papers in Microbiology* (2012–present); *Indian Journal of Microbiology* (2008–2012); guest editor for *Journal of Biomedicine and Biotechnology* (2012); "Food Biosensors and Food Safety"

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In 2003, Professor Bhunia received a prestigious Purdue Agricultural Research Award; in 2005, he was selected as University Faculty Scholar; his research team received the "2006 Agriculture Team Award" from Purdue University; he received the R&D Award from IFT (2009); and the Outstanding Graduate Educator Award from Department of Food Science (2010–2011) and College of Agriculture (2013) at Purdue University.

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# 1 INTRODUCTION TO MICROBES IN FOODS

Microorganisms are living entities of microscopic size and include bacteria, viruses, yeasts and molds (together designated as fungi), algae, and protozoa. For a long time, bacteria have been classified as prokaryotes (cells without definite nuclei) and the fungi, algae, and protozoa as eukaryotes (cells with nuclei); viruses do not have regular cell structures and are classified separately. In the 1990s, this classification changed and will be briefly mentioned in Chapter 2. Microorganisms are present everywhere on Earth, including in humans, animals, plants and other living creatures, soil, water, and the atmosphere, and they can multiply everywhere except in the atmosphere. Together, their numbers far exceed all other living cells on this planet. They were the first living cells to inhabit the Earth more than 3 billion years ago and since then have played important roles, many of which are beneficial to other living systems.

Among the microorganisms, some molds, yeasts, bacteria, and viruses play both desirable and undesirable roles in our food. In this section, the importance of microorganisms in food, the predominant microorganisms associated with food, sources from which they get into the food, and the microbiological quality of food under normal conditions are presented in the following chapters:

Chapter 1: History and Development of Food Microbiology
Chapter 2: Characteristics of Predominant Microorganisms in Food
Chapter 3: Sources of Microorganisms in Food
Chapter 4: Normal Microbiological Quality of Foods and Its Significance

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# Chapter 1

# History and Development of Food Microbiology

## Introduction

Except for a few sterile foods, all foods harbor one or more types of microorganisms. Some of them play desirable roles in food, such as in the production of naturally fermented foods, whereas others cause food spoilage and foodborne diseases. To study the role of microorganisms in food and to control them when necessary, it is important to isolate them in pure culture and study their morphological, physiological, biochemical, and genetic characteristics. Some of the simplest techniques in use today for these studies were developed over the past 300 years; a brief description is included here.

## Discovery of Microorganisms

The discovery of microorganisms[1–3] ran parallel with the invention and improvement of the microscope. Around 1658, Athanasius Kircher reported that, using a microscope, he had seen minute living worms in putrid meat and milk. The magnification power of his microscope was so low that he could not have seen bacteria. In 1664, Robert Hooke described the structure of molds. However, probably the first person to see different types of microorganisms, especially bacteria, under a microscope that probably did not have a magnification power above 300×, was Antony van Leeuwenhoek. He observed bacteria in saliva, rainwater, vinegar, and other materials; sketched the three morphological groups (spheroids or cocci, cylindrical rods or bacilli, and spiral or spirilla); and also described some as being motile. He called them animalcules, and between 1676 and 1683, he reported his observations to the newly formed leading scientific organization, The Royal Society of London, where his observations were read with fascination. As reasonably good microscopes were not easily available at the time, other interested individuals and scientists during the next 100 years only confirmed Leeuwenhoek's observations. In the 19th century, as an outcome of the Industrial Revolution, improved microscopes became more



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easily available, which stimulated many inquisitive minds to observe and describe the creatures they discovered under a microscope. By 1838, Ehrenberg (who introduced the term *bacteria*) had proposed at least 16 species in four genera, and by 1875, Ferdinand Cohn had developed the preliminary classification system of bacteria. Cohn was also the first to discover that some bacteria produced spores. Although, like bacteria, the existence of submicroscopic viruses was recognized in the mid-19th century, they were observed only after the invention of the electron microscope in the 1940s.

## Where Are They Coming from?

Following Leeuwenhoek's discovery, although there were no bursts of activity, some scientific minds were curious to determine from where the animalcules, observed to be present in many different objects, were emanating.[1–3] Society had just emerged from the Renaissance period, and science, known as experimental philosophy, was in its infancy. The theory of spontaneous generation, that is, the generation of some form of life from nonliving objects, had many powerful followers among the educated and elite classes. Since the time of the Greeks, the emergence of maggots from dead bodies and spoiled flesh was thought to be a result of spontaneous generation. However, in approximately 1665, Redi disproved that theory by showing that the maggots in spoiled meat and fish could only appear if flies were allowed to contaminate them. The advocates of the spontaneous generation theory argued that the animalcules could not regenerate by themselves (biogenesis), but they were present in different things only through abiogenesis (spontaneous generation). In 1749, Turbevill Needham showed that boiled meat and meat broth, following storage in covered flasks, could have the presence of animalcules within a short time. This was used to prove the appearance of these animalcules by spontaneous generation. Lazzaro Spallanzani (1765) showed that boiling the meat infusion in broth in a flask and sealing the flask immediately prevented the appearance of these microscopic organisms, thereby disproving Needham's theory.

This was the time when Antoine Laurent Lavoisier and his coworkers showed the need of oxygen for life. The believers of abiogenesis rejected Spallanzani's observation, suggesting that there was not enough vital force (oxygen) present in the sealed flask for animalcules to appear through spontaneous generation. Later, Schulze (1830), by passing air through acid; Theodore Schwann (1838), by passing air through red-hot tubes; and Schröeder (1854), by passing air through cotton showed that bacteria failed to appear in a boiled meat infusion even in the presence of air. Finally, in 1861, Louis Pasteur demonstrated that, in a boiled infusion, bacteria could grow only if the infusions were contaminated with bacteria carried by dust particles in the air.[1,4] His careful and controlled studies proved that bacteria were able to reproduce (biogenesis), and life could not originate by spontaneous generation. John Tyndall, in 1870, showed that a boiled infusion could be stored in dust-free air in a box without microbial growth.

## What Are Their Functions?

The involvement of invisible organisms in many diseases in humans was suspected as early as the 13th century by Roger Bacon. In the 16th century, Girolamo Fracastoro of Verona suggested that many human diseases were transmitted from person to person by small creatures. This was also indicated by Kircher in 1658. In 1762, von Plenciz of Vienna suggested that different invisible organisms were responsible for different diseases. Theodore Schwann (1837) and Hermann

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Helmholtz (1843) proposed that putrefaction and fermentation were connected with the presence of the organisms derived from air. Finally, Pasteur, in 1875, showed that wine fermentation from grapes and the souring of wine was caused by microorganisms. He also proved that spoilage of meat and milk was associated with the growth of microorganisms. Later, he showed the association of microorganisms with several diseases in humans, cattle, and sheep, and he also developed vaccines against a few human and animal diseases caused by microorganisms, including rabies. Robert Koch, in Germany (in the 1880s and 1890s), isolated pure cultures of bacteria responsible for anthrax, cholera, and tuberculosis. He also developed the famous Koch's postulates to associate a specific bacterium as a causative agent for a specific disease. Along with his associates, he also developed techniques of agar plating methods to isolate bacteria in pure cultures and to determine microbial numbers in a sample, the Petri dish (by Petri in his laboratory), staining methods for better microscopic observation of bacteria, and the use of steam to sterilize materials to grow bacteria.[1,5]

With time, the importance of microorganisms in human and animal diseases, soil fertility, plant diseases, fermentation, food spoilage and foodborne diseases, and other areas was recognized, and microbiology was developed as a specific discipline. Later, it was divided into several subdisciplines, such as medical microbiology, industrial microbiology, soil microbiology, plant pathology, and food microbiology.

## Development of Early Food Microbiology (Before A.D. 1900)

It is logical to comprehend that our early Homo ancestors, the hunters and gatherers, were aware of food spoilage and foodborne diseases. Even without any perception of the causative agents, they used ice and fire to preserve foods and make them safe. Around 8000 B.C., as agriculture and animal husbandry were adopted by the early civilizations, a food supply, especially agricultural produce, became available in abundance during the growing seasons. Preservation of foods became important for a uniform supply of food throughout the year. Between 8000 and 1000 B.C., many food preservation methods, such as drying, cooking, baking, smoking, salting, sugaring (with honey), low-temperature storage (in ice), storage without air (in pits), fermentation (with fruits, grains, and milk), pickling, and spicing were used, probably mainly to reduce spoilage. However, one cannot be sure whether society at that time recognized the implications of diseases transmitted through food. In later periods, however, the scriptural injunctions laid by many religions suggest that the societies recognized an association of diseases with some foods. Some of the regulations, such as not eating meat from a diseased animal or an animal killed by a scavenger or not eating a food that appeared unnatural or had been handled by an unclean person, were developed to safeguard the health of citizens against foodborne diseases. Fermentation was used extensively by many societies not only to preserve foods, but also as a method to produce various types of desirable foods from milk, meat, fish, eggs, grains, fruits, and vegetables.

Following the discovery of the ubiquitous existence of microorganisms (mainly bacteria and yeasts) by Leeuwenhoek around the 1670s, some individuals started associating the possible role of these organisms with food spoilage, food fermentation, and foodborne diseases. The major developments of ideas on the possible roles of microorganisms in foods and their scientific proof were initiated by Pasteur in the 1870s, followed by many other scientists before the end of the 19th century. This paved the way for the establishment of early food microbiology in the 20th century. Some of the major developments in the 19th century are briefly listed here.[1,6,7]

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Food fermentation

1822 C.J. Person named the microscopic organism found on the surface of wine during vinegar production as *Mycoderma mesentericum*. Pasteur, in 1868, proved that this organism was associated with the conversion of alcohol to acetic acid and named it *Mycoderma aceti*. In 1898, Martinus Beijerinck renamed it *Acetobacter aceti*.

1837 Theodor Schwann named the organism involved in sugar fermentation as *Saccharomyces* (sugar fungus).

1838 Charles Cogniard-Latour suggested that growth of yeasts was associated with alcohol fermentation.

1860 Louis Pasteur showed that fermentation of lactic acid and alcohol from sugar was the result of growth of specific bacteria and yeasts, respectively.

1883 Emil Christian Hansen used pure cultures of yeasts to ferment beer.

Food spoilage

1804 Francois Nicolas Appert developed methods to preserve foods in sealed glass bottles by heat in boiling water. He credited this process to Lazzaro Spallanzani (1765), who first used the method to disprove the spontaneous generation theory.

1819 Peter Durand developed canning preservation of foods in steel cans. Charles Mitchell introduced tin lining of metal cans in 1839.

1870 L. Pasteur recommended heating of wine at 145°F (62.7°C) for 30 minutes to destroy souring bacteria. F. Soxhlet advanced boiling of milk for 35 minutes to kill contaminated bacteria. Later, this method was modified and named pasteurization and used to kill mainly vegetative pathogens and many spoilage bacteria.

1895 Harry Russell showed that gaseous swelling with bad odors in canned peas was a result of the growth of heat-resistant bacteria (spores).

Foodborne diseases

1820 Justin Kerner described food poisoning from eating blood sausage (resulting from botulism). Fatal disease from eating blood sausage was recognized as early as A.D. 900.

1849 John Snow suggested the spread of cholera through drinking water contaminated with sewage. In 1854, Filippo Facini named the cholera bacilli as *Vibrio cholerae*, which was isolated in pure form by Robert Koch in 1884.

1856 William Budd suggested that water contamination with feces from infected persons spread typhoid fever and advocated the use of chlorine in the water supply to overcome the problem. In 1800, G. de Morveau and W. Cruikshank advocated the use of chlorine to sanitize potable water.

1885 Theodor Escherich isolated *Bacterium coli* (later named *Escherichia coli*) from the feces and suggested that some strains were associated with infant diarrhea.

1888 A.A. Gartner isolated *Bacterium* (later *Salmonella*) *enteritidis* from the organs of a diseased man as well as from the meat the man ate. In 1896, Marie von Ermengem proved that *Salmonella enterica serovar* enteritidis (modern nomenclature) caused a fatal disease in humans who consumed contaminated sausage.

1894 J. Denys associated pyogenic *Staphylococcus* with the death of a person who ate meat prepared from a diseased cow.

1894 Alexandre Yersin, a French scientist first reported a bacillus to be the causative agent for plague, and later it was named *Yersinia pestis*. *Y. pestis* can cause foodborne disease.

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*Yer. enterocolitica* and *Yer. pseudotuberculosis* are two members of the genus *Yersinia*, which are known foodborne pathogens.

1895 Marie von Ermengem isolated *Bacillus botulinus* (*Clostridium botulinum*) from contaminated meat and proved that it caused botulism.

1981 Stanley Prusiner characterized prion, a causative agent for bovine spongiform encephalopathy or "Mad Cow Disease."

Microbiology techniques

1854 Heinrich Schröder and Theodore von Dusch used cotton to close tubes and flasks to prevent microbial contamination in heated culture broths.

1876 Car Weigert used methylene blue (a synthetic dye) to stain bacteria in aqueous suspensions.

1877 Ferdinand Cohn showed the heat resistance of *Bacillus subtilis* endospores.

1878 Joseph Lister isolated *Streptococcus* (now *Lactococcus*) *lactis* in pure culture by serial dilution from sour milk.

1880s Robert Koch and his associates introduced many important methods that are used in all branches of microbiology, such as solid media (first gelatin, then agar) to purify and enumerate bacteria, the Petri dish, flagellar staining, steam sterilization of media above 100°C, and photography of cells and spores.

1884 Hans Christian Gram developed Gram staining of bacterial cells.

## Food Microbiology: Current Status

In the early 20th century, studies continued to understand the association and importance of microorganisms, especially pathogenic bacteria in food. Specific methods were developed for their isolation and identification. The importance of sanitation in the handling of food to reduce contamination by microorganisms was recognized. Specific methods were studied to prevent growth as well as to destroy the spoilage and pathogenic bacteria. There was also some interest in isolating beneficial bacteria associated with food fermentation, especially dairy fermentation, and studying their characteristics. However, after the 1950s, food microbiology entered a new era. Availability of basic information on the physiological, biochemical, and biological characteristics of diverse types of food; microbial interactions in food environments; and microbial physiology, biochemistry, genetics, and immunology has helped open new frontiers in food microbiology.[1,6–10] Now, understanding the microbial community and microbial interaction in a complex food environment through microbiome analysis, whole genome sequencing, and development of novel and natural intervention technologies are at the forefront of food microbiology.

### *Food Fermentation/Probiotics*

- Development of strains with desirable metabolic activities by genetic transfer among strains
- Development of bacteriophage-resistant lactic acid bacteria
- Metabolic engineering of strains for overproduction of desirable metabolites
- Development of methods to use lactic acid bacteria to deliver biologically relevant proteins or vaccines
- Sequencing genomes of important lactic acid bacteria and bacteriophages for better understanding of their characteristics
- Food biopreservation with desirable bacteria and their antimicrobial metabolites

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- Understanding of important characteristics of probiotic bacteria and development of desirable strains
- Effective methods to produce starter cultures for direct use in food processing
- Bioengineered probiotics for health promotion and disease control

## Food Spoilage

- Identification and control of new spoilage bacteria associated with the current changes in food processing and preservation methods
- Spoilage resulting from bacterial enzymes of frozen and refrigerated foods with extended shelf life
- Development of molecular methods, including nanotechnology, to identify metabolites of spoilage bacteria and predict the potential shelf life of foods
- Importance of environmental stress on the resistance of spoilage bacteria to antimicrobial preservatives
- Microbial community, ecology, and quorum sensing in food

## Foodborne Diseases

- Methods to detect emerging foodborne pathogenic bacteria from contaminated foods
- Application of molecular biology techniques, including nanotechnology and biotechnology, for rapid detection of pathogenic bacteria in food and the environment
- Effective detection and control methods of foodborne pathogenic viruses
- Transmission potentials of prion diseases from food animals to humans
- Importance of environmental stress on the detection and destruction of pathogens
- Factors associated with the increase in antibiotic-resistant pathogens in food
- Biofilm formation, microbial quorum sensing, and attachment of foodborne pathogens to food and equipment surfaces
- Mechanisms of pathogenicity of foodborne pathogens
- Effect of food or environment-related stress on gene regulation, pathogenicity, and survival
- Effective methods for the epidemiological study of foodborne diseases: Genome-based approaches to study epidemiology, including whole genome sequence and molecular fingerprinting techniques
- Application of bacteriophages and other natural antimicrobials for control of foodborne pathogens
- Control of pathogenic parasites in food
- Mold and mycotoxin detection and control
- Fish and shellfish toxin detection and control
- Foodborne disease control strategies in preharvest (on farms) and postharvest food production

## Miscellaneous

- Application of hazard analysis of critical control points (HACCP) in food production, processing, and preservation
- Novel food-processing technologies
- Microbiology of unprocessed and low-heat-processed ready-to-eat foods
- Microbial control of foods from farm to table (total quality management)
- Food safety legislation including the Food Safety Modernization Act of 2011

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## Food Microbiology and Food Microbiologists

From the above discussion, it is apparent what, as a discipline, food microbiology has to offer. Before the 1970s, food microbiology was regarded as an applied science mainly involved in the microbiological quality control of food. Since then, the technology used in food production and processing, distribution and retailing, and food consumption patterns have changed dramatically. These changes have introduced new problems that can no longer be solved by merely using applied knowledge. Thus, modern-day food microbiology needs to include a great deal of basic science to understand and effectively solve the microbiological problems associated with food. The discipline includes not only the microbiological aspects of food spoilage and foodborne diseases and their effective control and bioprocessing of foods, but also basic information of microbial ecology, physiology, metabolism, and genetics. This information is helping to develop methods for rapid and effective detection of spoilage and pathogenic bacteria, to develop desirable microbial strains by recombinant DNA technology, to produce fermented foods of better quality, to develop thermostable enzymes in enzyme processing of food and food additives, to develop methods to remove bacteria from food and equipment surfaces, and to combine several control methods for effective control of spoilage and pathogenic microorganisms in food.

An individual who has completed courses in food microbiology (both lecture and laboratory) should gain knowledge in the following areas:

- Determining the microbiological quality of foods and food ingredients by using appropriate techniques
- Determining the microbial types involved in spoilage and health hazards and identify the sources
- Understanding factors contributing to the emergence of new pathogens or the reemergence of old pathogens
- Understanding the basic mechanism of pathogenesis of foodborne microbes
- Designing corrective procedures to control the spoilage and pathogenic microorganisms in food
- Learning rapid methods of isolating and identifying pathogens and spoilage bacteria from food and the environment
- Identifying how new and novel technologies adapted for food processing can have specific microbiological problems and designing methods to overcome the problem
- Designing effective sanitation procedures to control spoilage and pathogen problems in food-processing facilities
- Effectively using desirable microorganisms to produce fermented foods
- Designing methods to produce better starter cultures for use in fermented foods and probiotics
- Knowing about food regulations (state, federal, and international)
- Understanding the microbiological problems of imported foods

To be effective, in addition to the knowledge gained, one has to be able to communicate with different groups of people about the subject (food microbiology and its relationship to food science, food processing, food biotechnology, and biomedical sciences). An individual with good common sense is always in a better position to sense a problem and correct it quickly.

## Conclusion

Human civilization began when hunters and gatherers adopted not only production, but also preservation of foods. Thus, long before the existence of microorganisms was discovered, their

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importance in food spoilage and health hazards was conceived of by our early ancestors. Once the microorganisms' association with and importance in food were proven, efforts were made to understand the basic principles associated with food and microbial interactions. This knowledge was used to control undesirable microbes and effectively use the desirable types. Current investigations are directed toward understanding microbes on the molecular level. A food microbiologist should have a good understanding of the current developments in food microbiology as well as the characteristics of microorganisms important in food. The latter aspect is discussed in Chapter 2.

### QUESTIONS

1. Describe briefly the contributions of the following scientists in the development of microbiology and food microbiology: (a) Leeuwenhoek, (b) Spallanzani, (c) Pasteur, (d) Koch, (e) Hess, (f) Cohn, (g) Lister, (h) Soxhlet, (i) Gartner, and (j) Appert.
2. Why did Needham's experiments fail to disprove spontaneous generation for microbes and Pasteur succeed in disproving that theory?
3. List three important areas (each) of current studies in food biotechnology, food spoilage, and foodborne diseases.
4. Briefly explain the major differences in the understanding of the importance of microorganisms in foods before and after the 1900s.
5. List the pathogens that were proven to be associated with foodborne diseases before 1900.
6. Briefly describe what a food microbiology student is expected to know.

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# *Chapter 2*

# Characteristics of Predominant Microorganisms in Food

## Introduction

The microbial groups important in foods consist of several species and types of bacteria, yeasts, molds, and viruses. Although some algae and protozoa as well as some worms (such as nematodes) are important in foods, they are not included among the microbial groups in this chapter. Some of the protozoa and worms associated with health hazards, and several algae (fish and shellfish poisoning) associated with health hazards and bioprocessing (sources of vitamins, single-cell proteins), are discussed in Chapters 17 and 28.

Bacteria, yeasts, molds, and viruses are important in food for their ability to cause foodborne diseases and food spoilage and to produce food and food ingredients. Many bacterial species and some molds and viruses, but not yeasts, are able to cause foodborne diseases. Most bacteria, molds, and yeasts, because of their ability to grow in foods (viruses cannot grow in foods), can potentially cause food spoilage. Several species of bacteria, molds, and yeasts are considered safe or food grade, or both, and are used to produce fermented foods and food ingredients. Among the four major groups, bacteria constitute the largest group. Because of their ubiquitous presence and rapid growth rate, even under conditions in which yeasts and molds cannot grow, they are considered the most important in food spoilage and foodborne diseases.

Prion or proteinaceous infectious particles have been identified as causing transmissible spongiform encephalopathies (TSEs) in humans and animals (see Chapter 28). However, their ability to cause foodborne diseases is not clearly understood.

In this chapter, a brief discussion is included initially on the methods currently used in the classification and nomenclature of microorganisms and later on the important characteristics of microorganisms predominant in food.



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## Classification of Microorganisms

Living cellular organisms, on the basis of phylogenetic and evolutionary relationships, were grouped originally in five kingdoms, in which bacteria belonged to prokaryotes (before nucleus), and the eukaryotic (with nucleus) molds and yeasts were grouped under fungi.[1] In the 1970s, the prokaryotic domain was changed to Eubacteria (with murein or peptidoglycan on cell wall) and Archaebacteria (without murein on cell wall). In the 1990s, this was changed to Bacteria and Archaea, respectively.[2,3] Figure 2.1 depicts a phylogenetic tree representing three major divisions: Bacteria, Archaea, and Eukarya. The Archaea include most extremophiles and are not important to food microbiology. Fungi and parasites are included under Eukarya and are responsible for foodborne disease and/or spoilage. Viruses are not included in this classification system.

For the classification of yeasts, molds, and bacteria, several ranks are used after the kingdom: divisions, classes, orders, families, genera (singular genus), and species. The basic taxonomic group is the species. Several species with similar characteristics form a genus. Among eukaryotes, species in the same genus can interbreed. This is not considered among prokaryotes although the conjugal transfer of genetic materials exists among many bacteria. Several genera make a family, and the same procedure is followed in the hierarchy. In food microbiology, ranks above species, genus, and family are seldom used. Among bacteria, a species is regarded as a collection of strains having many common features. A strain is the descendent of a single colony (single cell). Among

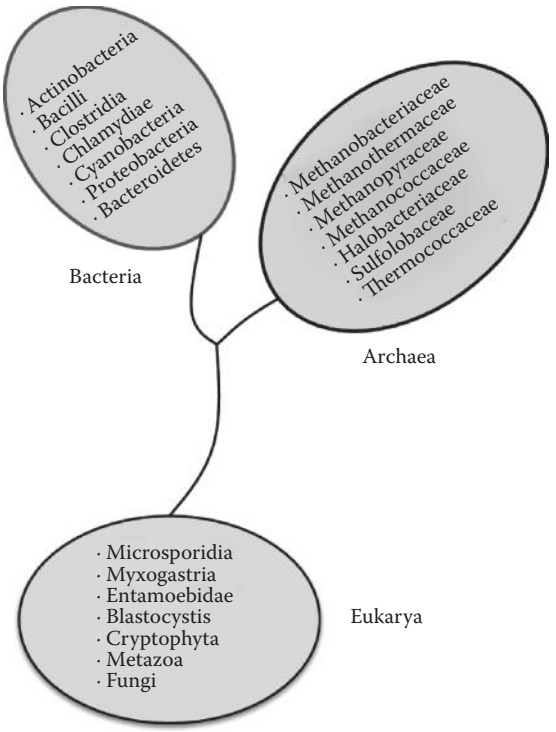



**Figure 2.1 Phylogenetic tree for microorganisms representing three major divisions (phyla): Bacteria, Archaea, and Eukarya. (Redrawn based on Eckburg, P.B. et al., *Infect. Immun.*, *71*, 591–596, 2003.) Representative classes of microorganisms are listed for each division (phylum).**

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the strains in a species, one is assigned as the type strain and is used as a reference strain while comparing the characteristics of an unknown isolate. However, by knowing the complete genome sequence, this system will change in the future.

Several methods are used to determine relatedness among bacteria, yeasts, and molds for taxonomic classification. In yeasts and molds, morphology, reproduction, the biochemical nature of macromolecules, and metabolic patterns are used along with other criteria. For bacteria, Gram-stain characteristics, protein profiles, amino acid sequences of some specific proteins, base composition (mol% G+C), nucleic acid (DNA and RNA) hybridization, nucleotide base sequence, and computer-assisted numerical taxonomy are used.[1–3] Protein profile, amino acid sequence, base composition, DNA and RNA hybridization, and nucleotide base sequence are directly or indirectly related to genetic makeup of the organisms and thus provide a better chance in comparing two organisms at the genetic level. In mol% G+C ratio, if two strains differ by 10% or more, they are most likely not related. Similarly, in a hybridization study, two strains are considered the same if their DNAs have 90% or more homology. For the nucleotide base sequence, the sequences in 16S rRNA among strains are compared. A sequence of approximately 1500 nucleotide bases over a stretch of 16S rRNA is most conserved, so related strains should have high homology. In numerical taxonomy, many characteristics are compared, such as morphology, physiology, and biochemistry. Each characteristic is given the same weight. Two strains in the same species should score 90% or more. Whole genome sequencing is now used for pathogen identification and classification, and it is anticipated this will be a routine microbial identification tool in the near future.

Evolutionary relationships among viruses, if any, are not known. Their classification system is rather arbitrary and based on the types of disease they cause (such as the hepatitis virus, causing inflammation of the liver cells), nucleic acid content (RNA or DNA, single stranded or double stranded), and morphological structures. In food, two groups of viruses are important: the bacterial viruses (bacteriophages) of starter culture bacteria and some foodborne pathogenic bacteria and the human enteric pathogenic viruses associated with foodborne diseases. Bacteriophages are also used as biocontrol to kill foodborne pathogens in meat animals during preharvest production and in food products during postharvest processing (see Chapter 38). Bacteriophages are also used as an elegant detection tool for pathogens because of their strong interaction with host pathogens (see Chapter 42).

## Nomenclature

The basic taxonomic group in bacteria, yeasts, and molds is the species, and each species is given a name. The name has two parts (binomial name): The first part is the genus name, and the second part is the specific epithet (adjective). Both parts are Latinized; when written, they are italicized (or underlined) with the first letter of the genus written with a capital letter (e.g., *Saccharomyces cerevisiae*, *Penicillium roquefortii*, *Listeria monocytogenes*, and *Lactobacillus acidophilus*). A bacterial species can be divided into several subspecies (subsp. or ssp.) if the members show minor but consistent differences in characteristics. Under such conditions, a trinomial epithet (subspecific epithet) is used (e.g., *Lactococcus lactis* ssp. *lactis* or *Lactococcus lactis* ssp. *cremoris*). In some instances, ranks below subspecies are used to differentiate strains recognized by specific characters (e.g., serovar, antigenic variation; biovar, producing a specific metabolite; and phagovar, sensitive to a specific phage types). Such ranks have no taxonomic importance but can be practically useful (e.g., *Lactococcus lactis* ssp. *lactis* biovar diacetilactis is a *Lactococcus lactis* ssp. *lactis* strain that produces diacetyl, an important flavor compound in some fermented dairy products). Each strain of a species should be identified with a specific strain number, which can be alphabetic or numeric

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or a mixture of both (e.g., *Pediococcus acidilactici* LB923; *Listeria monocytogenes* ATCC 19116). At the family level, bacterial names are used as plural adjectives in feminine gender and agree with the suffix "aceae" (e.g., Enterobacteriaceae). The species and strains in a genus can be represented collectively, either using "spp." after genus (e.g., *Lactobacillus* spp.) or plural forms of the genus (e.g., lactobacilli for *Lactobacillus*; lactococci for *Lactococcus*; leuconostocs for *Leuconostoc*, or salmonellae for *Salmonella*).

The scientific names of bacteria are given according to the specifications of the International Code of Nomenclature of Bacteria. The International Committee on Systematic Bacteriology of the International Union of Microbiological Association examines the validity of each name and then publishes the approved lists of bacterial names from time to time. A new name (species or genus) must be published in the *International Journal of Systematic Bacteriology* before it is judged for inclusion in the approved list. Any change in name (genus or species) has to be approved by this committee.

When writing the name of the same species more than once in an article, it is customary to use both genus and specific epithet the first time and abbreviate the genus name subsequently. In the *Bergey's Manual of Systematic Bacteriology*, only the first letter is used (e.g., *Listeria monocytogenes* and then *L. monocytogenes*). The same system is used in most publications in the United States. However, it creates confusion when one article has several species with the same first letter in the genus (e.g., *Lactobacillus lactis*, *Leuconostoc lactis*, and *Lactococcus lactis* as *L. lactis*). In some European journals, more than one letter is used, but there is no definite system (e.g., *Lact. lactis*, *Lc. lactis*, *Leu. lactis*, *Lb. lactis*, *List. monocytogenes*). In this book, to reduce confusion among readers, many of whom might not be familiar with the current rapid changes in bacterial nomenclature, a three-letter system is used (e.g., *Lis. monocytogenes*, *Leu. lactis*; *Shi. dysenterie* for *Shigella*; *Sta. aureus* for *Staphylococcus*). In rare cases, a slight modification is used (e.g., *Lactococcus lactis* and *Lactobacillus lactis* are written as *Lac. lactis* and *Lab. lactis*, respectively, for the two genera). The nomenclature system for *Salmonella* has been modified (Chapter 26).

The viruses, as indicated previously, have not been given specific taxonomic names as given for bacteria. They are often identified with alphabetic or numeric designations or a combination of both (e.g., T4 or T1 bacteriophages), the type of disease they produce (e.g., hepatitis A, causing liver disease), or by other methods, for example, Norwalk-like viruses (now called norovirus), causing a type of foodborne gastroenteritis in humans, originally isolated from the town called Norwalk in Ohio (United States).

## Morphology and Structure of Microorganisms in Foods

### *Yeasts and Molds*

Both yeasts and molds are eukaryotic, but yeasts are unicellular, whereas molds are multicellular.[5,6] Eukaryotic cells are generally much larger (20–100 μm) than prokaryotic cells (1–10 μm). Eukaryotic cells have rigid cell walls and thin plasma membranes. The cell wall does not have peptidoglycan, is rigid, and is composed of carbohydrates. The plasma membrane contains sterol. The cytoplasm is mobile (streaming) and contains organelles (mitochondria, vacuoles) that are membrane bound. Ribosomes are 80S type and attached to the endoplasmic reticulum. The DNA is linear (chromosomes), contains histones, and is enclosed in a nuclear membrane. Cell division is by mitosis (i.e., asexual reproduction); sexual reproduction, when it occurs, is by meiosis.

Molds are nonmotile, filamentous, and branched (Figure 2.2). The cell wall is composed of cellulose, chitin, or both. A mold (thallus) is composed of large numbers of filaments called hyphae.

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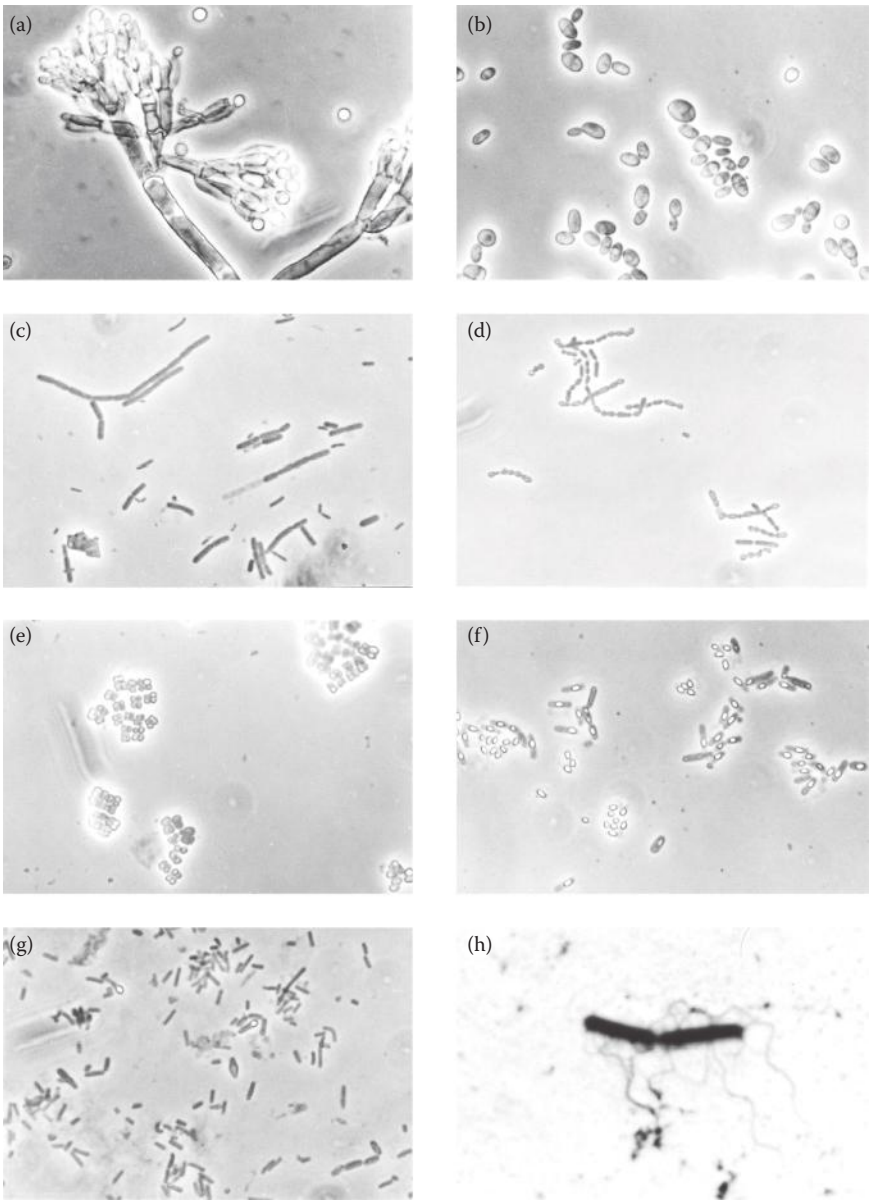



**Figure 2.2** Photograph of microbial morphology. (a) Molds: conidial head of *Penicillium* sp. showing conidiophore (stalk) and conidia. (b) Yeasts: *Saccharomyces cerevisiae*, some carrying buds. (c) Rod-shaped bacteria: *Bacillus* sp., single and chain. (d) Spherical-shaped bacteria: *Streptococcus* sp., chain. (e) Spherical-shaped bacteria: tetrads. (f) *Bacillus* cells carrying spores, center and off-center. (g) *Clostridium* cells, some carrying terminal spores (drumstick appearance). (h) Motile rod-shaped bacterium (*Clostridium* sp.) showing peretrichous flagella.

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An aggregate of hyphae is called mycelium. A hypha can be nonseptate, septate-uninucleate, or septate-multinucleate. A hypha can be vegetative or reproductive. The reproductive hypha usually extends in the air and forms exospores, either free (conidia) or in a sack (sporangium). Shape, size, and color of spores are used for taxonomic classification.

Yeasts are widely distributed in nature. The cells are oval, spherical, or elongated, about 5–30 × 2–10 μm in size (Figure 2.2).[4] They are nonmotile. The cell wall contains polysaccharides (glycans), proteins, and lipids. The wall can have scars, indicating the sites of budding. The membrane is beneath the wall. The cytoplasm has a fine granular appearance for ribosomes and organelles. The nucleus is well defined with a nuclear membrane.

## *Bacterial Cells*

Bacteria are unicellular, most approximately 0.5–1.0 × 2.0–10 μm in size, and have three morphological forms: spherical (cocci), rod shaped (bacilli), and curved (comma) (Figure 2.2). They can form associations, such as clusters, chains (two or more cells), or tetrads. They can be motile or nonmotile. Bacteria, when grown on a nutrient agar plate, form colonies that have circular or irregular edges, domed or flat shapes, rough or smooth surfaces, and a shiny (glistening) or dull appearance (Figure 2.3). Cytoplasmic materials are enclosed in a rigid wall on the surface and a membrane beneath the wall. Nutrients in the molecular and ionic form are transported from the environment through the membrane by several specific mechanisms. The membrane also contains energy-generating components. It also forms intrusions in the cytoplasm (mesosomes). The cytoplasmic material is immobile and does not contain organelles enclosed in a separate membrane. The ribosomes are 70S type and are dispersed in the cytoplasm. The genetic materials (structural and plasmid DNA) are circular; not enclosed in a nuclear membrane; and do not contain basic proteins, such as histones. Both gene transfer and genetic recombination occur but do not involve gamete or zygote formation. Cell division is by binary fission. Prokaryotic cells can also have flagella, capsules, surface layer proteins, and pili (fimbri) for specific functions. Some also form endospores (one per cell).

On the basis of Gram-stain behavior, bacterial cells are grouped as Gram-negative or Gram-positive (Figure 2.4). Gram-negative cells have an outer membrane (OM), a thin cell wall (CW) composed of peptidoglycan, and a cytoplasmic membrane (CM). The OM is composed of lipopolysaccharides (LPS), lipoproteins (LP), and phospholipids. Phospholipid molecules are arranged in a bilayer with the hydrophobic part (fatty acids) inside and the hydrophilic part (glycerol and phosphate) outside. LPS and LP molecules are embedded in the phospholipid layer. The OM has

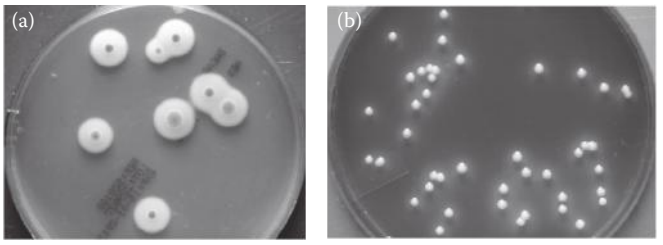



**Figure 2.3 (See color insert.) Colony formation by Gram-positive (a) *Bacillus thuringiensis* on BACARA chromogenic media from bioMérieux and Gram-negative (b) *Klebsiella pneumoniae* on XLT-4 (Xylose-Lysine-Tergitol-4) agar media representing different colony appearances.**

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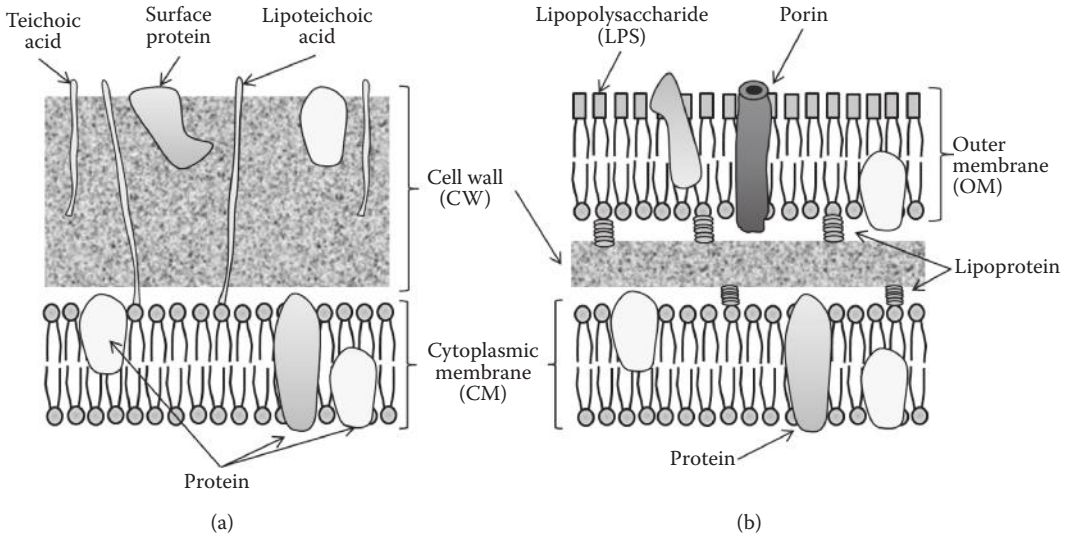



**Figure 2.4 Schematic representations of cell structure of bacteria. (a) Gram-positive bacteria: CW: cell wall showing thick mucopeptide backbone layers covalently linked to peptides, teichoic acids (or teichouronic acid); lipoteichoic acids (anchored to cytoplasmic membrane); CM: cytoplasmic membrane with lipid bilayers containing phospholipids, glycolipids, and embedded proteins. (b) Gram-negative bacteria: OM: outer membrane containing lipopolysaccharide (LPS) molecules, stabilized by divalent cations, phospholipids, and proteins; CW: middle membrane containing thin mucopeptide layers covalently linked to peptides and lipoproteins; CM: cytoplasmic membrane with phospholipid bilayers and proteins.**

limited transport and barrier functions. It contains porin, which allows the transport of selective small molecules. The resistance of Gram-negative bacteria to many enzymes (lysozyme, which hydrolyzes peptidoglycan), hydrophobic molecules (sodium dodecyl sulfate [SDS] and bile salts), and antibiotics (penicillin) is because of the barrier property of the OM. LPS molecules also have antigenic properties and are known as endotoxin. During infection with Gram-negative bacteria, LPS is responsible for a rise in body temperature (fever). Beneath the OM is the CW, composed of a thin layer of peptidoglycan or mucopeptide embedded in the periplasmic materials that contain several types of proteins, enzymes, and toxins. Beneath the periplasmic materials is the plasma or cytoplasmic membrane (CM) composed of a phospholipid bilayer in which many types of proteins are embedded.

Gram-positive cells (Figure 2.4) have a thick cell wall composed of several layers of peptidoglycan (mucopeptide, responsible for a thick, rigid structure) and two types of teichoic acids (teichoic acid and lipoteichoic acid). Peptidoglycan is a polymer consisting of *N*-acetyl muramic acid (NAM) and *N*-acetyl glucosamine (NAG) associated with short peptide chains. Some species also have a layer over the cell surface, called surface layer protein (SLP). The wall teichoic acid (TA) molecules are linked to mucopeptide layers, and the lipoteichoic acid (LTA) molecules are linked to both mucopeptide and cytoplasmic membrane. Teichoic acids are negatively charged (because of phosphate groups) and may bind to or regulate the movement of cationic molecules in and out of the cell. Teichoic acids have antigenic properties and can be used to identify Gram-positive bacteria serologically. Because of the complexity in the chemical composition of the cell wall, Gram-positive bacteria are considered to have evolved before Gram-negative bacteria.

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### Viruses

Viruses are regarded as noncellular entities or particles. Bacterial viruses (bacteriophages) important in food microbiology are widely distributed in nature.[5] They are composed of nucleic acids (DNA or RNA) and several proteins. The proteins forming the head (surrounding the nucleic acid) and tail are called capsids. Some also carry another layer called an envelope made of a lipid bilayer, and these viruses are called "enveloped viruses." Some viruses carry appendages or surface molecules for attachment to host cells. A bacteriophage attaches itself to the surface of a host bacterial cell and inoculates its nucleic acid into the host cell. Subsequently, many phages form inside a host cell and are released outside following lysis of the cell. This is discussed in Chapter 14.

Several pathogenic viruses have been identified as causing foodborne diseases in humans. The two most important viruses implicated in foodborne outbreaks are hepatitis A and norovirus. Both are single-stranded RNA viruses. Hepatitis A is a small, naked, polyhedral enteric virus approximately 30 nm in diameter. The RNA strand is enclosed in a capsid. In addition, other emerging viruses responsible for foodborne diseases include Hepatitis E virus (HEV) and Nipah virus. Viral diseases are discussed in Chapters 28 and 29.

## Important Microorganisms in Food

### Important Mold Genera

Molds are important in food because they can grow even in conditions in which many bacteria cannot grow, such as low pH, low water activity ($A_W$), and high osmotic pressure. Many types of molds are found in foods.[6,7] They are important spoilage microorganisms. Many strains also produce mycotoxins and have been implicated in foodborne intoxication (see Chapter 28). Some mycotoxins are carcinogenic or mutagenic and cause organ-specific pathology, such as hepatotoxic (liver toxicity) or nephrotoxic (kidney toxicity). Many are used in food bioprocessing. Finally, many are used to produce food additives and enzymes. Some of the most common genera of molds found in food are listed here (also see Figure 9.1).

1. *Aspergillus*. It is widely distributed and contains many species important in food. Members have septate hyphae and produce black-colored asexual spores on conidia. Many are xerophilic (able to grow in low $A_W$) and can grow in grains, causing spoilage. They are also involved in spoilage of foods such as jams, cured ham, nuts, and fruits and vegetables (rot). Some species or strains produce mycotoxins (e.g., *Aspergillus flavus* produces aflatoxin). Many species or strains are also used in food and food-additive processing. *Asp. oryzae* is used to hydrolyze starch by α-amylase in the production of sake. *Asp. niger* is used to process citric acid from sucrose and to produce enzymes such as β-galactosidase.
2. *Alternaria*. Members are septate and form dark-colored spores or conidia. They cause rot in tomatoes and rancid flavor in dairy products. Some species or strains produce mycotoxins (Alternaria toxin). Species: *Alternaria citri*.
3. *Fusarium*. Many types are associated with rot in citrus fruits, potatoes, and grains. They form cottony growth and produce septate, sickle-shaped conidia. They produce mycotoxins: fumonosins, zearalenone, tricothecenes, and dexoynivalenol (DON). Species: *Fusarium verticillioides*, *Fus. graminearum*, *Fus. proliferatum*, and so forth.

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4. *Geotrichum*. Members are septate and form rectangular arthrospores. They grow, forming a yeast-like cottony, creamy colony. They establish easily in equipment and often grow on dairy products (dairy mold). Species: *Geotrichum candidum*.
5. *Mucor*. It is widely distributed. Members have nonseptate hyphae and produce sporangiophores. They produce cottony colonies. Some species are used in food *Mucor rouxii*.
6. *Penicillium*. It is widely distributed and contains many species. Members have septate hyphae and form conidiophores on a blue-green, brush-like conidia head (Figure 2.2). Some species are used in food production, such as *Penicillium roquefortii* and *Pen. camembertii* in cheese. Many species cause fungal rot in fruits and vegetables. They also cause spoilage of grains, breads, and meat. Some strains produce mycotoxins (e.g., Ochratoxin A).
7. *Rhizopus*. Hyphae are aseptate and form sporangiophores in sporangium. They cause spoilage of many fruits and vegetables. *Rhizopus stolonifer* is the common black bread mold.

## Important Yeast Genera

Yeasts are important in food because of their ability to cause spoilage. Many are also used in food bioprocessing. Some are used to produce food additives. Several important genera are briefly described next.[4]

1. *Saccharomyces*. Cells are round, oval, or elongated. It is the most important genus and contains heterogenous groups (Figure 2.2). *Saccharomyces cerevisiae* variants are used in baking for leavening bread and in alcoholic fermentation. They also cause spoilage of food, producing alcohol and $CO_2$.
2. *Pichia*. Cells are oval to cylindrical and form pellicles in beer, wine, and brine to cause spoilage. Some are also used in oriental food fermentation. Species: *Pichia membranaefaciens*.
3. *Rhodotorula*. They are pigment-forming yeasts and can cause discoloration of foods such as meat, fish, and sauerkraut. Species: *Rhodotorula glutinis*.
4. *Torulopsis*. Cells are spherical to oval. They cause spoilage of milk because they can ferment lactose (e.g., *Torulopsis versatilis*). They also spoil fruit juice concentrates and acid foods.
5. *Candida*. Many species spoil foods with high acid, salt, and sugar and form pellicles on the surface of liquids. Some can cause rancidity in butter and dairy products (e.g., *Candida lipolyticum*).
6. *Zygosaccharomyces*. These cause spoilage of high-acid foods, such as sauces, ketchups, pickles, mustards, mayonnaise, and salad dressings, especially those with less acid and less salt and sugar (e.g., *Zygosaccharomyces bailii*).

## Foodborne Protozoan Parasites

Protozoan parasites[8,9] are eukaryotic cells and some are associated with water and foodborne outbreaks. Often wild animals, livestock, pets, or even humans carry these parasites and serve as a source. Contaminated soil and irrigation water are known causes for the contamination of fresh produce. The major organisms are *Giardia* (*Gia. lamblia*), *Cryptosporidium* (*Cry. parvum*), *Cyclospora* (*Cyc. cayetanensis*), *Cystoisospora* (*Cys. belli*), and *Toxoplasma* (*Tox. gondii*). Members of these organisms generally require two hosts to complete their life cycle. They produce an infective oocyst that generally contains two to four sporozoites. Both asexual and sexual reproduction allow

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their multiplication inside the host. These organisms cause typical gastroenteritis with abdominal cramps and sometimes muscle ache, fever, and headache.

## Important Viruses

Viruses are important in food for several reasons.[5,10–12]

1. Some are able to cause enteric disease and, thus, if present in a food, can cause foodborne diseases. Hepatitis A and norovirus have been implicated in foodborne outbreaks. Several other enteric viruses, such as poliovirus, adenovirus, echo virus, rota virus, saporovirus, hepatitis E virus, and Coxsackie virus, can cause foodborne diseases. In some countries where the level of sanitation is not very high, they can contaminate foods and cause disease.
2. Some bacterial viruses (bacteriophages) are used to detect and identify some pathogens (*Salmonella* serovars, *Listeria monocytogenes*, *Staphylococcus aureus*) on the basis of the sensitivity of the cells to a series of bacteriophages at appropriate dilutions.
3. Bacteriophages are used to transfer genetic traits in some bacterial species or strains by a process called transduction (e.g., in *Escherichia coli* or *Lactococcus lactis*).
4. Bacteriophages as biocontrol agents as antimicrobials to control pathogens such as *Listeria monocytogenes*, *Esc. coli* O157:H7, and *Salmonella* in foods or in food-production environments.
5. Finally, some bacteriophages can be very important because they can cause fermentation failure. Many lactic acid bacteria, used as starter cultures in food fermentation, are sensitive to different bacteriophages. They can infect and destroy starter-culture bacteria, causing product failure. Among the lactic acid bacteria, bacteriophages have been isolated for many species in the genera *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Lactobacillus*; no bacteriophage of *Pediococcus* is yet known. Methods are being devised to genetically engineer lactic starter cultures so that they become resistant to multiple bacteriophages (see Chapter 14).

## Important Bacterial Genera

Bacterial classification is changing rapidly.[1] In *Bergey's Manual of Systematic Bacteriology*, published between 1984 and 1988, more than 420 bacterial genera are listed in 33 sections on the basis of their differences in characteristics. Since then, many other genera have been created, such as *Lactococcus* (former N-group or dairy *Streptococcus*) and *Carnobacterium* (some species previously included in *Lactobacillus*). In the ninth edition of *Bergey's Manual of Determinative Bacteriology* (1993), more than 560 genera are listed in 35 groups. Of these, Table 2.1 lists 48 genera whose species are frequently associated with spoilage, health hazards, and bioprocessing of food. Species of other genera besides these 48 can also be found in food, but their relative significance is not well established. Many species names in several genera are also no longer valid and thus not included in the current *Bergey's Manual of Determinative Bacteriology*. In this text, only species and genera currently approved and listed in Bergey's Manual are used. Brief important characteristics of these genera and their importance in foods are described. Some descriptions are also presented in other chapters, such as pathogens in Chapters 25–29 and beneficial bacteria (bioprocessing) in Chapters 11 and 18.

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**Table 2.1 Genera of Bacteria Important in Foods**

| *Section*[a] *(Group*[b]*)* | *Description* | *Family* | *Genera* |
|---|---|---|---|
| 2 | Gram-negative, aerobic/ microaerophilic, motile, helical/vibrioid | Not indicated | *Campylobacter, Arcobacter, Helicobacter*[c] |
| 4 | Gram-negative, aerobic, rods and cocci | *Pseudomonadaceae* | *Pseudomonas, Xanthomonas* |
| | | *Acetobacteraceae* | *Acetobacter, Gluconobacter* |
| | | *Nisseriaceae* | *Acinetobacter, Morexella* |
| | | Not indicated | *Alteromonas, Flavobacterium, Alcaligenes, Brucella, Psychrobacter* |
| 5 | Gram-negative, facultative anaerobic, rods | *Enterobacteriaceae* | *Citrobacter, Cronobacter, Escherichia, Enterobacter, Edwardsiella, Erwinia, Hafnia, Klebsiella, Morganella, Proteus, Salmonella, Shigella, Serratia, Yersinia* |
| | | *Vibrionaceae* | *Vibrio, Aeromonas, Plesiomonas* |
| 9 | Rickettsias | *Rickettsiaceae* | *Coxiella* |
| 12 (17) | Gram-positive, cocci | *Micrococcaceae* | *Micrococcus, Staphylococcus* |
| | | Not indicated | *Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Pediococcus, Sarcina* |
| 13 (18) | Gram-positive, endospore-forming rods and cocci | Not indicated | *Bacillus, Sporolactobacillus, Clostridium, (Desulfotomaculum*[d]*)* |
| 14 (19) | Gram-positive, nonsporing, regular rods | Not indicated | *Lactobacillus, Carnobacterium, Brochothrix, Listeria* |
| 15 (20) | Gram-positive, nonsporing, irregular rods | Not indicated | *Corynebacterium, Brevibacterium, Propionibacterium, Bifidobacterium* |

[a] Sections in *Bergey's Manual of Systematic Bacteriology*.
[b] Groups in *Bergey's Manual of Determinative Bacteriology*. Only those sections (or groups) containing bacteria important in food are listed in this table.
[c] Included in this group and contain pathogenic species that can be foodborne.
[d] *Desulfotomaculum* cells stain Gram-negative.

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## Gram-Negative Aerobes

*Acetobacter*. Ellipsoid to rod-shaped (0.6 × 4 μm); occur singly or in short chains; motile or nonmotile; aerobes; oxidize ethanol to acetic acid; mesophiles. Cause souring of alcoholic beverages and fruit juices and used to produce vinegar (acetic acid). Can also spoil some fruits (rot). Widely distributed in plants and in places where alcohol fermentation occurs. Important species: *Acetobacter aceti*.

*Acinetobacter*. Rods (1 × 2 μm); occur in pairs or small chains; show twitching motility because of the presence of polar fimbriae; strictly aerobic and grow between 20°C and 35°C. Found in soil, water, and sewage. Important species: *Acinetobacter calcoaceticus*.

*Alcaligenes*. Rods or coccobacilli (0.5 × 1 μm); motile; present in water, soil, or fecal material; mesophiles. Cause spoilage of protein-rich foods. Species: *Alcaligenes faecalis*.

*Alteromonas*. Most currently assigned *Alteromonas* species are of marine origin and might be present in foods of marine origin. Need 100 mM NaCl for optimum growth (unlike *Pseudomonas*). Because *Alteromonas putrefacience* (species reclassified as *Shewanella putrefaciens*) has many characters similar to those of *Pseudomonas*, it was previously designated as *Pseudomonas putrefacience*. Strains important in fish and meat spoilage. Psychrotrophs.

*Brucella*. Coccobacilli (0.5 × 1.0 μm); mostly single; nonmotile. Different species cause disease in animals, including cattle, pigs, and sheep. They are also human pathogens and have been implicated in foodborne brucellosis. *Brucella abortus* causes abortion in cows.

*Campylobacter*. Two species, *Campylobacter jejuni* and *Cam. coli*, are foodborne pathogens. Small (0.2 × 1 μm) microaerophilic, helical, motile cells found in the intestinal tract of humans, animals, and birds. Mesophiles.

*Flavobacterium*. Rods with parallel sides (0.5 × 3 μm); nonmotile; colonies colored; some species psychrotrophic. Cause spoilage of milk, meat, and other protein foods. Species: *Flavobacterium aquatile*. *Gluconobacter*. Many characteristics of this group are similar to those of *Acetobacter*. *Gluconobacter oxydans* causes spoilage of pineapples, apples, and pears (rot).

*Moraxella*. Very short rods, frequently approaching coccoid shape (1 × 1.5 μm); occur singly, in pairs or short chains; may be capsulated; twitching motility may be present in some cells; optimum growth at 30°C–35°C. Found in the mucous membrane of animals and humans. Important species: *Moraxella lacunata*.

*Pseudomonas*. Straight or curved (0.5 × 5 μm); aerobes; motile rods; psychrotrophs (grow at low temperatures). Found widely in the environment. This genus includes large numbers of species. Some important species in foods are *Pseudomonas fluorescens*, *Pse. aeruginosa*, and *Pse. putida*. Important spoilage bacteria, can metabolize a wide variety of carbohydrates, proteins, and lipids in foods.

*Psychrobacter*. The genus was created in 1986 and contains one species: *Psychrobacter immobilis*. Coccobacilli (1 × 1.5 μm) and nonmotile. Can grow at 5°C or below, show optimum growth at 20°C, and are unable to grow at 35°C. Found in fish, meat, and poultry products.

*Xanthomonas*. Most characteristics of this group are similar to those for *Pseudomonas*. Plant pathogens, can thus cause spoilage of fruits and vegetables. *Xanthomonas campestris* strains used to produce xanthan gum, which is used as a food stabilizer.

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## Gram-Negative Facultative Anaerobes

*Aeromonas*. Small rods (0.5 × 1.0 μm); occur singly or in pairs; motile; psychrotrophs. Found in aquatic environments; generally a fish or marine pathogen. *Aeromonas hydrophila* has been suspected as a potential foodborne pathogen.

*Citrobacter*. Straight rods (1 × 4 μm); single or in pairs; usually motile; mesophiles. Found in the intestinal contents of humans, animals, and birds and in the environment. Included in the coliform group as an indicator of sanitation. Important species: *Citrobacter freundii*.

*Cronobacter*. (Previously known as *Enterobacter*) rods (1 × 3 μm): oxidase-negative, catalase-positive, facultatively anaerobic, motile (peritrichous flagella); mesophiles. Found in the intestinal contents of humans, animals, and birds and in the environment. Involved with powdered infant formula. Important species: *Cronobacter sakazakii*.

*Escherichia*. Straight rods (1 × 3 μm); motile or nonmotile; mesophiles. Found in the intestinal contents of humans, warm-blooded animals, and birds. Many strains are nonpathogenic, but some strains are pathogenic to humans and animals and involved in foodborne diseases. Used as an indicator of sanitation (theoretically nonpathogenic strains) in coliform and fecal coliform groups. Important species: *Escherichia coli*.

*Enterobacter*. Straight rods (1 × 2 mm); motile; mesophiles. Found in the intestinal contents of humans, animals, and birds and in the environment. Included in the coliform group as an indicator of sanitation. Important species: *Enterobacter aerogenes*.

*Edwardsiella*. Small rods (1 × 2 μm); motile. Found in the intestines of cold-blooded animals and in fresh water. Can be pathogenic to humans, but involvement in foodborne disease is not shown.

*Erwinia*. Small rods (1 × 2 μm); occur in pairs or short chains; motile; facultative anaerobes; optimum growth at 30°C. Many are plant pathogens and cause spoilage of plant products. Species: *Erwinia amylovora*.

*Hafnia*. Small rods (1 × 2 μm); motile; mesophiles. Found in intestinal contents of humans, animals, and birds and in the environment. Associated with food spoilage and may cause food poisoning. Species: *Hafnia alvei*.

*Klebsiella*. Medium rods (1 × 4 μm); occur singly or in pairs; motile; capsulated; mesophiles. Found in the intestinal contents of humans, animals, and birds; soil; water; and grains. Included in the coliform group as an indicator of sanitation. Important species: *Klebsiella pneumoniae*.

*Morganella*. Small rods (0.5 × 1 μm); motile; mesophiles. Found in the intestinal contents of humans and animals. Can be pathogenic but has not been implicated in foodborne disease. Species: *Morganella morganii*.

*Plesiomonas*. Small rods (0.5 × 1.0 μm); motile. Found in fish and aquatic animals. *Plesiomonas shigelloides* has been suspected as a potential foodborne pathogen.

*Proteus*. Straight, small rods (0.5 × 1.5 μm); highly motile; form swarms on agar media; some grow at low temperatures. Occur in the intestinal contents of humans and animals and the environment. Many involved in food spoilage. Species: *Proteus vulgaris*.

*Salmonella*. Medium rods (1 × 3 μm); usually motile; mesophiles. There are more than 2500 serovars, and all are regarded as pathogens. Found in the intestinal contents of humans, animals, birds, and insects. Major cause of foodborne diseases. Species: *Salmonella enterica* ssp. *enterica*. (See Chapter 26 for the new naming system.)

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*Shigella*. Medium rods; nonmotile; mesophiles. Found in the intestine of humans and primates. Associated with foodborne diseases. Species: *Shigella dysenteriae*.

*Serratia*. Small rods (0.5 × 1.5 μm); motile; colonies white, pink, or red; some grow at refrigerated temperature. Occur in the environment. Cause food spoilage. Species: *Serratia liquefaciens*.

*Yersinia*. Small rods (0.5 × 1 μm); motile or nonmotile; can grow at 1°C. Present in the intestinal contents of animals. *Yersinia enterocolitica* has been involved in foodborne disease outbreaks.

*Vibrio*. Curved rods (0.5 × 1.0 μm); motile; mesophiles. Found in freshwater and marine environments. Some species need NaCl for growth. Several species are pathogens and have been involved in foodborne disease (*Vibrio cholerae*, *Vib. parahaemolyticus*, and *Vib. vulnificus*), whereas others can cause food spoilage (*Vib. alginolyticus*).

## Rickettsia

*Coxiella*. Gram-negative; nonmotile; very small cells (0.2 × 0.5 μm); grow on host cells. Relatively resistant to high temperature (killed by pasteurization). *Coxiella burnetii* causes infection in cattle and has been implicated with Q fever in humans (especially on consuming unpasteurized milk).

## Gram-Positive Cocci

*Enterococcus*. Spheroid cells (1 μm); occur in pairs or chains; nonmotile; facultative anaerobes; some strains survive low heat (pasteurization); mesophiles. Normal habitat is the intestinal contents of humans, animals, and birds, and the environment. Can establish on equipment surfaces. Used as an indicator of sanitation. Important in food spoilage. Species: *Enterococcus faecalis*.

*Lactococcus*. Ovoid elongated cells (0.5–1.0 μm); occur in pairs or short chains; nonmotile; facultative anaerobes; mesophiles, but can grow at 10°C; produce lactic acid. Used to produce many bioprocessed foods, especially fermented dairy foods. Species: *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*; present in raw milk and plants, and several strains produce bacteriocins, some with a relatively wide host range against Gram-positive bacteria and have potential as food biopreservatives.

*Leuconostoc*. Spherical or lenticular cells; occur in pairs or chains; nonmotile; facultative anaerobes; heterolactic fermentators; mesophiles, but some species and strains can grow at or below 3°C. Some are used in food fermentation. Psychrotrophic strains are associated with spoilage (gas formation) of vacuum-packaged refrigerated foods. Found in plants, meat, and milk. Species: *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leu. lactis*, *Leu. carnosum*; *Leu. mesenteroides* subsp. *dextranicum* produces dextran while growing in sucrose. Several strains produce bacteriocins, some with a wide spectrum against Gram-positive bacteria, and these have potential as food biopreservatives.

*Micrococcus*. Spherical cells (0.2–2 μm); occur in pairs, tetrads, or clusters; aerobes; nonmotile; some species produce yellow colonies; mesophiles, resistant to low heat. Found in mammalian skin. Can cause spoilage. Species: *Micrococcus luteus*.

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*Pediococcus*. Spherical cells (1 μm); form tetrads; mostly present in pairs; nonmotile; facultative anaerobes; homolactic fermentators; mesophiles, but some can grow at 50°C; some survive pasteurization. Some species and strains are used in food fermentation. Some can cause spoilage of alcoholic beverages. Found in vegetative materials and in some food products. Species: *Pediococcus acidilactici* and *Ped. pentosaceus*. Several strains produce bacteriocins, some with a wide spectrum against Gram-positive bacteria, and they can be used as food biopreservatives.

*Sarcina*. Large, spherical cells (1–2 μm); occur in packets of eight or more; nonmotile; produce acid and gas from carbohydrates; facultative anaerobes. Present in soil, plant products, and animal feces. Can be involved in spoilage of foods of plant origin. Species: *Sarcina maxima*.

*Staphylococcus*. Spherical cells (0.5–1 μm); occur singly, in pairs, or in clusters; nonmotile; mesophiles; facultative anaerobes; grow in 10% NaCl. *Staphylococcus aureus* strains are frequently involved in foodborne diseases. *Sta. carnosus* is used for processing some fermented sausages. Main habitat is skin of humans, animals, and birds.

*Streptococcus*. Spherical or ovoid (1 μm); occur in pairs or chains; nonmotile; facultative anaerobes; mesophiles. *Streptococcus pyogenes* is pathogenic and has been implicated in foodborne diseases; present as commensals in human respiratory tract. *Str. thermophilus* is used in dairy fermentation; can be present in raw milk; can grow at 50°C.

## Gram-Positive, Endospore-Forming Rods

*Bacillus*. Rod-shaped, straight cells; vary widely in size (small, medium, or large; 0.5–1 × 2–10 μm) and shape (thick or thin); single or in chains; motile or nonmotile; mesophiles or psychrotrophic; aerobes or facultative anaerobes; all form endospores that are spherical or oval and large or small (one per cell); spores are highly heat resistant. Includes many species, some of which are important in foods, because they can cause foodborne disease (*Bacillus cereus*) and food spoilage, especially in canned products (*Bac. coagulans*, *Bac. stearothermophilus*). Enzymes of some species and strains are used in food bioprocessing (*Bac. subtilis*). Present in soil, dust, and plant products (especially spices). Many species and strains can produce extracellular enzymes that hydrolyze carbohydrates, proteins, and lipids.

*Clostridium*. Rod-shaped cells that vary widely in size and shape; motile or nonmotile; anaerobes (some species extremely sensitive to oxygen); mesophiles or psychrotrophic; form endospores (oval or spherical) usually at one end of the cell, some species sporulate poorly, spores are heat resistant. Found in soil, marine sediments, sewage, decaying vegetation, and animal and plant products. Some are pathogens and important in food (*Clostridium botulinum*, *Clo. perfringens*) and others are important in food spoilage (*Clo. tyrobutyricum*, *Clo. saccharolyticum*, *Clo. laramie*). Some species are used as sources of enzymes to hydrolyze carbohydrates and proteins in food processing.

*Sporolactobacillus*. Slender, medium-sized rods (1 × 4 μm); motile; microaerophilic; homolactic fermentors; form endospores (spore formation is rare in most media), but the spores are less heat resistant than *Bacillus* spores. Found in chicken feed and soil. Importance in food is not clearly known. Species: *Sporolactobacillus inulinus*.

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## Gram-Negative, Endospore-Forming Rods

*Desulfotomaculum*. One species important in food is *Desulfotomaculum nigrificans*. The medium-sized cells are rod-shaped, motile, thermophilic, strictly anaerobes, and produce H2S. Endospores are oval and resistant to heat. Found in soil. Cause spoilage of canned food.

## Gram-Positive, Nonsporulating Regular Rods

*Brochothrix*. Similar in many characteristics to lactobacilli; facultative anaerobes; homofermentative; nonmotile; found in meat. It can grow in refrigerated vacuum-packaged meat and meat products. Species: *Brochothrix thermosphacta*.

*Carnobacterium*. Similar in many characteristics to lactobacilli cells; found in meat and fish; facultative anaerobes; heterofermentative; nonmotile; can grow in foods, especially in meat products; stored at refrigerated temperature. Some strains produce bacteriocins. Species: *Carnobacterium piscicola*.

*Listeria*. Short rods (0.5 × 1 μm); occur singly or in short chains; motile; facultative anaerobes; can grow at 1°C; cells killed by pasteurization. The species are widely distributed in the environment and have been isolated from different types of foods. Some *Listeria monocytogenes* strains are important foodborne pathogens.

*Lactobacillus*. Rod-shaped cells that vary widely in shape and size, some are very long, whereas others are coccobacilli, appear in single or in small and large chains; facultative anaerobes; most species are nonmotile; mesophiles (but some are psychrotrophs); can be homo- or heterolactic fermentors. Found in plant sources, milk, meat, and feces. Many are used in food bioprocessing (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lab. helveticus*, *Lab. plantarum*) and some are used as probiotics (*Lab. acidophilus*, *Lab. reuteri*, *Lab. casei* subsp. *casei*). Some species can grow at low temperatures in products stored at refrigerated temperature (*Lab. sake*, *Lab. curvatus*). Several strains produce bacteriocins, of which some having a wide spectrum can be used as food biopreservatives.

## Gram-Positive, Non-Spore-Forming Irregular Rods

*Bifidobacterium*. Rods of various shapes; present singly or in chains; arranged in V or starlike shape; nonmotile; mesophiles; anaerobes. Metabolize carbohydrates to lactate and acetate. Found in colons of humans, animals, and birds. Some species are used in probiotics (*Bifidobacterium bifidum*, *Bif. infantis*, *Bif. adolescentis*).

*Brevibacterium*. Cells can change from rod to coccoid shape; aerobes; nonmotile; mesophiles. Two species, *Brevibacterium linens* and *Bre. casei*, have been implicated in the development of the aroma in several cheese varieties (surface ripened) because of the production of sulfur compounds (such as methanethiol). In other protein-rich products, they can cause spoilage (in fish). They are found in different cheeses and raw milk.

*Corynebacterium*. Slightly curved rods; some cells stain unevenly; facultative anaerobes; nonmotile; mesophiles; found in the environment, plants, and animals. Some species cause food spoilage. *Corynebacterium glutamicum* is used to produce glutamic acid.

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*Propionibacterium*. Pleomorphic rods (0.5 × 2 μm); can be coccoid, bifid, or branched; present singly or in short chains; V and Y configuration and in clumps with Chinese-character-like arrangement; nonmotile; anaerobes; mesophiles. Dairy propionibacteria are used in food fermentation (*Propionibacterium freudenreichii* in Swiss cheese). Produce proline and propionic acid. Found in raw milk, Swiss cheese, and silage.

### Some New Genera

On the basis of nucleic acid homology studies, several new genera have been created from the existing genera. Some that are important in food microbiology are listed here. Most of their characteristics are similar to those of the species in the respective old genera. Examples include *Tetragenococcus* (from *Pediococcus*), *Vagococcus* (from N-group *Streptococcus*), *Weissella* and *Oenococcus* (both from *Leuconostoc*), *Kocuria* (from *Micrococcus*), *Shewanella* (from *Pseudomonas*), and *Alicyclobacillus* (from *Bacillus*).

## Important Bacterial Groups in Foods

Among the microorganisms found in foods, bacteria constitute major important groups. This is not only because many different species can be present in foods, but also because of their rapid growth rate, ability to utilize food nutrients, and ability to grow under a wide range of temperatures, aerobiosis, pH, and water activity as well as to better survive adverse situations, such as survival of spores at high temperatures. For convenience, bacteria important in foods have been arbitrarily divided into several groups on the basis of similarities in certain characteristics. This grouping does not have any taxonomic significance. Some of these groups and their importance in foods are listed here.

### *Lactic Acid Bacteria*

These bacteria produce relatively large quantities of lactic acid from carbohydrates. Species mainly from genera *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, and *Streptococcus thermophilus* are included in this group.

### *Acetic Acid Bacteria*

These are bacteria that produce acetic acid, such as *Acetobacter aceti*.

### *Propionic Acid Bacteria*

These bacteria produce propionic acid and are used in dairy fermentation. Species such as *Propionibacterium freudenreichii* are included in this group.

### *Butyric Acid Bacteria*

These bacteria produce butyric acid in relatively large amounts. Some *Clostridium* spp., such as *Clostridium butyricum*, are included in this group.

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### Proteolytic Bacteria

These bacteria can hydrolyze proteins because they produce extracellular proteinases. Species in genera *Micrococcus*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Alcaligenes*, some in *Enterobacteriaceae*, and *Brevibacterium* are included in this group.

### Lipolytic Bacteria

These bacteria hydrolyze triglycerides because they produce extracellular lipases. Species in genera *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Alteromonas*, and *Flavobacterium* are included in this group.

### Saccharolytic Bacteria

These are bacteria that are able to hydrolyze complex carbohydrates. Species in genera *Bacillus*, *Clostridium*, *Aeromonas*, *Pseudomonas*, and *Enterobacter* are included in this group.

### Thermophilic Bacteria

These are bacteria that are able to grow at 50°C and above. Species from genera *Bacillus*, *Clostridium*, *Pediococcus*, *Streptococcus*, and *Lactobacillus* are included in this group.

### Psychrotrophic Bacteria

These are bacteria that are able to grow at refrigerated temperature (≤5°C). Some species from *Pseudomonas*, *Alteromonas*, *Alcaligenes*, *Flavobacterium*, *Serratia*, *Bacillus*, *Clostridium*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Brochothrix*, *Listeria*, *Yersinia*, and *Aeromonas* are included in this group.

### Thermoduric Bacteria

They are bacteria that are able to survive pasteurization temperature treatment. Some species from *Micrococcus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Bacillus* (spores), and *Clostridium* (spores) are included in this group.

### Halotolerant Bacteria

They are bacteria that are able to survive high salt concentrations (≥10%). Some species from *Bacillus*, *Micrococcus*, *Staphylococcus*, *Pediococcus*, *Vibrio*, and *Corynebacterium* are included in this group.

### Aciduric Bacteria

These are bacteria that are able to survive at low pH (<4.0). Some species from *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, and *Streptococcus* are included in this group.

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### Osmophilic Bacteria

These are bacteria that can grow in a relatively higher osmotic environment than what is needed for other bacteria. Some species from genera *Staphylococcus*, *Leuconostoc*, and *Lactobacillus* are included in this group. They are much less osmophilic than yeasts and molds.

### Gas-Producing Bacteria

These are bacteria that produce gas ($CO_2$, $H_2$, $H_2S$) during metabolism of nutrients. Species from genera *Leuconostoc*, *Lactobacillus*, *Propionibacterium*, *Escherichia*, *Enterobacter*, *Clostridium*, and *Desulfotomaculum* are included in this group.

### Slime Producers

These are bacteria that produce slime because they synthesize polysaccharides. Some species or strains from *Xanthomonas*, *Leuconostoc*, *Alcaligenes*, *Enterobacter*, *Lactococcus*, and *Lactobacillus* are included in this group.

### Spore Formers

These are bacteria having the ability to produce spores. Species from *Bacillus*, *Clostridium*, and *Desulfotomaculum* are included in this group. They are further divided into aerobic spore formers, anaerobic spore formers, flat sour spore formers, thermophilic spore formers, and sulfide-producing spore formers.

### Aerobes

These are bacteria that require oxygen for growth and multiplication. Species from *Pseudomonas*, *Bacillus*, and *Flavobacterium* are included in this group.

### Anaerobes

These are bacteria that cannot grow in the presence of oxygen. Species from *Clostridium* are included in this group.

### Facultative Anaerobes

These are bacteria that are able to grow in both the presence and absence of oxygen. *Lactobacillus*, *Pediococcus*, *Leuconostoc*, enteric pathogens, and some species of *Bacillus*, *Listeria*, *Salmonella*, *Serratia*, and coliforms are included in this group.

### Coliforms

Species from *Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella* are included in this group. They are used as an index of sanitation.

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### Fecal Coliforms

Mainly *Escherichia coli* is included in this group. They are also used as an index of sanitation.

### Enteric Pathogens

Pathogenic *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Escherichia*, *Vibrio*, hepatitis A, and others that can cause gastrointestinal infection are included in this group.

Because of the importance of these bacterial groups in food, many laboratory methods are designed to detect a specific group instead of a specific genus or species. Similarly, control methods are sometimes designed to destroy or prevent growth of a specific group.

## Conclusion

Two major aspects have been discussed in this chapter: the nomenclature system of bacteria, which is rapidly changing because of the development of new molecular biology techniques, and the morphological and physiological characteristics of the microorganisms important in food. A food microbiologist should be knowledgeable about these facets. Chapter 3 discusses the sources of these microorganisms in food.

**QUESTIONS**

1. List five methods that are used in the classification of bacteria. Why are nucleotide sequences in 16S rRNA used as an important technique in the classification?
2. Explain the following terms and give one example for each in relation to bacterial nomenclature: (a) family, (b) genus, (c) species, (d) subspecies, and (e) biovar (use scientific method in the examples). Give the plural of *Lactobacillus*, *Staphylococcus*, *Enterococcus*, *Leuconostoc*, *Listeria*, and *Salmonella*.
3. List the general differences in the morphology of yeasts, molds, bacteria, and bacteriophages important in food.
4. List the differences in the chemical nature and function of cell wall structures between Gram-positive and Gram-negative bacteria. How does this help determine the Gram characteristics of an unknown bacterial isolate?
5. List four species of molds and two species of yeasts most important in food.
6. List four protozoan species that are responsible for water and foodborne outbreaks.
7. Discuss the importance of bacteriophages in food. Briefly explain how prions are different from the bacteriophages.
8. List two genera from each of the following groups: (a) Gram-negative aerobic rods, (b) Gram-negative facultative anaerobic rods, (c) Gram-positive cocci, (d) Gram-positive endospore-forming rods, and (e) Gram-positive nonsporulating rods.
9. Briefly mention the common characteristics used to group bacterial genera or species in the following groups and give one example of genus for each: (a) lactic acid bacteria, (b) coliforms, (c) proteolytic bacteria, (d) psychrotrophic bacteria, and (e) enteric pathogens.

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# *Chapter 3*

# Sources of Microorganisms in Foods

## Introduction

The internal tissues of healthy plants (fruits and vegetables) and animals (meat) are essentially sterile. Yet raw and processed (except sterile) foods contain different types of molds, yeasts, bacteria, and viruses. Microorganisms get into foods from both natural (including internal) sources and from external sources to which a food comes into contact from the time of production until the time of consumption. Natural sources for foods of plant origin include the surfaces of fruits, vegetables, nuts, grains, and spices, and the damaged tissues and the pores in some tubers (e.g., radish and onion). Natural sources for foods of animal origin include skin, hair, feathers, the gastrointestinal tract, the urogenital tract, the respiratory tract, and milk ducts (teat canal) in the udders of milk animals. Natural microflora exist in ecological balance with their hosts, and their types and levels vary greatly with the type of plants and animals as well as their geographical locations and environmental conditions. Besides natural microorganisms, a food can be contaminated with different types of microorganisms coming from outside sources, such as air, soil, sewage, water, feed, humans, food ingredients, equipment, packages, and insects. Microbial types and their levels from these sources getting into foods vary widely and depend on the degree of sanitation used during the handling of foods.

An understanding of the sources of microorganisms in food is important to develop methods to control access of some microorganisms to the food, to develop processing methods to kill them in food, and to determine the microbiological quality of food as well as to set up microbiological standards and specifications of foods and food ingredients (Figure 3.1). The predominant types of microorganisms that can get into food from each of these sources and methods to reduce the levels of microorganisms are briefly discussed here.



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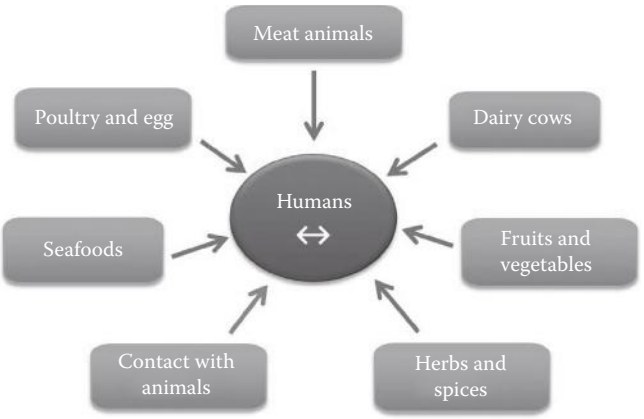



**Figure 3.1 Diagram showing various sources for foodborne pathogens. ↔, indicates person-to-person transmission.**

## Predominant Microorganisms in Different Sources

### *Plants (Fruits and Vegetables)*

The inside tissue of foods from plant sources are essentially sterile, except for a few porous vegetables (e.g., radishes and onions) and leafy vegetables (e.g., cabbage and Brussels sprouts).[1–3] Some plants produce natural antimicrobial metabolites that can limit the presence of microorganisms. Fruits and vegetables harbor microorganisms on the surface; their type and level vary with soil condition, type of fertilizers and water used, and air quality. Molds, yeasts, lactic acid bacteria, and bacteria from genera *Pseudomonas*, *Alcaligenes*, *Micrococcus*, *Erwinia*, *Bacillus*, *Clostridium*, and *Enterobacter* can be expected from this source. Pathogens, especially of enteric types (*Salmonella*, *Escherichia coli*, *Campylobacter*, *Shigella*, *Cyclospora*, *Giardia*), can be present if the soil is contaminated with untreated sewage. Diseases of the plants, damage of the surface (before, during, and after harvest), long delay between harvesting and washing, and unfavorable storage and transport conditions after harvesting and before processing can greatly increase microbial numbers as well as predominant types. Improper storage conditions following processing can also increase their numbers.

Proper methods used during growing (such as use of treated sewage or other types of fertilizers), damage reduction during harvesting, quick washing with good-quality water to remove soil and dirt, and storage at low temperatures before and after processing can be used to reduce microbial load in foods of plant origin.

### *Animals, Birds, Fish, and Shellfish*

Food animals and birds normally carry many types of indigenous microorganisms in the digestive, respiratory, and urogenital tracts; the teat canal in the udder; and in the skin, hooves, hair, and feathers.[4–6] Their numbers, depending on the specific organ, can be very high (large intestinal contents can have as high as $10^{12}$ microbes/g). Many, as carriers, can harbor pathogens, such as *Salmonella* serovars, pathogenic *Escherichia coli*, *Campylobacter jejuni*, *Yersinia enterocolitica*, and *Listeria monocytogenes*, without showing symptoms. Laying birds have been suspected

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of asymptomatically carrying *Salmonella* Enteritidis in their ovaries and contaminating the yolk during ovulation. Disease situations, such as mastitis in cows and intestinal, respiratory, and uterine infections as well as injury can change the ecology of normal microflora. Similarly, poor husbandry resulting in fecal contamination on the body surface (skin, hair, feathers, and udder) and supplying contaminated water and feed (e.g., contaminated with salmonellae) can also change their normal microbial flora. In addition, stress in farm animals during transportation or resulting from environmental conditions also can increase the pathogen load in animals before slaughter and contribute to increased foodborne diseases.[6]

Fish and shellfish also carry normal microflora in their scales, skin, and digestive tracts. Water quality, feeding habits, and diseases can change the normal microbial types and levels. Pathogens, such as *Vibrio parahaemolyticus*, *Vib. vulnificus*, and *Vib. cholerae*, are of major concern from these sources. Many spoilage and pathogenic microorganisms can get into foods of animal origin (milk, eggs, meat, and fishery products) during production and processing. Milk can be contaminated by fecal materials on the udder surface, eggshells with fecal material during laying, meat with the intestinal contents during slaughtering, and fish with intestinal contents during processing. Because of its specific nature, contamination of foods of animal sources with fecal materials is viewed with concern (possible presence of enteric pathogens).

In addition to enteric pathogens from fecal materials, meat from food animals and birds can be contaminated with several spoilage and pathogenic microorganisms from skin, hair, and feathers, namely *Staphylococcus aureus*, *Micrococcus* spp., *Propionibacterium* spp., *Campylobacter*, *Corynebacterium* spp., and molds and yeasts.

Prevention of food contamination from these sources needs the use of effective husbandry of live animals and birds, which include good housing, avoiding overcrowding, a supply of uncontaminated food and water, and prevention of access to wild animals and birds. Also, testing animals and birds for pathogens and culling the carriers are important in reducing the incidence of pathogenic microorganisms in foods. Thorough cleansing using good-quality water for washing carcasses (preferably with acceptable antimicrobial agents); hair removal; feather removal; careful removal of digestive, urogenital, and respiratory organs without contaminating tissues; removal of contaminated parts; and proper sanitation during the entire processing stage are necessary during slaughter to keep microbial quantity and quality at desirable levels. Proper cleaning of the udder before milking, cooling milk immediately after milking, processing as soon as possible, and sanitization at all stages are important to keep microbial levels low in milk. Eggs should be collected soon after laying and washed and stored as per recommended procedures.

Fish and marine products should be harvested from unpolluted and recommended water. Proper sanitation should be used during processing. They should be stored properly to prevent further contamination and microbial growth. Ice to be used for storage should be produced from potable water.

## Air

Microorganisms are present in dust and moisture droplets in the air. They do not grow in dust but are transient and variable, depending on the environment. Their levels are controlled by the degree of humidity, size, and level of dust particles, temperature and air velocity, and resistance of microorganisms to drying. Generally, dry air with low dust content and higher temperature has a low microbial level. Spores of *Bacillus* spp., *Clostridium* spp., and molds and cells of some Gram-positive bacteria (e.g., *Micrococcus* spp. and *Sarcina* spp.), as well as yeasts, can be predominantly present in air. If the surroundings contain a source of pathogens (e.g., animal and poultry farms or

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a sewage-treatment plant), different types of bacteria, including pathogens and viruses (including bacteriophages), can be transmitted via the air.

Microbial contamination of food from the air can be reduced by removing the potential sources, controlling dust particles in the air (using filtered air), using positive air pressure, reducing humidity levels, and installing UV light.

## Soil

Soil, especially the type used to grow agricultural produce and raise animals and birds, contains several varieties of microorganisms. Because microorganisms can multiply in soil, their numbers can be very high (billions/g). Many types of molds, yeasts, and bacterial genera (e.g., *Enterobacter*, *Pseudomonas*, *Proteus*, *Micrococcus*, *Enterococcus*, *Bacillus*, and *Clostridium*) can enter foods from the soil. Soil contaminated with fecal materials can be the source of enteric pathogenic bacteria and viruses in food. Sediments in which fish and marine foods are harvested can also be a source of microorganisms, including pathogens, in those foods. Different types of parasites can also get in food from soil. Removal of soil (and sediments) by washing and avoiding soil contamination can reduce microorganisms in foods from this source.

## Sewage and Manure

Sewage, especially when used as fertilizer in crops, can contaminate food with microorganisms, the most significant of which are different enteropathogenic bacteria and viruses. This can be a major concern with organically grown food and many imported fruits and vegetables, in which untreated sewage and manure might be used as fertilizer. Pathogenic parasites can also get into food from sewage.

To reduce incidence of microbial contamination of foods from sewage, it is better not to use sewage as fertilizer. If used, it should be efficiently treated to kill the pathogens. Also, effective washing of foods following harvesting is important.

## Water

Water is considered "environmental commons" and essential in the entire food production chain and can spread disease easily. Water is used to produce, process, and, under certain conditions, store foods. It is used for irrigation of crops, drinking by food animals and birds, raising fishery and marine products, washing foods, processing (pasteurization, canning, and cooling of heated foods) and storage of foods (e.g., fish on ice), washing and sanitation of equipment, and processing and transportation facilities. Water is also used as an ingredient in many processed foods. Thus, water quality can greatly influence the microbial quality of foods. Contamination of foods with pathogenic bacteria, viruses, and parasites from water has been recorded.

Wastewater can be recycled for irrigation. However, chlorine-treated potable water (drinking water) should be used in processing, washing, sanitation, and as an ingredient. Although potable water does not contain coliforms and pathogens (mainly enteric types), it can contain other bacteria capable of causing food spoilage, such as *Pseudomonas*, *Alcaligenes*, and *Flavobacterium*. Improperly treated water can contain pathogenic and spoilage microorganisms. To overcome the problems, many food processors use water, especially as an ingredient, that has a higher microbial quality than that of potable water.

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### Humans

Between production and consumption, foods come in contact with different people handling the foods. They include not only people working on farms and in food-processing plants, but also those handling foods at restaurants, catering services, retail stores, and at home. Human carriers have been the source of pathogenic microorganisms in foods that later caused foodborne diseases, especially with ready-to-eat foods. Improperly cleaned hands, lack of aesthetic sense and personal hygiene, and dirty clothes and hair can be major sources of microbial contamination in foods. The presence of minor cuts and infection on the hands and face and mild generalized diseases (e.g., flu, strep throat, or hepatitis A in an early stage) can amplify the situation. In addition to spoilage bacteria, pathogens, such as *Staphylococcus aureus*, *Salmonella* serovars, *Shigella* spp., pathogenic *Esc. coli*, norovirus, and hepatitis A, can be introduced into foods from human sources sometimes through fecal-oral contamination.

Proper training of personnel in personal hygiene, regular checking of health, and maintaining efficient sanitary and aesthetic standards are necessary to reduce contamination from this source.

### Food Ingredients

In prepared or fabricated foods, many ingredients or additives are included in different quantities. Many of these ingredients can be the source of both spoilage and pathogenic microorganisms. Various spices generally have very high populations of mold and bacterial spores. Starch, sugar, and flour might have spores of thermophilic bacteria. Pathogens have been isolated from dried coconut, eggs, nuts, flours, and chocolate, and many of these ingredients were responsible for foodborne outbreaks.

The ingredients should be produced under sanitary conditions and given antimicrobial treatments. In addition, setting up acceptable microbial specifications for the ingredients will be important in reducing microorganisms in food from this source.

### Equipment

A wide variety of equipment is used in harvesting, transporting, slaughtering, processing, and storing foods. Many types of microorganisms from air, raw foods, water, and personnel can get into the equipment and contaminate foods. Depending on the environment (moisture, nutrients, and temperature) and time, microorganisms can multiply and, even from a low initial population, reach a high level and contaminate large volumes of foods. Also, when processing equipment is used continuously for a long period of time, microorganisms initially present can multiply and act as a continuous source of contamination in the product produced subsequently. In some equipment, small parts, inaccessible sections, and certain materials might not be efficiently cleaned and sanitized. These dead spots can serve as sources of both pathogenic and spoilage microorganisms in food. Small equipment, such as cutting boards, knives, spoons, and similar articles, because of improper cleaning, can be sources of cross contamination. *Salmonella*, *Listeria*, *Escherichia*, *Enterococcus*, *Micrococcus*, *Pseudomonas*, *Lactobacillus*, *Leuconostoc*, *Clostridium*, *Bacillus* spp., and yeasts and molds can get into food from equipment (see Chapter 21).

Proper cleaning and sanitation of equipment at prescribed intervals are important to reduce microbial levels in food. In addition, developing means to prevent or reduce contamination from air, water, personnel, and insects is important. Finally, in designing the equipment, potential microbiological problems need to be considered.

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### Miscellaneous

Foods might be contaminated with microorganisms from several other sources, namely packaging and wrapping materials, containers, flies, vermin, birds, house pets, and rodents. Many types of packaging materials are used in food. Because they are used in products ready for consumption and, in some cases, without further heating, proper microbiological standards (or specifications) for packaging materials are necessary. Any failure to produce microbiologically acceptable products can reduce the quality of food. Flies, vermin, birds, and rodents in food processing and food preparation and storage facilities should be viewed with concern as they can carry pathogenic microorganisms. House pets can also harbor pathogens; proper care should be taken not to contaminate food from these source.

## Conclusion

Microorganisms enter foods from both internal and external sources. Their levels and types depend on the care used during production, processing, and storage of foods. Recontaminations of heat-processed foods with pathogens have been associated with many foodborne disease outbreaks. Proper sanitation at every stage helps reduce the microbial level normally expected in a food. Normal microbiological quality of foods is discussed in Chapter 4.

**QUESTIONS**

1. Briefly discuss how an understanding of the microbial sources in food can be helpful to a food microbiologist.
2. List five major sources of foodborne pathogens in foods and indicate the measures that should be implemented to reduce their incidence in foods.
3. A *Salmonella* outbreak from the consumption of a national brand of ice cream, involving more than 50,000 consumers, was found to be related to contamination of heat-treated ice cream mix with raw liquid egg containing the pathogen. Both products were transported by the same truck at different times. Briefly indicate how this could have been avoided.
4. A batch of turkey rolls (10 lb—approximately 4.5 Kg—each) were cooked to 165°F internal temperature in bags, opened, sliced, vacuum-packaged, and stored at 40°F. The product was expected to have a refrigerated shelf life of 50 days. However, after 40 days, the packages contained gas and approximately $10^7$ bacterial cells/g of meat. The bacterial species involved in the spoilage was found to be *Leuconostoc carnosum*, which is killed at 165°F. What could be the sources of the bacterial species in this cooked product?
5. Consumption of vacuum-packaged refrigerated hotdogs has been frequently incriminated with listeriosis although the internal temperature used for cooking the raw products (160°F) is high enough to kill the pathogen. Discuss the possible sources of the pathogen in the cooked products.
6. A child consumed watermelon in a restaurant and was thereafter infected with *Esc. coli* O157:H7. Investigations revealed that the knife used to slice the melon was also used to cut raw beef (and then washed). What could have been done to prevent the situation?

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# Chapter 4

# Normal Microbiological Quality of Foods and Its Significance

## Introduction

Although many types (genera, species, strains) of microorganisms are present in nature, under normal conditions, a food generally harbors only a few types. These types include those that are naturally present in raw foods (which provide the ecological niche) and those that enter from outside sources to which the foods are exposed from the time of production until consumption. The relative numbers (population level) of a specific type of microorganism initially present (without growth) in a food depend on the intrinsic and extrinsic conditions to which the food is exposed. If growth occurs, the predominant types will be the ones for which the optimum growth condition is present in the food. This aspect is discussed in Chapter 6. The objective of this chapter is to develop an understanding of the microbial types (and their levels where possible) that can be expected under normal conditions in different food groups. It has to be recognized that the microbial load in a food results from initial contamination from different sources and growth of the contaminants before testing; normally, it is difficult to separate the two.

## Raw and Ready-to-Eat Meat Products

Following slaughter and dressing, the carcasses of animals and birds contain many types of microorganisms, predominantly bacteria, coming from the skin, hair, feathers, gastrointestinal tract, and so forth; the environment of the feedlot and pasture (feed, water, soil, and manure); and the environment at the slaughtering facilities (equipment, air, water, and humans). Normally, carcasses contain an average of $10^{1-3}$ bacterial cells/in$^2$. Different enteric pathogens, *Salmonella*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Escherichia coli*, *Clostridium perfringens*, and *Staphylococcus aureus*, both from animals or birds and humans, can be present, but normally at a low level.



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Carcasses of birds, as compared with those of animals, generally have a higher incidence of *Salmonella* contamination coming from fecal matter.[1–3]

Following boning, chilled raw meat and ground meat contain microorganisms coming from the carcasses as well as from different equipment used during processing, personnel, air, and water. Some of the equipment used can be important sources of microorganisms, such as conveyors, grinders, slicers, and similar types that can be difficult to clean. Chilled meat has mesophiles, such as *Micrococcus*, *Enterococcus*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Lactobacillus*, coliforms, and other *Enterobacteriaceae*, including enteric pathogens. However, because the meats are stored at low temperatures (–1°C to 5°C), the psychrotrophs constitute major problems. The predominant psychrotrophs in raw meats are some lactobacilli and leuconostocs, *Brochothrix thermosphacta*, *Clo. laramie*, some coliforms, *Serratia*, *Pseudomonas*, *Alteromonas*, *Achromobacter*, *Alcaligenes*, *Acinetobacter, Moraxella, Aeromonas*, and *Proteus*. Psychrotrophic pathogens include *Listeria monocytogenes* and *Yer. enterocolitica*. The microbial load of fresh meat varies greatly with bacteria predominating. Ground meat can have $10^{4–5}$ microorganisms/g; *Salmonella* can be present at approximately 1 cell/25 g. As indicated previously, the frequency of the presence of *Salmonella* is higher in chicken than in red meats. If the products are kept under aerobic conditions, psychrotrophic aerobes will grow rapidly, especially Gram-negative rods, such as *Pseudomonas*, *Alteromonas*, *Proteus*, and *Alcaligenes*, as well as yeasts. Under anaerobic packaging, growth of psychrotrophic facultative anaerobes and anaerobes (e.g., *Lactobacillus*, *Leuconostoc*, *Brochothrix*, *Serratia*, some coliforms, and *Clostridium*) predominate. The pH of the meat (which is low in beef, approximately 5.6, but high in birds, approximately 6.0), high protein content, and low carbohydrate level, along with the environment, determine the types that predominate during storage.

Low-heat-processed red meat and poultry products include perishable cured or uncured products that have been subjected to heat treatment to approximately 160°F (71°C), packaged aerobically or anaerobically, and stored at refrigerated temperature. They include products such as franks, bologna, lunchmeats, and hams. The products, especially those packaged anaerobically and cured, are expected to have a long storage life (50 days or more). The microbial sources before heat treatment include the raw meat, ingredients used in formulation, processing equipment, air, and personnel. Heat treatment, especially at an internal temperature of 160°F or higher, kills most microorganisms except for some thermodurics, which include *Micrococcus*; some *Enterococcus*; and maybe some *Lactobacillus* and spores of *Bacillus* and *Clostridium*. The microbial level can be $10^{1–2}$/g. Following heating, the products, some of which are further processed (such as removing casings or slicing), come in contact with equipment, personnel, air, and water before final packaging. Different types of bacteria, yeasts, and molds, including pathogens, can enter these products, depending on the conditions of the processing plants. Although the initial bacterial level normally does not exceed $10^2$/g, some of them can be psychrotrophic facultative anaerobic and anaerobic bacteria (*Lactobacillus*, *Leuconostoc*, some coliforms, *Serratia*, *Listeria*, *Clostridium* spp.). During extended storage in vacuum or modified-air packages, even from a low initial level, the bacterial population can rise and adversely affect the safety and shelf life of products. This is aggravated by fluctuation in storage temperature and in products having low fat, high pH, and high $A_W$.

## Raw and Pasteurized Milk

Raw milk comes from cows, buffalo, sheep, and goats although the largest volume comes from cows. Pasteurized or market milk includes whole, skim, low-fat, and flavored milks, as well as cream, which are heat treated (pasteurized) according to regulatory specifications.[4,5] Milk is high

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in proteins and carbohydrates (lactose), which many microorganisms can utilize for growth. Because both raw milk and pasteurized milk contain many types of bacteria as predominant microorganisms, they are refrigerated, yet they have limited shelf life.

In raw milk, microorganisms come from inside the udder, animal body surfaces, feed, air, water, and equipment used for milking and storage. The predominant types from inside a healthy udder are *Micrococcus*, *Streptococcus*, and *Corynebacterium*. Normally, raw milk contains $<10^3$ microorganisms/mL. If a cow has mastitis, *Streptococcus agalactiae*, *Sta. aureus*, coliforms, and *Pseudomonas* can be excreted in relatively high numbers. Contaminants from animals, feed, soil, and water predominantly have lactic acid bacteria; coliforms; *Micrococcus*, *Staphylococcus*, *Enterococcus*, *Bacillus*, and *Clostridium* spores; and Gram-negative rods. Pathogens, such as *Salmonella*, *Mycobacterium bovis*, *Lis. monocytogenes*, *Yersinia enterocolitica*, *Brucella abortus*, *Coxiella burnetii*, Shiga toxigenic *Escherichia coli* (STEC), and *Cam. jejuni*, can also come from some of these sources and from milk. Equipment can be a major source of Gram-negative rods, such as *Pseudomonas*, *Alcaligenes*, and *Flavobacterium*, as well as Gram-positive *Micrococcus* and *Enterococcus*.

During refrigerated storage (at dairy farms and processing plants) before pasteurization, only psychrotrophs can grow in raw milk. They include *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, and some coliforms and *Bacillus* spp. They can affect the acceptance quality of raw milk (e.g., by making flavor and texture undesirable). Some of them can produce heat-stable enzymes (proteinases and lipases), which can also affect the product quality, even after pasteurization of raw milk (see Chapter 21). Psychrotrophic pathogens (*Lis. monocytogenes* and *Yer. enterocolitica*) can multiply in refrigerated raw milk during storage.

The microbiological quality of raw and pasteurized milk is monitored in many countries by regulatory agencies. In the United States, the standard plate counts of raw milk for use as market milk are $1–3 \times 10^5$/mL and for use in product manufacturing are $0.5–1 \times 10^6$/mL. Grade A pasteurized milk can have standard plate counts of 20,000/mL and ≤10 coliforms/mL.

Microorganisms present in pasteurized milk are those that survive the pasteurization of raw milk (e.g., the thermodurics) and those that enter after heating and before packaging (i.e., post-pasteurization contaminants). Thermodurics surviving pasteurization include *Micrococcus*, some *Enterococcus* (e.g., *Ent. faecalis*), *Streptococcus*, some *Lactobacillus* (e.g., *Lab. viridescens*), and spores of *Bacillus* and *Clostridium*. Postheat contaminants can be coliforms as well as *Pseudomonas*, *Alcaligenes*, and *Flavobacterium*. Some heat-sensitive pathogens can also enter pasteurized milk following heat treatment. Psychrotrophs can grow during refrigerated storage.

## Shell Eggs and Liquid Egg

Shell eggs are contaminated with microorganisms on the outer surface from fecal matter, nesting materials, feed, air, and equipment.[6,7] Eggshells, depending on the contamination level, can have $10^7$ bacteria. Washing helps reduce the bacterial level considerably. Eggshells can harbor different types of bacteria, namely *Pseudomonas*, *Alcaligenes*, *Proteus*, *Citrobacter*, *Esc. coli*, *Enterobacter*, *Enterococcus*, *Micrococcus*, and *Bacillus*. They can also have *Salmonella* from fecal contamination. Infected ovaries of laying hens can be the source of *Salmonella enterica* serovar Enteritidis in the yolk. Liquid egg can be contaminated with bacteria from the shell of washed eggs as well as from the breaking equipment, water, and air. Pasteurization can reduce the numbers to $10^3$cells/mL. Bacteria, especially motile Gram-negative bacteria, can enter through the pores of eggshells, particularly if the shells are wet. Several antimicrobial factors present in egg albumin, such as lysozyme, conalbumin (binds iron), avidin (binds biotin), or alkaline pH (8.0–9.0), can control

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bacterial growth. However, if the storage temperature is favorable, they can grow in yolk that is rich in nutrients and has a pH of 7.0. Pasteurization of liquid egg has been designed to destroy pathogens (especially *Salmonella*) and other Gram-negative rods. Thermoduric bacteria, namely *Micrococcus*, *Enterococcus*, and *Bacillus*, present in the raw liquid egg survive pasteurization.

## Fish and Shellfish

This group includes finfish, crustaceans (shrimp, lobster, crabs), and mollusks (oysters, mussels, clams, scallops) harvested from aquatic environments (marine and freshwater).[8] Fish and shellfish are harvested from natural sources and aquacultures. In general, they are rich in protein and nonprotein nitrogenous compounds; their fat content varies with type and season. Except for mollusks, they are very low in carbohydrates; mollusks contain about 3% glycogen.

The microbial population in these products varies greatly with the pollution level and temperature of the water. Bacteria from many groups, as well as viruses, parasites, and protozoa and helminths, can be present in the raw materials. Muscles of fish and shellfish are sterile, but scales, gills, and intestines harbor microorganisms. Finfish and crustaceans can have $10^{3-8}$ bacterial cells/g. During feeding, mollusks filter large volumes of water and can thus concentrate bacteria and viruses. Products harvested from marine environments can have halophilic vibrios as well as *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Enterococcus*, *Micrococcus*, coliforms, and pathogens, such as *Vib. parahaemolyticus*, *Vib. vulnificus*, *Shigella* spp. and *Clo. botulinum* type E. Freshwater fish generally have *Pseudomonas*, *Flavobacterium*, *Enterococcus*, *Micrococcus*, *Bacillus*, and coliforms. Fish and shellfish harvested from water polluted with human and animal waste can contain *Salmonella*, *Shigella*, *Clo. perfringens*, *Vib. cholerae*, and hepatitis A and norovirus. They can also contain opportunistic pathogens, such as *Aeromonas hydrophila* and *Plesiomonas shigelloides*. The harvesting of seafoods, especially shellfish, is controlled by regulatory agencies in the United States. Water with high coliform populations is forbidden to harvest. Protozoan species, such as *Giardia* spp., and large, worm-like parasites, such as nematodes, trematodes, and cestodes, are also associated with seafoods (see Chapter 28).

If harvested from polluted water, microorganisms can grow rapidly in fish and crustaceans because of high $A_W$, high pH of the tissue, and availability of large amounts of nonprotein nitrogenous compounds. As many of the bacterial species are psychrotrophs, they can grow at refrigerated temperatures. Pathogens can remain viable for a long time during storage. Microbial loads are greatly reduced during their subsequent heat processing to produce different products.

## Vegetables, Fruits, and Nuts

Vegetables include edible plant components, such as leaves (i.e., spinach, lettuce, cilantro), stalks (i.e., celery, sprouts), roots, tubers, bulbs, and flowers. In general, they are relatively high in carbohydrates, with pH values of 5.0–7.0. Thus, different types of bacteria, yeasts, and molds can grow if other conditions are favorable.[9] Fruits are high in carbohydrates and have a pH of 4.5 or below because of the presence of organic acids, and some also have antimicrobial essential oils. Nuts can be from the ground (peanuts) or from trees (pecans) and have protective shells and low $A_W$ (0.7). They are converted to nutmeats for further use or to products, such as peanut butter.

Fruits and vegetables are perceived as healthy, and these are consumed raw or minimally processed. However, recently, these products have been responsible for numerous fatal outbreaks,

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and their association with outbreaks is on the rise.[9] Microorganisms in vegetables can come from several sources, such as soil, water, air, wild or domestic animals, insects, birds, or equipment, and vary with the types of vegetables. A leafy vegetable has more microorganisms from the air, whereas a tuber has more from the soil. Microbial levels and types in these products also vary greatly, depending on environmental conditions and conditions of farming and harvesting. Generally, vegetables have $10^{3-5}$ microorganisms/cm$^2$ or $10^{4-7}$/g. Some of the predominant bacterial types are lactic acid bacteria, *Corynebacterium*, *Enterobacter*, *Proteus*, *Pseudomonas*, *Micrococcus*, *Enterococcus*, and spore formers. They also have different types of molds, such as *Alternaria*, *Fusarium*, and *Aspergillus*. Vegetables can have enteric pathogens, especially if animal and human wastes and polluted water are used for fertilization and irrigation. They include *Lis. monocytogenes*, *Salmonella*, *Shigella*, pathogenic *Eschrichia coli* (O157:H7 and O104:H4), *Campylobacter*, *Clo. botulinum*, and *Clo. perfringens*. They can also have pathogenic protozoa and parasites (*Cyclospora*, *Cystoisospora*, *Giardia*). If the vegetables are damaged, then plant pathogens (e.g., *Erwinia*) can also predominate. Many of the microorganisms can cause different types of spoilage (different types of rot) of raw products. Pathogens can grow in plant products and cause foodborne diseases (e.g., listeriosis or botulism). Lactic acid bacteria have important roles in the natural fermentation of vegetables (e.g., sauerkraut). Different methods used to process vegetables and vegetable products greatly reduce the microbial population.

Fruits, because of their high carbohydrate content and low pH, favor the growth of different types of molds, yeasts, and lactic acid bacteria. Microorganisms generally come from air, soil, insects, and harvesting equipment. In general, microbial populations are $10^{3-6}$/g. Improperly harvested and processed fruits can have pathogens that survive, grow, and cause foodborne disease. Molds, yeasts, and bacteria can cause different types of spoilage. Natural flora, especially yeasts in fruits, can be important in alcohol fermentation.

Microorganisms enter nuts from the soil (peanuts) and air (tree nuts). During processing, air, equipment, and water can also be the sources. Nuts are protected by shells, but damage to the shell can facilitate microbial contamination. Raw nuts and nutmeats can have $10^{3-4}$ microorganisms/g with *Bacillus* and *Clostridium* spores, *Leuconostoc*, *Pseudomonas*, and *Micrococcus* predominating. Because of a low $A_W$, bacteria do not grow in the products. However, when used as ingredients, they can cause microbiological problems in the products. Molds can grow in nuts and nutmeats and produce mycotoxins (from toxin-producing strains, such as aflatoxins by *Aspergillus flavus*).

## Cereal, Starches, and Gums

Cereal includes grains, flour, meals, breakfast cereals, pasta, baked products, dry mixes, and frozen and refrigerated products of cereal grains (also beans and lentils). Starches include flours of cereals (e.g., corn, rice), tapioca (from a plant), potatoes, and other tubers. Gums are used as stabilizers, gelling agents, and films and are obtained from plants, seaweeds, and microorganisms (e.g., tragacanth, pectin, xanthan, agar, and carrageenan) and as modified compounds (e.g., carboxymethyl cellulose). They are rich in amylose and amylopectin but can also have simple sugars (e.g., in grains) and protein (e.g., in lentils). Microbial sources are mainly the soil, air, insects, birds, and equipment.

Unprocessed products (grains) may contain high bacterial levels (aerobic plate count $\leq 10^4$/g, coliform $\leq 10^2$/g, yeasts and molds $\leq 10^3$/g). They may also contain mycotoxins produced by toxicogenic molds. Processed products may also contain a wide variety of yeasts, molds, and bacteria. Flours and starches may have higher microbial counts, similar to those of grains, whereas

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processed products (such as breakfast cereals and pasta) may contain an aerobic plate count of $10^{2-3}$/g, coliform of $<10^{1-2}$/g, and yeasts and molds of $<10^{1-2}$/g. They can contain bacterial spores and psychrotrophs. Some pathogens, such as *Salmonella*, *Sta. aureus*, and *Clo. perfringens*, have also been isolated. Depending on the product, they can either grow (such as in dough) or be the source (when used as ingredients) of spoilage and pathogenic microorganisms as well as mycotoxins. Gums also may be the source of yeasts, molds (also mycotoxins), bacterial spores, and lactic acid bacteria.

## Canned Foods

This group of foods includes those packed in hermetically sealed containers and given high heat treatment. The products with a pH of 4.6 or above are given heat treatments to obtain commercial sterility, but those with a pH below 4.6 are given heat treatments of approximately 100°C.

Canned foods prepared and processed to obtain commercial sterility can have spores of thermophilic spoilage bacteria, namely *Bacillus stearothermophilus*, *Clo. thermosaccharolyticum*, and *Desulfotomaculum nigrificans*. Their major sources in the products are soil and blanching water as well as sugar and starches that are used as ingredients. In canned products stored at 30°C or below, thermophilic spores do not germinate to cause spoilage. However, if the cans are temperature-abused to 40°C or higher, the spores germinate; subsequently, the cells multiply and spoil the products.

If the canned products are given a lower heat treatment (approximately 100°C), spores of mesophilic bacteria that include both spoilage (*Bac. coagulans*, *Bac. licheniformis*, *Clo. sporogenes*, *Clo. butyricum*) and pathogenic types (*Bac. cereus*, *Clo. perfringens*, *Clo. botulinum*), along with the spores of thermophiles, survive. In low-pH products, particularly in tomato products, *Bac. coagulans* spores can germinate, and cells can multiply and cause spoilage. Other spore formers can germinate and grow in high-pH products. *Sta. aureus* toxins, if present in raw products, are not destroyed by the heat treatment of the canned products and can thus cause food poisoning following consumption of the products.

## Sugars and Confectioneries

Refined sugar is obtained from sugarcane and beets. Sugar can have thermophilic spores of *Bac. stearothermophilus*, *Bac. coagulans*, *Clo. thermosaccharolyticum*, and *Des. nigrificans* as well as mesophilic bacteria (e.g., *Lactobacillus* and *Leuconostoc*), yeasts, and molds.[10] When sugars are used as ingredients in food products, the spores can survive and cause spoilage of products. Pathogens are not present in refined sugar unless contaminated. In liquid sugar, mesophiles can grow. Refined sugar, used in canned products or to make liquid sugar, has strict microbiological standards (for spores).

Confectioneries include a large variety of products with a sweet taste. In general, these products have low $A_W$ (0.84 or less) and some have low pH. They may contain many types of bacteria, yeasts, and molds, but their microbiological standards are well regulated. Although they may harbor *Lactobacillus*, *Leuconostoc*, spores of *Bacillus* and *Clostridium*, and yeasts and molds, only a few osmotolerant yeasts and molds can grow. However, when used as additives in other foods, confectioneries can be a source of these microbes. If ready-to-consume products are contaminated with pathogens, either from raw materials, the environment, or personnel, they can cause foodborne diseases.

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## Soft Drinks, Fruit and Vegetable Drinks, Juices, and Bottled Water

Soft drinks are nonalcoholic beverages containing water, sweeteners, acids, flavoring, coloring and emulsifying agents, and preservatives. Some may contain fruit juices and be carbonated or noncarbonated and have a pH of 2.5–4.0. Fruit juices (100%) have a pH of 4.0 or below. Vegetable juices (e.g., tomato) can have a pH of 4.5 or above. Bottled water is obtained from either natural springs or drilled wells and handled under conditions that prevent contamination.

Soft drinks can have different types of microorganisms, but only aciduric microorganisms, such as molds, yeasts, lactic acid bacteria, and acetic acid bacteria, can multiply.[11] In carbonated beverages, some yeasts are microaerophilic and can grow; in beverages with fruit juices, *Lactobacillus* and *Leuconostoc* species can grow. In noncarbonated beverages, molds (*Geotrichum*) and *Acetobacter* and *Gluconobacter* spp. can also grow. Most of these come from the processing environment and equipment. In fruit juices, molds, yeasts, *Lactobacillus* spp. (*Lactobacillus fermentum*, *Lactobacillus plantarum*), *Leuconostoc* spp. (*Leu. mesenteroides*), and acetic acid bacteria can grow. Spoilage of fruit juices by acid-resistant spore-forming species from genus *Alicyclobacillus* has currently been recognized. Some pathogens (e.g., acid-tolerant *Salmonella* and *Esc. coli* O157:H7 strains in orange juice and apple cider) can remain viable for a long time (≥30 days) in the acid products.[11] Vegetable juices can have molds, yeasts, and lactic acid bacteria along with *Bac. coagulans*, *Clo. butyricum*, and *Clo. pasteurianum*.

Bottled water should not contain more than 10–100 bacteria and >10 coliforms/100 mL. The indigenous flora are mainly *Flavobacterium*, *Alcaligenes*, and *Micrococcus*. They may also have some *Pseudomonas* as contaminants from outside. They should not have pathogens unless produced under poor sanitation.

## Mayonnaise and Salad Dressings

Water-in-oil emulsion products formulated with oil, water, vinegar (approximately 0.25% acetic acid) or lemon juice, sugar, salt, starch, gum, egg, spices, and vegetable pieces, such as mayonnaise and salad dressings, have a pH between 3.5 and 4.0. Some low-calorie and less sour products containing less acid, less oil, and more water may have a pH of 4.5 or above.

Microorganisms are introduced into the products through ingredients, equipment, and air. However, except for aciduric microorganisms, most others die, especially when stored for a long time at room temperature.[12] Among aciduric microorganisms, molds (*Geotrichum* and *Aspergillus* spp.), yeasts (*Saccharomyces* spp.), and several species of *Lactobacillus* (*Lab. fructivorans*, *Lab. brevis*) and some *Bacillus* spp. (*Bac. subtilis*, *Bac. mesentericus*) have been isolated. Normally, their numbers should not exceed 10/g. If pathogens are introduced (e.g., *Salmonella* through eggs), they are expected to be killed rapidly; however, they may survive longer in low-calorie, high-pH products kept at refrigerated temperatures.

## Spices and Condiments

Spices are plant products (seeds, flowers, leaves, bark, roots, or bulbs) used whole or ground, singly or mixed. The top 12 spices used in the United States include dehydrated onion/garlic, mustard seed, red pepper, sesame seeds, black pepper, paprika, cinnamon, cumin seeds, white pepper, oregano, poppy seeds, and ginger. Other spices include allspice, anise, basil, bay leaves, cloves,

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fennel seeds, sage, thyme, and turmeric. Typically, the moisture levels for spices range from 8% to 12%.[13] Condiments are spices blended with other components and have a sauce-like consistency (catsup, mustard). They are used in relatively small amounts for aroma and color.

Some spices, unless given antimicrobial treatments, may contain microorganisms as high as $10^{6-7}$/g. The most important are spores of molds, such as *Aspergillus*, *Fusarium*, *Alternaria*, *Penicillium, Rhizopus*, *Cladosporium*, and *Trichoderma*, and bacterial spores, such as *Bacillus*, and *Clostridium* spp. Also, micrococci, enterococci, yeasts, and several pathogens, such as *Salmonella enterica* serovars, *Staphylococcus aureus*, and *Bacillus cereus*, have been found. Untreated spices may have mold counts of $10^4$ CFU/g or higher. They can also have mycotoxins. Although used in small amounts, they can be the source of spoilage and pathogenic microorganisms in food. Some spices, such as cloves, allspice, and garlic, have antimicrobial properties.[13]

Because spices are becoming a major source for pathogens that have contributed to several outbreaks, various processing treatments are employed to reduce pathogens. Those include ionizing (gamma irradiation) and nonionizing (UV, microwave and infrared radiation) radiation, and fumigation with ethylene oxide (ETO) gas.[14,15] In addition, the use of heat treatment and high-pressure $CO_2$ (supercritical $CO_2$) have been proposed.[15,16] According to the American Spice Trade Association (ASTA), approximately, 40%–85% of spices are treated with ethylene oxide in the United States; however, use of ETO is prohibited in Europe and Australia because of a concern about toxicity. ETO-mediated toxicity could be minimized by using appropriate dosage and exposure time. In addition, efforts are made by the spice growers and processors to minimize the risk of mold growth and spoilage through the use of Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), and improvement in the storage environment.

## Conclusion

A normal microbial population in a food comes from those that enter from different sources as well as from growth of the contaminants before a food is examined. It is expected that a food that is produced under proper sanitary conditions and preserved properly will have a lower microbial load. Information on normal microbial load helps in determining the microbiological quality of a food and also in setting up microbiological standards and specifications. Mere microbial presence does not reduce the quality of food except in the case of some pathogens. It is necessary for microorganisms to grow or multiply in a food to bring definite changes in quality. This is discussed in Chapter 5.

### QUESTIONS

1. List the psychrophilic microorganisms in raw meat. Discuss the significance of psychrophilic and facultative anaerobic microorganisms in raw chilled meats.
2. Discuss the sources of postheat contamination of low-heat-processed meat products by bacteria and their significance on the quality of these products.
3. List and discuss the significance of (a) psychrotrophic bacteria in raw milk, (b) high thermoduric bacteria in raw milk to be used for pasteurized grade A milk, and (c) >10 coliforms/mL in pasteurized grade A milk.
4. Discuss the significance of *Salmonella* contamination with emphasis on the presence of *Salmonella* Enteriditis in shell eggs.
5. "A large volume of seafoods consumed in the United States and other developed countries are obtained from countries where the level of sanitation is not very stringent." Explain how

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the situation in this statement can affect the microbiological quality and microbiological safety of these seafoods.

6. Many vegetables are eaten raw. Discuss what microbiological concerns the consumer should have for these vegetables.
7. Molds can grow on cereal grains, peanuts, and spices at different stages of their production and processing. What concerns should consumers, regulatory agencies, and food processors have for the use of these products in foods?
8. Discuss the normal microbial quality of soft drinks, fruit juices, vegetable juices, and bottled water.
9. "Low-pH products, such as mayonnaise, can cause foodborne disease." Describe under what conditions this is possible.
10. List the microorganisms that are able to survive in properly processed canned foods and discuss their significance as to the product quality.
11. Some food ingredients are generally used in small quantities in the preparation of processed foods. Even then, their microbiological quality is considered important for the quality of the processed product. Name two such ingredients and briefly discuss why their microbiological quality is important.
12. Briefly explain the importance of knowing the normal microbiological quality of food.
13. What methods are used for controlling microbes in spices?

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# II

# MICROBIAL GROWTH RESPONSE IN THE FOOD ENVIRONMENT

Microorganisms present in raw and processed (nonsterile and commercially sterile) foods are important for their involvement in foodborne diseases, food spoilage, and food bioprocessing. These are generally accomplished through the growth of microorganisms (except for viruses and protozoa) in foods. Growth or cell multiplication of bacteria, yeasts, and molds is influenced by the intrinsic and extrinsic environments of the food. Microbial growth is also facilitated through the metabolism of some food components that provide the needed energy and cellular materials and substrates for many byproducts. Microbial growth in laboratory media is also important for quantitative and qualitative detection of the microbiological quality of a food. In this section, the nature of microbial growth and influencing factors associated with microbial growth are discussed. Microbial attachment and biofilm formation on food processing equipment and food contact surfaces are important strategies for microbial survival and persistence. The process and importance of spore formation by some bacterial species and alteration in characteristics of bacterial cells under stress are also discussed. An understanding of these factors is helpful in designing methods to control (as against spoilage and health hazard), stimulate (as in bioprocessing), or detect the microorganisms in food. In this section, the following aspects are discussed:

Chapter 5: Microbial Growth Characteristics
Chapter 6: Factors Influencing Microbial Growth in Food
Chapter 7: Microbial Attachments and Biofilm Formation
Chapter 8: Microbial Metabolism of Food Components
Chapter 9: Microbial Sporulation and Germination
Chapter 10: Microbial Stress Response in the Food Environment

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# Chapter 5

# Microbial Growth Characteristics

## Introduction

Microorganisms grow or multiply in numbers when exposed to a favorable environment, such as food. Their growth is associated with food spoilage, foodborne diseases, and food bioprocessing. Growth is also important in isolating an unknown microbial strain involved in food spoilage, foodborne diseases, or food bioprocessing in pure form and studying its morphological, physiological, biochemical, and genetic characteristics in order to design methods to control or stimulate its growth in food, destroy it, or improve its genetic makeup for better use.

## Microbial Reproduction or Growth

### *Binary Fission*

An increase in the number or mass of vegetative cells of bacteria, yeasts, and molds is customarily used to reflect growth for microorganisms. Bacteria reproduce by a process called transverse binary fission or, simply, binary fission. The process includes several steps, such as DNA replication and separation, partition between the DNA, invagination and septum formation of a wall, and cell separation. In this process, one cell asexually divides into two cells, each an essentially true replica of the original cell. However, when growing for a long time, the population from a single cell may contain a few variant cells. In bacteria, a form of sexual recombination can occur that involves transfer of genetic materials from a donor to a recipient cell (e.g., conjugation). However, this is quite different from sexual reproduction, which is facilitated through the union of two cells (gametes) of opposite mating type.[1,2]

A bacterial cell has a specific surface area-to-volume ($s/v$) ratio. A newly divided cell has a higher ($s/v$) ratio, which helps in the rapid transport of nutrients from the environment. A young cell predominantly uses nutrients to synthesize energy and cellular components, leading to an increase in cell size. As the cell size increases, the ($s/v$) ratio decreases, which adversely affects the transport of



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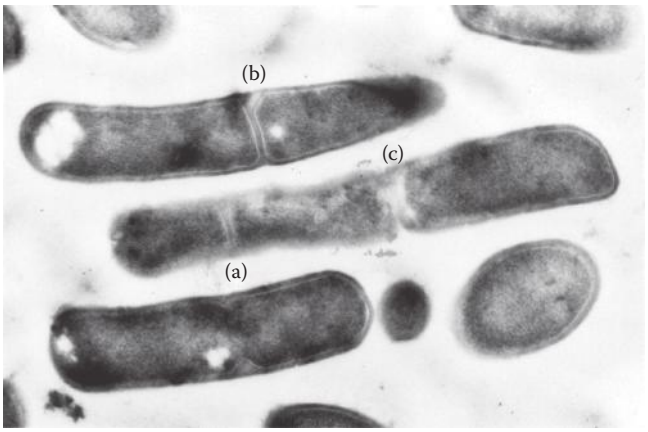



**Figure 5.1 Photograph of thin sections of *Lactobacillus* cells by transmission electron microscopy showing cell wall formation during cell division at earlier stage (a), later stage (b), and final stage with partial separation of cells (c).**

nutrients into and byproducts out of the cell. To increase the (*s*/*v*) ratio, the cell initiates division by forming constriction on the cell surface, followed by formation of a transverse wall, separating the cellular materials (especially the genetic materials) equally between two cells (Figure 5.1). The division can occur in one or more planes, depending on the species and the arrangement of the cells. After division, the two cells may separate from each other. In some species, the two cells remain attached to each other and continue to divide in one plane (e.g., in *Streptococcus* and *Bacillus* spp.). If the cells divide into two or more planes and the cells remain close to each other, they form a cluster (e.g., in *Staphylococcus* spp.). In some species, such as *Pediococcus*, cell division occurs in two or more planes, and the cells have a tendency to stay together for some time, giving rise to four- (tetrad) or eight-cell arrangements.

Yeasts and molds can also reproduce asexually. A yeast cell produces a bud that initially is much smaller in size and remains attached to the surface of the original cell. As it grows in size, it also can produce a bud, giving the appearance of a chain of buds growing on the surface of the original yeast cell. A yeast cell can have several buds forming on its surface. As the buds mature, they separate from the original cell. Molds can grow in size by cell division or elongation at the tip of a hypha. They can also form large numbers of asexual spores on specialized structures. Both yeasts and molds can also reproduce sexually. Viruses do not reproduce by themselves. Instead, they attach on the surface of and inject their nucleic acid inside specific host cells, which then replicate the viral nucleic acid and produce viral particles. The viral particles are released into the environment following lysis of the host cells. Viruses associated with foodborne diseases do not increase in numbers by replication in food. However, if the specific host bacterial cells and the bacteriophages are present in a food, replication can occur.

## *Generation Time (or Doubling Time)*

The time that a single cell takes to divide into two is called generation time.[1] However, in practice, generation time is referred to as the doubling time for the entire population. In a population of a microbial species, not all cells divide at the same time or at the same rate. The generation time of a

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microbial species under different conditions provides valuable information for developing methods to preserve foods. In general, under optimum conditions of growth, bacteria have the shortest generation time, followed by yeasts and molds. Also, among bacterial species and strains, generation time under optimum conditions varies greatly; some species, such as *Vibrio parahaemolyticus*, under optimum conditions, can have a generation time as low as 10–12 minutes. Generally, in food systems, microorganisms have longer generation times than in a nutritionally rich bacteriological broth.

The generation time of a microbial population can be calculated mathematically from the differences in population during a given time period. Because of large numbers, the calculation is done in logarithmatics ($\log_{10}$) by using the following formula:

$$G = \frac{0.3t}{\log_{10} z - \log_{10} x}$$

in which $G$ is generation time (with time unit in minutes; also expressed as doubling time, $t_d$, with time unit usually in hours), 0.3 is a constant (value of $\log_{10} 2$ and indicates doubling), $t$ is the duration of study (minutes), $\log_{10} x$ is initial, and $\log_{10} z$ is the final cell numbers per milliliter or colony-forming units (CFUs), per milliliter. For example, if, under a given growth condition, the initial population of $10^4$ cells/mL of a bacterial species increases to $10^6$ cells/mL in 120 minutes, its generation time will be

$$G = \frac{0.3 \times 120}{6 - 4} = 18\,\text{min}$$

For the same bacterial strain, this value changes by changing the growth conditions.

## *Specific Growth Rate*

The rate of growth of microorganisms during the exponential growth phase can also be determined mathematically by measuring cell numbers, $OD_{600}$ nm, cell mass (wet or dry weight), or cell constituents (proteins, RNA, or DNA). If $N_0$ at time $t_0$ and $N_t$ at time $t$ are the quantity and time of any of these components, respectively; the growth rate of microorganisms is determined by the equation:

$$N_t = N_0 e^{\mu(t - t_0)}$$

in which μ is the specified growth rate in unit time (e.g., $h^{-1}$). The equation can be logarithmically expressed as follows:

$$\ln \frac{N_t}{N_0} = \mu(t - t_0)$$

$$\log N_t - \log N_0 = \frac{\mu}{2.3}(t - t_0)$$

$$\mu = \frac{2.3(\log N_t - \log N_0)}{t - t_0}$$

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$\mu(h^{-1})$ varies with microbial types, species, and the growth environment. Normally, it is approximately 0.2 for molds and yeasts. A fast-growing bacterial strain under optimum conditions can have a $\mu(h^{-1})$ of 2.5 or higher. Under nonoptimal growth conditions, $\mu(h^{-1})$ can range between 0.2–0.02. The value of doubling time ($t_d$) can also be determined from the $\mu$ value by the relationship $t_d/h = 0.69/\mu$ (0.69 is the value of In2). These equations are important for determining predictable growth rates and population levels (or of other components) in fermentation and shelf life of foods.

## Optimum Growth

Many environmental parameters of food, such as storage temperature, acidity (pH), water activity ($A_W$), oxidation-reduction (O-R) potential, and nutrients, influence the microbial growth rate. This aspect is discussed in Chapter 6. If one of the factors (e.g., temperature) is varied, keeping all other parameters constant during the growth of a microbial strain, and its growth rate is measured, it is evident that the growth rate is fastest (or generation time is shortest) at a certain temperature. This temperature is referred to as the optimum growth temperature for the strain under a given condition. The growth rate slows down on either side of the optimum growth temperature until the growth stops. The area under the two points on both sides of an optimum growth condition when minimum growth occurs is the growth temperature range. When the cells of a microbial species are exposed to a factor (e.g., temperature) beyond the growth range, the cells not only stop growing but, depending on the situation, may be injured or may lose viability. The growth range and optimum growth of a microorganism under a specific parameter provide valuable information for its inhibition, reduction, or the stimulation of growth in a food.

## Growth Curve

The growth rate and growth characteristics of a microbial population under a given condition can be graphically represented by counting cell numbers, enumerating CFUs, or measuring optical density in a spectrophotometer at a given wavelength (above 300 nm, usually at 600 nm) of a cell suspension. Cell mass or specific cell components, such as proteins, RNA, or DNA, can also be measured to determine growth rate. Each method has several advantages and disadvantages. If the CFU values are enumerated at different times of growth and a growth curve is plotted using $\log_{10}$ CFU versus time ($\log_{10}$ CFU is used because of high cell numbers), a plot similar to the one presented in Figure 5.2 is obtained. The plot has several features that represent the conditions of the cells at different times. Initially, the population does not change (lag phase). During this time, the cells assimilate nutrients and increase in size. Although the population remains unchanged because of a change in size, both cell mass and optical density show some increase. Following this, the cell number starts increasing, first slowly and then very rapidly. The cells in the population differ initially in metabolic rate and only some multiply, and then almost all cells multiply. This is the exponential phase (also called the logarithmic phase). The growth rate at the exponential phase follows first-order reaction kinetics and can be used to determine generation time. Following this, the growth rate slows down, and finally, the population enters the stationary phase. At this stage, because of nutrient shortage and accumulation of waste products, a few cells die, and a few cells multiply, keeping the living population stable. However, if one counts the cells under a microscope or measures cell mass, both may show an increase as dead cells may remain intact. After the stationary phase, the population enters the death phase in which the rate of cell death is higher than the rate of cell multiplication. Depending on the strain and conditions of the environment, after a long period of time (which may even be a few years) some cells may still remain viable. This

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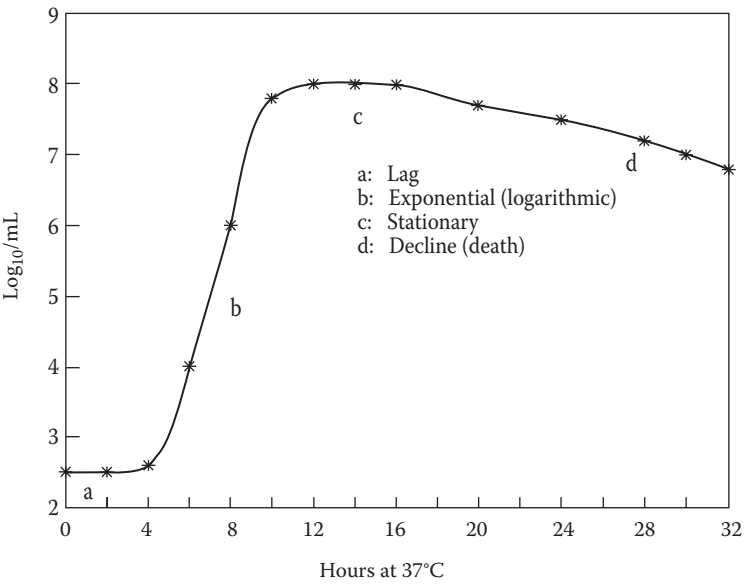



**Figure 5.2 Bacterial growth curve showing changes in cell numbers of *Pediococcus acidilactici* H during 32 hours of incubation at 37°C in a broth. After a four-hour lag, the cells grow exponentially up to approximately 10 hours and then remain in stationary phase up to approximately 16 hours before entering the death phase.**

information is important to determine some microbiological criteria of food, especially in controlling spoilage and pathogenic microorganisms in food. It is important to note that by changing some environmental parameters (e.g., refrigeration), the growth rate of some microbial species can be slowed down, but after a long time, the population can reach high numbers and cause problems in a food. Dead cells, by the action of autolytic enzymes, may lyse and release the cellular enzymes in a food, which then can act on food components.

## Nature of Microbial Growth in Food

### *Mixed Population*

Normally, a food harbors a mixed population of microorganisms that can include different species and strains of bacteria, yeasts, and molds.[3,4] Some species can be present in relatively higher numbers than others. The growth characteristics of a mixed population differ in several respects from that of a pure culture (a single strain of a species). Depending on the environment, which includes both the food environment (intrinsic) and the environment in which the food is stored (extrinsic), some of the species or strains can be in an optimum or near-optimum growth condition. Because of a rapid rate of growth during storage, they will outnumber the others and become predominant. This can occur even if they are initially present in low numbers. This is often the case in foods kept for a long time under a specific condition, such as at refrigerated temperatures. If the product is enumerated initially, it may show that the majority of microbial populations is able to grow at 35°C, and only a few grow at 4°C (refrigerated temperature). If the product is enumerated after a few weeks of refrigerated storage, usually populations that grow at 4°C outnumber those that grow at 35°C, but not those

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that grow at 4°C. Another situation can arise if a food contains, among the mixed population, two species initially present in equal numbers with both growing optimally under the specific intrinsic and extrinsic environments of the food but one having a shorter generation time than the other. After a storage period, the one with the shorter generation time becomes predominant. Many foodborne pathogens are poor competitors, and some of them have a longer generation time than the resident flora; thus their growth is very poor in a mixed culture environment (e.g., *Listeria monocytogenes*). Many foods, most often, are spoiled by bacteria rather than by yeasts and molds because, in general, bacteria have shorter generation times. Thus, in a mixed population, the intrinsic and extrinsic environments dictate which one, two, or few in the initial mixed population will become predominant and produce specific changes in a food. These aspects are very important in the control of microbial spoilage of foods and in the production of bioprocessed (or fermented) foods.[3,4]

## Sequence of Growth

Among the different microbial types normally present in a food, different species (strains) can become predominant in sequence during storage.[3,4] Initially, depending on the environment, one or two types may grow optimally and then create an environment in which they can no longer grow rapidly. However, another type in the mixed population can find this changed environment favorable for growth and grow rather rapidly. This shift in predominance can occur several times during the storage of a food. The sequential microbial growth can be seen particularly in foods stored for a long time. If a food is packaged in a bag with a little air (e.g., ground meat stored in a vacuum package), the aerobes grow first and utilize the oxygen. The environment then becomes anaerobic, in which the anaerobes (or facultative anaerobes) grow favorably. In the natural fermentation of some foods, such as sauerkraut, four different bacterial species grow in succession, one creating the favorable growth condition for the next. The desirable characteristics of the final product are dependent on the growth of all four species in specific sequence. To identify the existence of such a situation, it is necessary to enumerate and determine the microbial types at different stages of storage or fermentation.[3,4]

## Growth in Succession or Diauxic Growth

Microorganisms that can metabolize two or more nutrients in a food, one preferred over the other and present in limiting concentrations, show growth in stages separated by a short lag phase.[1] Initially, a bacterial strain grows by utilizing the preferred nutrient and, after a short lag of adaptation, grows by utilizing the other nutrient. During each stage, the growth curve has exponential and stationary phases with a lag phase in between. An example is the growth of certain bacterial strains (such as some lactic acid bacteria and Gram-negative bacteria) in fresh meat. A strain grows initially by utilizing the limiting concentrations of carbohydrate present, followed by utilization of nonprotein nitrogenous (NPN, such as amino acids) substances.

## Symbiotic Growth

Symbiosis, or helping one another, during growth often occurs in food containing two or more types of microorganisms.[3,4] One type may produce metabolic products that the second type needs for proper growth but cannot produce by itself. In turn, the second species produces a nutrient that stimulates the first one to grow better. This is found in the production of some fermented foods, such as yogurt. Initially, *Streptococcus thermophilus* hydrolyzes milk proteins by its extracellular

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proteinases and generates amino acids that are necessary for good growth of *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Lactobacillus*, in turn, produces formate, which stimulates the growth of *Streptococcus* species. Both are necessary to produce a desirable product.[3,4]

### *Synergistic Growth*

This is observed during symbiotic growth of two or more microbial types in a food.[3,4] In synergistic growth, each type is capable of growing independently and producing some metabolites at lower rates. However, when the types are allowed to grow in a mixed population, both the growth rate and the level of byproduct formation greatly increase. The increase is more than the additive of the amounts produced by growing the two separately. For example, both *Str. thermophilus* and *Lab. delbrueckii* subsp. *bulgaricus*, when growing in milk independently, produce approximately 8–10 ppm acetaldehyde, the desirable flavor component of yogurt. However, when growing together in milk, 30 ppm or more of acetaldehyde is produced, which is much higher than the additive amounts produced independently by the two species and is necessary for a good-quality yogurt. In the production of a desirable fermented food, two separate strains and species can be used to induce synergistic growth.[3,4]

### *Antagonistic Growth*

Two or more types of microorganisms present in a food can adversely affect the growth of each other, or one can interfere with the growth of one or more types; sometimes one can kill the other. This has been found among many bacterial species or strains, between bacteria and yeasts, between yeasts and molds, and between bacteria and molds. This occasionally occurs as a result of the production of one or more antimicrobial compounds by one or more strains in the mixed population. Some Gram-positive bacteria produce antibacterial proteins or bacteriocins or metabolic byproducts that can kill or suppress the growth of other types of Gram-positive bacteria. Similarly, some yeasts can produce wall-degrading enzymes and reduce the growth of molds. Some strains have probably developed these specific traits for a growth advantage in a mixed-population situation. There is now an interest in using this phenomenon to control growth and enhance the viability loss of undesirable spoilage and pathogenic microorganisms in food.

## Conclusion

The study of microbial growth provides basic information that is important to understand the mechanisms of food spoilage, foodborne diseases, food bioprocessing and strain improvement, and their detection in food. Microorganisms are present in mixed cultures in food and can interact with each other during growth. Their growth is influenced by the environment of a food, which is discussed in Chapter 6.

**QUESTIONS**

1. Describe the process of microbial growth with a diagram.
2. Define generation time as related to bacterial growth. If a pure culture of a bacterial population during incubation at 35°C in a nutritionally rich broth increases to $5 \times 10^6$/mL from an initial population of $2.5 \times 10^2$ in 300 minutes, what is the generation time of the strain?

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3. Using the data in question 2, determine the specific growth rate of the bacterial population.
4. A ground beef sample has $10^5$ CFUs/g of a Gram-negative psychrotrophic bacterial population. The specific growth rate of the population at 5°C is 0.2. In how many hours will the population reach $10^7$ CFUs/g? Determine its $t_d$ value.
5. "In foods, microorganisms are present as a mixed population." What disadvantage does this situation impose in applying the results of pure-culture study in food systems?
6. Explain synergistic growth, antagonistic growth, and symbiotic growth of microorganisms in food. What are their advantages and disadvantages?
7. A food contains two nutrients that can be used by one bacterial strain present in it. Indicate how the bacterial strain will use the two nutrients.
8. List one advantage and one disadvantage of measuring microbial growth by (a) CFU counts, (b) OD at 600 nm, and (c) cell mass.

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# Chapter 6

# Factors Influencing Microbial Growth in Food

## Introduction

The ability of microorganisms (except viruses) to grow or multiply in a food is determined by the food environment as well as the environment in which the food is stored, designated as the intrinsic and extrinsic environments of food, respectively. It is not possible to study the influence of any one factor on growth independently as the factors are interrelated. Instead, the influence of any one factor on different levels of growth is compared keeping other factors unchanged. The influence of these factors is discussed here.

## Intrinsic Factors or Food Environment

The intrinsic factors of a food include nutrients, growth factors, inhibitors (or antimicrobials), water activity, pH, and oxidation–reduction potential. The influence of each factor on growth is discussed separately. But, as indicated previously, in a food system, the factors are present together and exert effects on microbial growth in combination, either favorably or adversely.

### *Nutrients and Growth*

Microbial growth is accomplished through the synthesis of cellular components and energy.[1] The necessary nutrients for this process are derived from the immediate environment of a microbial cell and, if the cell is growing in a food, receives its nutrients from the food. These nutrients include carbohydrates, proteins, lipids, minerals, and vitamins. Water is not considered a nutrient, but it is essential as a medium for the biochemical reactions necessary for the synthesis of cell mass and energy. All foods contain these five major nutrient groups, either naturally or added, and the amount of each nutrient varies greatly with the type of food.[2] In general, meat and fish are rich in proteins, lipids, minerals, and vitamins but poor in carbohydrates. Foods from plant sources



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are rich in carbohydrates but can be poor sources of proteins, minerals, and some vitamins. Some foods, such as milk, and many prepared foods have all five nutrient groups in sufficient amounts for microbial growth.

Microorganisms normally present in food vary greatly in their nutrient requirements with bacteria requiring the most, followed by yeasts and molds. Microorganisms also differ greatly in their ability to utilize large and complex carbohydrates (e.g., starch and cellulose), large proteins (e.g., casein in milk), and lipids. Microorganisms are capable of using these molecules do so by producing specific extracellular enzymes (or exoenzymes) and hydrolyzing the complex molecules to simpler forms outside before transporting them inside the cell. Molds are the most capable of doing this. However, this provides an opportunity for a species to grow in a mixed population even when it is incapable of metabolizing the complex molecules. Microbial cells, following death and lysis, release intracellular enzymes that can also catalyze breakdown of complex food nutrients to simpler forms, which can then be utilized by other microorganisms.

### *Carbohydrates in Foods*

Major carbohydrates present in different foods, either naturally or added as ingredients, can be grouped on the basis of chemical nature as follows:

- Monosaccharides
  - Hexoses: glucose, fructose, mannose, galactose
  - Pentoses: xylose, arabinose, ribose, ribulose, xylulose
- Disaccharides
  - Lactose (galactose + glucose)
  - Sucrose (fructose + glucose)
  - Maltose (glucose + glucose)
- Oligosaccharides
  - Raffinose (glucose + fructose + galactose)
  - Stachyose (glucose + fructose + galactose + galactose)
- Polysaccharides
  - Starch (glucose units)
  - Glycogen (glucose units)
  - Cellulose (glucose units)
  - Inulin (fructose units)
  - Hemicellulose (xylose, galactose, mannose units)
  - Dextrans (α-1,6 glucose polymer)
  - Pectins
  - Gums and mucilages

Lactose is found only in milk and thus can be present in foods made from or with milk and milk products. Glycogen is present in animal tissues, especially in liver. Pentoses, most oligosaccharides, and polysaccharides are naturally present in foods of plant origin.

All microorganisms normally found in food metabolize glucose, but their ability to utilize other carbohydrates differs considerably. This is because of the inability of some microorganisms to transport the specific monosaccharides and disaccharides inside the cells and the inability to hydrolyze polysaccharides outside the cells. Molds are the most capable of using polysaccharides.

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Food carbohydrates are metabolized by microorganisms principally to supply energy through several metabolic pathways. Some of the metabolic products can be used to synthesize cellular components of microorganisms (e.g., to produce amino acids by amination of some keto acids). Microorganisms also produce metabolic byproducts associated with food spoilage ($CO_2$ to cause gas defect) or food bioprocessing (lactic acid in fermented foods). Some are also metabolized to produce organic acids, such as lactic, acetic, propionic, and butyric acids, which have an antagonistic effect on the growth and survival of many bacteria. Some of these metabolic pathways are discussed in Chapters 8 and 12. Microorganisms can also polymerize some monosaccharides to produce complex carbohydrates, such as dextrans, capsular materials, and cell walls (or outer membranes in Gram-negative bacteria). Some of these carbohydrates from pathogens may cause health hazards (the Gram-negative lipopolysaccharide is endotoxin and causes septicemia), some may cause food spoilage (such as slime defect), and some can be used in food production (such as dextrans as stabilizers). Carbohydrate and amino acid metabolism profiles are extensively used in the laboratory for the biochemical identification of unknown microorganisms isolated from foods.

### *Proteins in Foods*

The major proteinaceous components in foods are simple proteins, conjugated proteins, peptides, and nonprotein nitrogenous (NPN) compounds (amino acids, urea, ammonia, creatinine, trimethylamine). Proteins and peptides are polymers of different amino acids without or with other organic (e.g., a carbohydrate) or inorganic (e.g., iron) components and contain approximately 15%–18% nitrogen. Simple food proteins are polymers of amino acids, such as albumins (in eggs), globulins (in milk), glutelins (gluten in cereal), prolamins (zein in grains), and albuminoids (collagen in muscle). They differ greatly in their solubility, which determines the ability of microorganisms to utilize a specific protein. Many microorganisms can hydrolyze albumin, which is soluble in water. In contrast, collagens, which are insoluble in water or weak salt-and-acid solutions, are hydrolyzed only by a few microorganisms. As compared with simple proteins, conjugated proteins of food on hydrolysis produce metals (metalloproteins, such as hemoglobin and myoglobin), carbohydrates (glycoproteins, such as mucin), phosphates (phosphoproteins, such as casein), and lipids (lipoproteins, such as some in liver). Proteins are present in higher quantities in foods of animal origin than in foods of plant origin. But plant foods, such as nuts and legumes, are rich in proteins. Proteins as ingredients can also be added to foods.

Microorganisms differ greatly in their ability to metabolize food proteins. Most transport amino acids and small peptides in the cells; small peptides are then hydrolyzed to amino acids inside the cells. Microorganisms also produce extracellular proteinases and peptidases to hydrolyze large proteins and peptides to small peptides and amino acids before they can be transported inside the cells. Soluble proteins are more susceptible to this hydrolytic action than are the insoluble proteins. Hydrolysis of food proteins can be either undesirable (texture loss in meat or production of off-flavor) or desirable (flavor in cheese). Microorganisms can also metabolize different NPN compounds found in foods.

Amino acids inside microbial cells are metabolized via different pathways to synthesize cellular components, energy, and various byproducts. Many of these byproducts can be undesirable (e.g., $NH_3$ and $H_2S$ production causes spoilage of food, and toxins and biological amines (such as histamine) cause health hazards) or desirable (e.g., some sulfur compounds give cheddar cheese flavor). Production of specific metabolic products is used for the laboratory identification of microbial isolates from food. An example of this is the ability of *Escherichia coli* to produce indole from

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tryptophan, which is used to differentiate this species from non-indole-producing related species (e.g., *Enterobacter* spp.).

### Lipids in Foods

Lipids in foods include compounds that can be extracted by organic solvents, some of which are free fatty acids, glycerides, phospholipids, waxes, and sterols. Lipids are relatively higher in foods of animal origin than in foods of plant origin although nuts, oil seeds, coconuts, olives, and avocados have high amounts of lipids. Fabricated or prepared foods can also vary greatly in lipid content. Cholesterols are present in foods of animal origin or foods containing ingredients from animal sources. Lipids are, in general, less preferred substrates for the microbial synthesis of energy and cellular materials. Many microorganisms can produce extracellular lipases that can hydrolyze glycerides to fatty acids and glycerol. Fatty acids can be transported in cells and used for energy synthesis, whereas glycerol can be metabolized separately. Some microorganisms also produce extracellular lipid oxidases, which can oxidize unsaturated fatty acids to produce different aldehydes and ketones. In general, molds are more capable of producing these enzymes. However, certain bacterial genera, such as *Pseudomonas*, *Achromobacter*, and *Alcaligenes*, can produce these enzymes. Lysis of dead microbial cells in foods causes release of intracellular lipases and oxidases, which then can carry out these reactions. In many foods, the action of these enzymes is associated with spoilage (such as rancidity), whereas in other foods, the enzymes are credited for desirable flavors (such as in mold-ripened cheeses). Some beneficial intestinal microorganisms, such as *Lactobacillus acidophilus* strains, can metabolize cholesterol and are believed to be capable of reducing serum cholesterol levels in humans.

### Minerals and Vitamins in Foods

Microorganisms need several elements in small amounts, such as phosphorous, calcium, magnesium, iron, sulfur, manganese, and potassium. Most foods have these elements in sufficient amounts. Many microorganisms can synthesize B vitamins, and foods also contain most B vitamins.

In general, most foods contain different carbohydrates, proteins, lipids, minerals, and vitamins in sufficient amounts to supply necessary nutrients for the growth of molds, yeasts, and bacteria, especially Gram-negative bacteria normally present in foods. Some foods may have limited amounts of one or a few nutrients for rapid growth of some Gram-positive bacteria, especially some fastidious *Lactobacillus* species. When their growth is desired, some carbohydrates, essential amino acids, and B vitamins may be added to a food. It is not possible or practical to control microbial growth in a food by restricting nutrients.

## Growth Factors and Inhibitors in Food

Foods can also have some factors that either stimulate growth or adversely affect growth of microorganisms.[3] The exact nature of growth factors is not known, but they are naturally present in some foods. An example is the growth factors in tomatoes that stimulate growth of some *Lactobacillus* species. These factors can be added to raw materials during food bioprocessing or to media to isolate some fastidious bacteria from foods.

Foods also contain many chemicals, either naturally or added, that adversely affect microbial growth. Some of the natural inhibitors are lysozyme in egg, agglutinin in milk, and eugenol in

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cloves. The inhibitors, depending on their mode of action, can prevent or reduce growth and kill microorganisms. This aspect is discussed in Chapter 38.

## Water Activity and Growth

### Principle

Water activity ($A_W$) is a measure of the availability of water for biological functions and relates to water present in a food in free form. In a food system, total water or moisture is present in free and bound forms. Bound water is the fraction used to hydrate hydrophilic molecules and to dissolve solutes, and is not available for biological functions; thus, it does not contribute to $A_W$. The $A_W$ of a food can be expressed by the ratio of water vapor pressure of the food (*P*, which is <1) to that of pure water (Po, which is 1), that is, P/Po. It ranges between 0 and 1 or, more accurately, >0 to <1 because no food can have a water activity of either 0 or 1. The $A_W$ of a food can be determined from its equilibrium relative humidity (ERH) by dividing ERH by 100 (because ERH is expressed as a percentage).[4–7]

### $A_W$ of Food

The $A_W$ of food ranges from approximately 0.1 to 0.99. The $A_W$ values of some food groups are as follows: cereals, crackers, sugar, salt, dry milk, 0.10–0.20; noodles, honey, chocolate, dried egg, <0.60; jam, jelly, dried fruits, parmesan cheese, nuts, 0.60–0.85; fermented sausage, dry cured meat, sweetened condensed milk, maple syrup, 0.85–0.93; evaporated milk, tomato paste, bread, fruit juices, salted fish, sausage, processed cheese, 0.93–0.98; and fresh meat, fish, fruits, vegetables, milk, eggs, 0.98–0.99. The $A_W$ of foods can be reduced by removing water (desorption) and increased by the adsorption of water, and these two parameters can be used to draw a sorption isotherm graph for a food (Figure 6.1). The desorption process gives relatively lower $A_W$

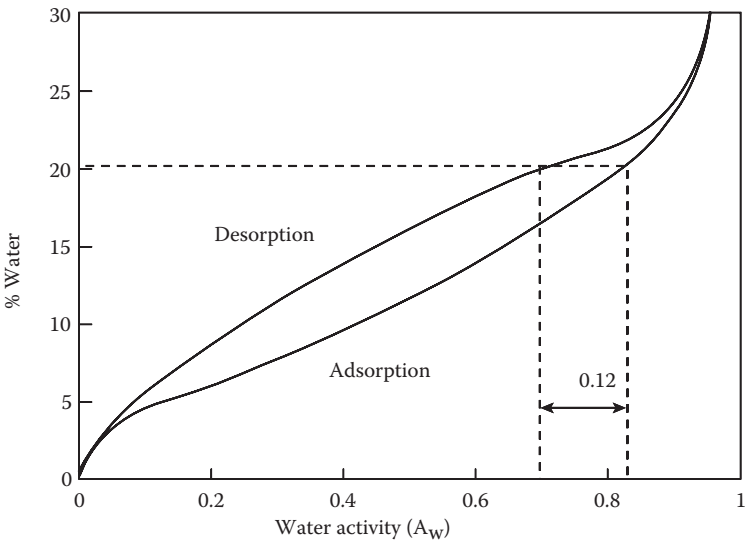



**Figure 6.1 Water sorption isotherm showing hysterisis. At the same percentage of water, $A_W$ is lower by desorption than by adsorption.**

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values than the adsorption process does at the same moisture content of a food. This has important implications in the control of a microorganism by reducing the $A_W$ of a food. The $A_W$ of a food can be reduced by several means, such as adding solutes, ions, hydrophilic colloids, and freezing and drying.[4–7]

### *$A_W$ and Microbial Growth*

The free water in a food is necessary for microbial growth. It is necessary to transport nutrients and remove waste materials, carry out enzymatic reactions, synthesize cellular materials, and take part in other biochemical reactions, such as hydrolysis of a polymer to monomers (proteins to amino acids). Each microbial species (or group) has an optimum, maximum, and minimum $A_W$ level for growth. In general, the minimum $A_W$ values for growth of microbial groups are as follows: most molds, 0.8, with xerophilic molds as low as 0.6; most yeasts, 0.85, with osmophilic yeasts, 0.6–0.7; most Gram-positive bacteria, 0.90; and Gram-negative bacteria, 0.93. Some exceptions are growth of *Staphylococcus aureus* at 0.85 and halophilic bacteria at 0.75. The $A_W$ needed for spore-forming bacteria to sporulate, for the spores to germinate, and for the toxin-producing microorganisms to produce toxins is generally higher than the minimum $A_W$ needed for their growth. Also, the minimum $A_W$ for growth in an ideal condition is lower than that in a nonideal condition. As an example, if the minimum $A_W$ for growth of a bacterial strain at pH 6.8 is 0.91, then at pH 5.5, it can be 0.95 or more. When the $A_W$ is reduced below the minimum level required for growth of a microorganism, the cells remain viable for a while. But if the $A_W$ is reduced drastically, microbial cells in a population lose viability, generally rapidly at first and then more slowly. This information is used to control spoilage and pathogenic microorganisms in food as well as enhance the growth of desirable types in food bioprocessing (such as adding salt in the processing of cured ham, see Chapter 35) and in laboratory detection of microorganisms (adding salt to media to create a selective environment for enumeration of *Sta. aureus*, *Listeria monocytogenes*, etc.).

## *pH and Growth*

### *Principle*

pH indicates the hydrogen ion concentrations in a system and is expressed as $-\log [H^+]$, the negative logarithm of the hydrogen ion or proton concentration. It ranges from 0 to 14, with 7.0 being neutral pH. $[H^+]$ concentrations can differ in a system, depending on what acid is present. Some strong acids used in foods, such as HCl and phosphoric acid, dissociate completely. Weak acids, such as acetic or lactic acids, remain in equilibrium with the dissociated and undissociated forms:

$$[HCl] \rightarrow [H^+] + [Cl^-], \text{ pH of 0.1 NHCl is 1.1}$$

$$CH_3COOH \rightarrow [H^+] + [CH_3COO^-], \text{ pH of 0.1 N } CH_3COOH \text{ is 2.9}$$

Acidity is inversely related to pH: A system with high acidity has a low pH and vice versa.[8,9]

### *pH of Food*

Depending on the type, the pH of a food can vary greatly. On the basis of pH, foods can be grouped as high-acid foods (pH below 4.6) and low-acid foods (pH 4.6 and above). Most fruits,

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fruit juices, fermented foods (from fruits, vegetables, meat, and milk), and salad dressings are high-acid (low-pH) foods, whereas most vegetables, meat, fish, milk, and soups are low-acid (high-pH) foods. Tomato, however, is a high-acid vegetable (pH 4.1–4.4). The higher pH limit of most low-acid foods remains below 7.0; only in a few foods, such as clams (pH 7.1) and egg albumen (pH 8.5), does the pH exceed 7.0. Similarly, the low-pH limit of most high-acid foods remains above 3.0 except in some citrus fruits (lemon, lime, grapefruit) and cranberry juice in which the pH can be as low as 2.2. The acid in the foods can either be present naturally (as in fruits), produced during fermentation (as in fermented foods), or added during processing (as in salad dressings). Foods can also have compounds that have a buffering capacity. A food such as milk or meat, because of good buffering capacity, does not show pH reduction when compared with a vegetable product in the presence of the same amount of acid.

### *pH and Microbial Growth*

The pH of a food has a profound effect on the growth and viability of microbial cells. Each species has an optimum and a range of pH for growth. In general, molds and yeasts are able to grow at lower pH levels than do bacteria, and Gram-negative bacteria are more sensitive to low pH than are Gram-positive bacteria. The pH range of growth for molds is 1.5–9.0; for yeasts, 2.0–8.5; for Gram-positive bacteria, 4.0–8.5; and for Gram-negative bacteria, 4.5–9.0. Individual species differ greatly in lower pH limit for growth; for example, *Pediococcus acidilactici* can grow at pH 3.8, and *Sta. aureus* and *Salmonella* can grow at pH 4.5, but normally, *Campylobacter* cannot. The lower pH limit of growth of a species can be a little higher if the pH is adjusted with a strong acid instead of a weak acid (because of its undissociated molecules). Acid-resistant or acid-tolerant strains can acquire resistance to lower pH compared with the other strains of a species (e.g., acid-resistant *Salmonella*; *Escherichia coli* O157:H7).

When the pH in a food is reduced to below the lower limit for growth of a microbial species, the cells not only stop growing but also lose viability, the rate of which depends on the extent of pH reduction. This is more apparent with weak acids, especially with those that have higher dissociation constants (pK), such as acetic acid versus lactic acid (with pK values 4.8 and 3.8, respectively). This is because at the same pH, acetic acid has more undissociated molecules than lactic acid does. The undissociated molecules, being lipophilic, enter into the cell and dissociate to generate $H^+$ in the cytoplasm. This causes a reduction in internal pH, which ultimately destroys the proton gradient between the inside and the outside of the cells and dissipates proton motive force as well as the ability of the cells to generate energy. The information on the influence of pH on the growth and viability of microbial cells is important to develop methods to prevent the growth of undesirable microorganisms in food (e.g., in acidified foods; see Chapter 36), to produce some fermented foods (e.g., sequential growth of lactic acid bacteria in sauerkraut fermentation), and to selectively isolate aciduric microorganisms from food (e.g., yeasts and molds in a medium with pH 3.5).[8,9] Acquired acid tolerance by pathogens and spoilage bacteria can impose problems in their control in low-pH foods; this aspect is discussed in Chapter 10.

## *Redox Potential, Oxygen, and Growth*

### *Principle*

The redox or oxidation–reduction (O-R) potential measures the potential difference in a system generated by a coupled reaction in which one substance is oxidized and a second substance is

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reduced simultaneously. The process involves the loss of electrons from a reduced substance (thus it is oxidized) and the gain of electrons by an oxidized substance (thus it is reduced). The electron donor, because it reduces an oxidized substance, is also called a reducing agent. Similarly, the electron recipient is called an oxidizing agent. The redox potential, designated as Eh, is measured in electrical units of millivolts (mV). In the oxidized range, it is expressed in +mV and in the reduced range in –mV. In biological systems, the oxidation and reduction of substances are the primary means of generating energy. If free oxygen is present in the system, then it can act as an electron acceptor. In the absence of free oxygen, oxygen bound to some other compound, such as $NO_3$ and $SO_4$, can accept the electron. In a system where no oxygen is present, other compounds can accept the electrons. Thus, the presence of oxygen is not a requirement of O-R reactions.[10]

### Redox Potential in Food

The redox potential of a food is influenced by its chemical composition, specific processing treatment given, and its storage condition (in relation to air). Fresh foods of plant and animal origin are in a reduced state because of the presence of reducing substances, such as ascorbic acid, reducing sugars, and the –SH group of proteins. Following stoppage of respiration of the cells in a food, oxygen diffuses inside and changes the redox potential. Processing, such as heating, can increase or decrease reducing compounds and alter the Eh. A food stored in air will have a higher Eh (+mV) than when it is stored under vacuum or in modified gas (such as $CO_2$ or $N_2$). Oxygen can be present in a food in the gaseous state (on the surface, trapped inside) or in dissolved form.

### Redox Potential and Microbial Growth

On the basis of their growth in the presence and absence of free oxygen, microorganisms have been grouped as aerobes, anaerobes, facultative anaerobes, or microaerophiles. Aerobes need free oxygen for energy generation as the free oxygen acts as the final electron acceptor through aerobic respiration (see Chapter 8). Facultative anaerobes can generate energy if free oxygen is available, or they can use bound oxygen in compounds such as $NO_3$ or $SO_4$ as final electron acceptors through anaerobic respiration. If oxygen is not available, then other compounds are used to accept the electron (or hydrogen) through (anaerobic) fermentation. An example of this is the acceptance of hydrogen from $NADH_2$ by pyruvate to produce lactate. Anaerobic and facultative anaerobic microorganisms can only transfer electrons through fermentation. Many anaerobes (obligate or strict anaerobes) cannot grow in the presence of even small amounts of free oxygen as they lack the superoxide dismutase necessary to scavenge the toxic oxygen free radicals. Addition of scavengers, such as thiols (e.g., thiolglycolate), helps overcome the sensitivity to these free radicals. Microaerophiles grow better in the presence of less oxygen.

Growth of microorganisms and their ability to generate energy by the specific metabolic reactions depend on the redox potential of foods. The Eh range at which different groups of microorganisms can grow are as follows: aerobes, +500 to +300 mV; facultative anaerobes, +300 to +100 mV; and anaerobes, +100 to –250 mV or lower. However, this varies greatly with concentrations of reducing components in a food and the presence of oxygen. Molds, yeasts, and *Bacillus*, *Pseudomonas*, *Moraxella*, and *Micrococcus* genera are some examples that have aerobic species. Some examples of facultative anaerobes are the lactic acid bacteria and those in the family *Enterobacteriaceae*, such as *Salmonella*, *Escherichia coli*, and *Yersinia* spp. The most important anaerobe in food is *Clostridium*. An example of a microaerophile is *Campylobacter* spp. The Eh range indicates that in each group some species are stricter in their Eh need than others. Although

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most molds are strict aerobes, a few can tolerate less aerobic conditions. Similarly, yeasts are basically aerobic, but some can grow under low Eh (below +300 mV). Many clostridial species can grow at Eh +100 mV, but some need –150 mV or less.

The presence or absence of oxygen and the Eh of food determine the growth capability of a particular microbial group in a food and the specific metabolic pathways used during growth to generate energy and metabolic byproducts. This is important in microbial spoilage of a food (such as putrification of meat by *Clostridium* spp. under anaerobic conditions) and to produce desirable characteristics of fermented foods (such as growth of *Penicillium* species in blue cheese under aerobic conditions). This information is also important to isolate microorganisms of interest from foods (such as *Clostridium laramie*, a strict anaerobe from spoiled meat) in the laboratory.

## Extrinsic Factors

Extrinsic factors important in microbial growth in a food include the environmental conditions in which it is stored. These are temperature, relative humidity, and gaseous environment. The relative humidity and gaseous condition of storage, respectively, influence the $A_W$ and Eh of the food. The influence of these two factors on microbial growth has been discussed previously. In this section, the influence of the storage temperature of food on microbial growth is discussed.

### *Temperature and Growth*

#### *Principle*

Microbial growth is accomplished through enzymatic reactions. It is well known that within a certain range with every 10°C (50°F) rise in temperature, the catalytic rate of an enzyme doubles. Similarly, the enzymatic reaction rate is reduced to half by decreasing the temperature by 10°C. This relationship changes beyond the growth range. Because temperature influences enzyme reactions, it has an important role in microbial growth in food.

#### *Food and Temperature*

Foods are exposed to different temperatures from the time of production until consumption. Depending on processing conditions, a food can be exposed to high heat, from 65°C (149°F) (roasting of meat) to more than 100°C (in ultrahigh temperature processing). For long-term storage, a food can be kept at 5°C (refrigeration) to –20°C or below (freezing). Some relatively stable foods are also kept between 10 and 35°C (cold to ambient temperature). Some ready-to-eat foods are kept at warm temperature (50–60°C) for several hours (e.g., in the supermarket deli). Different temperatures are also used to stimulate desirable microbial growth in food fermentation.[11]

#### *Microbial Growth and Viability*

Microorganisms important in foods are divided into three groups on the basis of their temperature of growth, each group having an optimum temperature and a temperature range of growth: (1) thermophiles (which grow at relatively high temperatures) with an optimum at approximately 55°C (131°F) and a range of 45°C–70°C; (2) mesophiles (which grow at ambient temperatures), with an optimum at 35°C and a range of 10°C–45°C; and (3) psychrophiles (which grow at cold

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temperatures), with an optimum at 15°C and a range of –5°C to 20°C. However, these divisions are not clear cut and overlap each other.

Two other terms used in food microbiology are very important with respect to microbial growth at refrigerated temperatures and survival of microorganisms to low heat treatment or pasteurization because both methods are widely used in the storage and processing of foods. Psychrotrophs are microorganisms that grow at refrigerated temperature (0°C–5°C) irrespective of their optimum range of growth temperature. They usually grow rapidly between 10°C and 30°C. Molds; yeasts; many Gram-negative bacteria from genera *Pseudomonas*, *Achromobacter*, *Yersinia*, *Serratia*, and *Aeromonas*; and Gram-positive bacteria from genera *Leuconostoc*, *Lactobacillus*, *Bacillus*, *Clostridium*, and *Listeria* are included in this group (see Chapter 21). Microorganisms that survive pasteurization temperatures are designated as thermodurics. They include species from genera *Micrococcus*, *Bacillus*, *Clostridium*, *Lactobacillus*, *Pediococcus*, and *Enterococcus*. Bacterial spores are also included in this group. They have different growth temperatures, and many can grow at refrigerated temperatures as well as thermophilic temperatures.

When foods are exposed to temperatures beyond the maximum and minimum temperatures of growth, microbial cells die rapidly at higher temperatures and relatively slowly at lower temperatures. Influence of temperature on microbial growth and viability is important in reducing food spoilage, enhancing safety against pathogens, and in food bioprocessing. Temperature of growth is also effectively used in the laboratory to enumerate and isolate microorganisms from foods.

## *Quorum Sensing and Bacterial Growth*

The intrinsic and extrinsic factors control bacterial growth in food, but little was known about the molecular mechanism of bacterial behavior in these conditions until recently. Bacterial quorum sensing (QS) is thought to play a major role in the bacterial response to a changing environment in food. QS is described as a bacterial cell-to-cell communication system to develop a strategy for the bacterial population to adapt to the harsh environment in food or the host.[12] QS is cell-density dependent, that is, when bacteria reach a certain cell number, they produce small diffusible molecules, termed autoinducers (AIs) as signaling molecules that are used for cell-to-cell communication during growth. For example, under stressful conditions, such as nutrient starvation, low water activity, high temperatures, antimicrobial compounds, acid, or other microbes, bacterial AIs bind to receptors and activate gene expression to alter behavior (i.e., adaptation to harsh environment) and morphological characteristics (i.e., biofilm formation, sporulation). There are four major categories of AIs: (i) autoinducer-1 (AI-1), for example, *N*-acyl homoserine lactone (Figure 6.2) is produced by Gram-negative bacteria and is used for intraspecies communication; (ii) autoinducer-2 (AI-2) is produced by both Gram-positive and Gram-negative bacteria and is used as a universal signaling molecule; (iii) autoinducer-3 (AI-3) is a signaling molecule used by enterohaemorrhagic *Escherichia coli* during infection; and (iv) autoinducing peptides (AIPs) that are produced and used by Gram-positive bacteria. AI-1 and AI-2 have been found in many different food systems, including milk, meat, and vegetables, when these foods are associated with pseudomonads, *Enterobactericeae*, and lactic acid bacteria.

QS has been recognized as a key regulatory system in both food safety and food spoilage. In pathogenic bacteria, such as *Salmonella*, *Escherichia coli* O157:H7, and *Staphylococcus aureus*, QS is involved in virulence gene expression, promoting increased pathogen survival and colonization in the host.[13] QS is also a regulatory spoilage mechanism.[12] Food spoilage is characterized by

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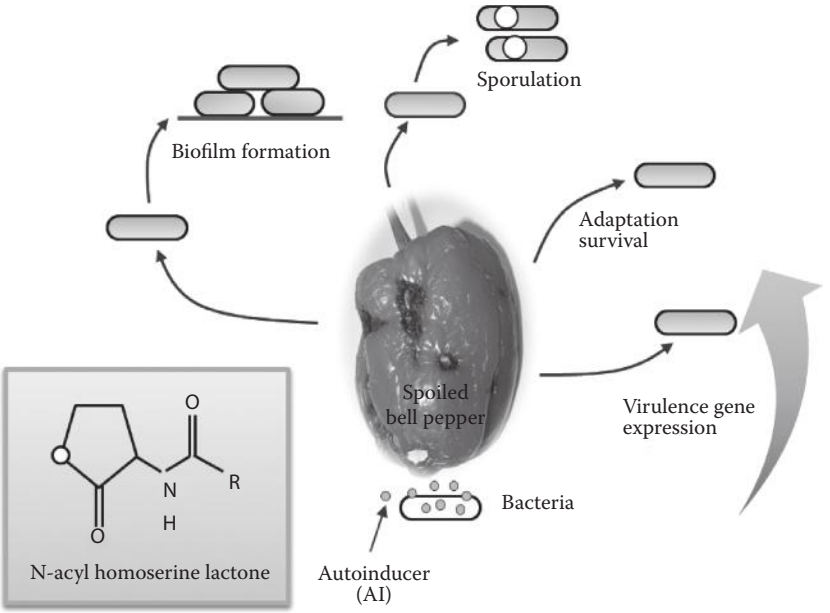



**Figure 6.2 (See color insert.) Bacterial quorum sensing network and adaptation strategy on food. Bacteria secrete an autoinducer (AI), such as *N*-acyl homoserine lactone, to communicate with others in response to a changing food environment.**

excessive microbial growth rendering food undesirable for consumption (see Chapter 19). Foods of animal origin are rich in protein, which is degraded by proteolytic enzymes produced by pseudomonads, *Enterobactericeae*, and lactic acid bacteria, rendering spoiled food. Small molecules, such as glucose, lactate, and free amino acids, regulate growth of these microorganisms in these foods. Spoilage of fruits and vegetables depends on the pectinolytic activity (enzymes involved are pectin lyases, pectate lyase, polygalacturonase, pectin methylesterage) of members of the *Pseudomonadaceae* and *Enterobactericeae* families resulting in enzymatic browning, off-flavor, and texture breakdown. Production of these enzymes is regulated by QS; thus chemicals (i.e., garlic-extract plant-derived *p*-coumaric acid) that inhibit QS can be used to control bacterial growth in foods. Another strategy to control pathogen or spoilage bacterial growth in food would be to add QS compounds in foods to create a false sense indicating there are already too many bacteria present in food; thus growth can be ceased.[12]

## Conclusion

The physical and chemical environments control microbial growth within the growth range mainly by influencing their metabolic process associated with synthesis of energy and cellular components. Beyond the growth range, these factors, either individually or in combination, can be used to control microbial growth and even to destroy them. Actual growth is accomplished through the metabolism of various nutrients present in a food. The processes by which the food nutrients are transported inside the microbial cells and then metabolized to produce energy, cellular molecules, and byproducts are briefly discussed in Chapter 8.

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### QUESTIONS

1. List the intrinsic and extrinsic factors necessary for growth of microorganisms in a food.
2. What are the major nutrients in food that are metabolized by the microorganisms? List the major groups of carbohydrates present in foods. List the carbohydrates in milk and meat, the pentoses in plant foods, and an oligosaccharide in plant foods.
3. Discuss how bacteria are able to metabolize large molecules of carbohydrates, proteins, and lipids. How do molds differ from bacteria in the metabolism of these molecules?
4. Discuss the importance of antimicrobials in foods that can adversely affect microbial growth.
5. Define $A_W$ and explain the desorption and adsorption processes of moisture in a food. Discuss the importance of $A_W$ in microbial growth. How do halophilic, osmophilic, and xerophilic microorganisms differ in minimum $A_W$ needed for growth?
6. Define pH and discuss factors that influence the pH of a food. Discuss the role of pH on microbial growth. How does a bacterial cell maintain a high intracellular pH (6.0) while growing in a low-pH (5.0) environment? Give examples of an aciduric bacterium and an acid-tolerant bacterium.
7. Define redox potential and discuss how it influences microbial growth in a food. How can microorganisms be grouped on the basis of their growth capabilities at different redox potentials and oxygen availabilities?
8. How are microorganisms grouped on the basis of their temperature of growth and survival? Discuss the significance of psychrotrophic and thermoduric microorganisms in the processing and refrigerated storage of foods.
9. What is quorum sensing? How does quorum sensing affect bacterial growth in foods?

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# Chapter 7

# Microbial Attachments and Biofilm Formation

## Introduction

The normal tendency of a microbial cell (bacteria, yeast, molds) when it comes in contact with a solid surface is to attach itself to the surface in an effort to compete efficiently with other microbial cells for space and the nutrient supply and to resist any unfavorable environmental conditions. The solid surface may be comprised of a biotic, that is, biological, such as meat, fruits, etc., or abiotic surface (nonbiological), such as floors, walls, pipes, drains, equipment, or food contact surfaces (conveyor belts, pasteurizers, gaskets). Under suitable conditions, almost all microbial cells can attach to a solid surface, which is achieved through their ability to produce extracellular polysaccharides. As the cells multiply, they form a three-dimensional scaffold, forming a biofilm on the surface containing a community of microbial cells, a polyanionic extracellular polymeric substance (EPS), and entrapped debris. The EPS may contain polysaccharide-glycocalyx, proteins, phospholipids, teichoic and nucleic acids, and other polymeric substances.[1] In some situations, instead of forming a biofilm, the cells may attach to contact surfaces and other cells by thin, threadlike exopolysaccharide materials, also called fimbriae. Biofilms are comprised of a single species or a mixed species culture with one being dominant.

The attachment of microorganisms to solid surfaces has several implications for the overall microbiological quality of food. Advantages of microbial attachment to and biofilm formation on solid surfaces are to provide some protection to the cells against desiccation and a resistance to antibiotics or biocides, ultraviolet radiation, metallic cations, and physical removal of the cells by washing and cleaning. These cells seem to have greater resistance to sanitizers and heat. Thus, spoilage and pathogenic microorganisms attached to food surfaces, such as carcasses, fish, meat, and cut fruits and vegetables, cannot be easily removed by washing, and later they can multiply and reduce the safety and quality of the foods. Similarly, microbial cells attached to equipment surfaces, especially those that come in contact with food, may not be easily killed by chemical sanitizers or heat designed to be effective against unattached microbial cells (like in a suspension), and thus they can contaminate food. This problem increases if cleaning and sanitation are



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delayed following equipment use. Finally, microbial attachment and biofilm formation in the food-processing environment, such as floors, walls, hoses, and drains, enable the cells to establish in the environment, and they become difficult to control effectively by the methods developed and designed to work against the unattached microorganisms from a culture broth. These places, in turn, can be a constant source of undesirable microorganisms for foods handled in the environment.

The concept and importance of microbial attachment and biofilm formation in solid food, equipment, and food environments are now being recognized.[1–4] Limited studies have shown that under suitable conditions, many of the microorganisms important in food can form a biofilm. Several species and strains of *Pseudomonas* were found to attach to stainless steel surfaces, some within 30 minutes at 25°C to within two hours at 4°C. *Listeria monocytogenes* was found to attach to stainless steel, glass, and rubber surfaces within 20 minutes of contact. Attachment of several pathogenic and spoilage bacteria has also been demonstrated on meat and carcasses of poultry, beef, pork, and lamb. The microorganisms found to attach to meat surfaces include *Listeria monocytogenes*, *Micrococcus* spp., *Staphylococcus* spp., *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Brochothrix thermosphacta*, *Salmonella*, *Escherichia coli*, *Serratia* spp., *Campylobacter*, and *Pseudomonas* spp. It is apparent from the limited data that microbial attachment to solid food and food contact surfaces is quite wide and needs to be considered in controlling the microbiological quality of food.

## Mechanisms of Attachment

Several possible mechanisms by which microbial cells attach and form a biofilm on solid surfaces have been suggested.[2–4] The life cycle of a biofilm consists of several stages: (i) attachment, (ii) microcolony and cell cluster formation, (iii) maturation, and (iv) detachment or dispersion (Figure 7.1). The initial attachment occurs in two stages. In the first stage, which is reversible, the free-floating planktonic cell is held to the surface by weak forces, such as Van der Waals and

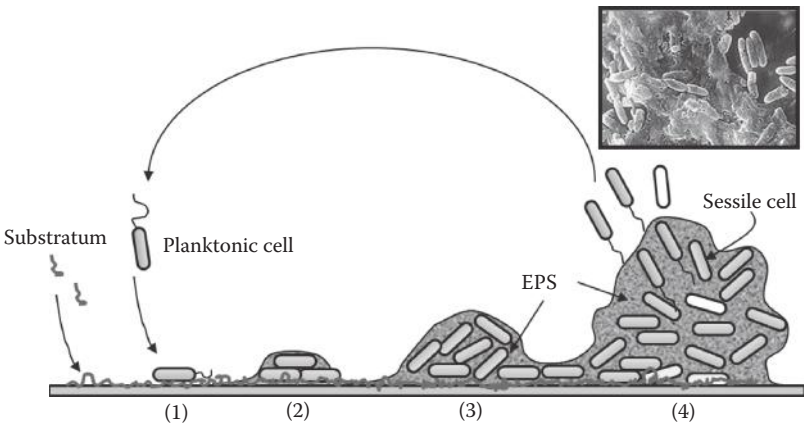



**Figure 7.1 (See color insert.) Schematic of a bacterial biofilm life cycle. The steps involve (1) attachment, (2) microcolony and cell cluster formation, (3) maturation, and (4) detachment or dispersion. Inset: Scanning electron microscopy photograph showing matured biofilm with EPS matrix.**

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**Table 7.1 Role of Extracellular Polymeric Substances (EPS) in a Biofilm**

| *Function* | *Relevance for Biofilms* |
|---|---|
| Adhesion | Initial attachment and colonization to solid surface, long-term attachment of biofilm to surfaces |
| Aggregation of cells | Bridging of cells, high cell density, cell-to-cell communication |
| Cohesion of biofilms | Forms hydrated polymer network, mechanical stability, three-dimensional configuration, biofilm architecture |
| Retention of water | Maintains hydrated microenvironment, resistance to desiccation |
| Protective barrier | Resistant to antimicrobial agents, antibiotics, biocides, sanitizers, and host defense (during infection) |
| Sorption of organic and inorganic compounds | Accumulation of nutrients and toxic metal ions |
| Enzymatic activity | Digestion of exogenous macromolecules and EPS for nutrients |
| Nutrient source | Source of carbon, nitrogen, and phosphorous-containing compounds |
| Genetic exchange | Horizontal gene transfer between biofilm cells |

*Source:* Adapted from Flemming, H.-C. and Wingender, J., *Nat. Rev. Microbiol.*, *8*, 623–633, 2010.

electrostatic forces. In the second stage, the cell produces EPS molecules to attach its outer surface to the surface of a food (biotic) or equipment (abiotic), and the process is irreversible. The adherent cells in the biofilm are referred to as sessile cells. Besides attachment, EPS contributes to multiple tasks within the biofilm, which are summarized in Table 7.1. A three-step process for attachment that includes adsorption, consolidation, and colonization has also been suggested. In the reversible adsorption stage, which can occur in 20 minutes, the cells attach loosely to the surface. During the consolidation stage, the microorganisms produce fimbriae, curli, flagella, adhesion protein, and capsules and firmly attach to the surface. Cells grow in close proximity and exhibit intense interaction, including cell-to-cell communication (quorum sensing) and formation of synergistic microconsortia. Autoinducers, such as *N*-acyl homoserine lactone AI-2, help in cell-to-cell communication and regulate gene expression for survival, growth, cell density, resistance to antimicrobials, and tolerance to desiccation (see Chapter 6). At this stage, the cells cannot be removed by rinsing. In the colonization stage, which is also irreversible, the EPS or complex polysaccharides may bind to metal ions on equipment surfaces, and the cells may metabolize products that can damage the surfaces.[1–5] As a microcolony continues to grow, cells accumulate, forming a matured biofilm with three-dimensional scaffolds. Loose cells are then sloughed off from the matured biofilm and convert into planktonic cells, which again attach to a new surface preconditioned by food particles or substrates completing the life cycle of a biofilm.

## Influencing Factors

The level of attachment of microorganisms to food-processing equipment surfaces is found to be directly related to contact time. As the contact time is prolonged, more cells attach to the

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surface, the size of the microcolony increases, and attachment between cells increases. Fimbriae formation by the cells occurs faster at optimum temperature and pH of growth. Limited studies also showed that when microorganisms, such as *Pseudomonas fragi* and *Listeria monocytogenes*, are grown together they form a more complex biofilm than when either is grown separately.[1–4]

The factors associated with the attachment of spoilage and pathogenic bacteria to meat and carcass surfaces can be divided into three groups: associated with bacteria, associated with meat, and extrinsic factors.[1–4] Many Gram-positive bacteria (*Clostridium*, *Micrococcus*, *Staphylococcus*, *Lactobacillus*, and *Brochothrix*) and Gram-negative bacteria (*Escherichia*, *Proteus*, *Pseudomonas*, *Serratia*, *Salmonella*, *Enterobacter*, *Shewanella*, and *Acinetobacter* spp.) can attach to skin and meat surfaces of chicken, pork, beef, and lamb. Some studies showed that several Gram-negatives, such as *Proteus* and *Pseudomonas* spp., attach more rapidly and in higher numbers than some Gram-positive bacteria, such as *Lactobacillus*, *Staphylococcus*, and *Micrococcus* spp. However, there are differences in observations, and some researchers think that the more negative charge the cells of species or strains have, the faster and higher their ability to attach to muscle surfaces is. Similarly, results differ among researchers with respect to the influence of types of food animals, birds, and microbial species to attachment. Bacterial attachment to lean tissues is generally higher than to adipose tissues; however, there is probably no difference in the attachment of both Gram-positive and Gram-negative bacterial species in the skin and muscle of different types of food animals and birds. Among the extrinsic factors, the number of attached cells on meat surfaces is directly related to contact time and cell concentrations used. At optimum growth temperature, a bacterial species shows a higher attachment rate. Electrical stimulation of the carcasses may favor attachment, but opinions differ on this.[2–4]

Intimate attachment of pathogens to fresh vegetables and fruits and their resistance to cleaning and washing are thought to be responsible for increasing produce-related outbreaks. *Escherichia coli* O157:H7 are found to attach to edges, grooves, or damaged tissues of lettuce, sprouts, or spinach. *Salmonella enterica* was also found on intact or broken skins of tomatoes, cantaloupes, and apples and damaged areas of cilantro leaves.[6]

## Assessment of Biofilm Formation

Various methods are used to assess the formation of biofilms on solid surfaces. The direct microbiological plating method involves swabbing of the surface by using moistened cotton swabs or sponges, and then diluted samples are spread on agar plates. To learn about the type of pathogens involved, selective agar media are used. Swabs are also enriched in selective enrichment broths and a polymerase chain reaction (PCR) is used for identification of the microorganisms. Contact plating is another method that allows direct imprinting of the surface onto the agar plate to determine the microbes in biofilms. However, the rough surface of test materials, oils (lipids), pressure on agar, and contact times may affect the result.

Indirectly, biofilm formation is also assessed by monitoring the ATP level of the surface swabs by luminescence assay because ATP is present in living microbes (see Chapter 42). However, food residues on the test surface may be a source of ATP, giving rise to false results. Traditional microscopic techniques involving both light microscopy and scanning electron microscopy are also used to examine biofilm structure and organization of a microbial community of cells (Figure 7.1). In addition, more sophisticated tools, such as laser scanning microscopy (LSM), magnetic resonance imaging (MRI), and scanning transmission microscopy (STXM), are also used to assess structure and composition of biofilms.[7]

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## Control and Removal of Biofilms

To control and remove biofilm in the food industry different strategies may be needed, including physical, chemical, and biological approaches.[8] Microbial biofilm formation on equipment surfaces and attachment to solid food surfaces can resist their removal and killing by using some of the traditional methods designed to remove or kill unattached microbial cells from a culture broth. Therefore, modified as well as novel methods must be applied because biofilms are difficult to remove if they are left to grow. They should be removed when the biofilms are young. The planktonic cells at the early stage of biofilm formation are more sensitive to cleaning and sanitizers than the sessile cells in the matured biofilms. In a food-processing operation, cleaning, rinsing, and sanitation, in that order, have to be done every few hours. Cleaning is essential to remove food residues, which interfere with sanitization. Removal and control of biofilms also depend on sanitizer exposure time, temperature, and mechanical action to break down the matrix to facilitate sanitizer penetration.

Several sanitization methods have been used such as enzyme-based detergents to break down the glycocalyx. EDTA, along with treatment by quaternary ammonium compounds following enzyme treatment, can be helpful. However, this may prove to be expensive because of the high price of enzymes compared to other chemical sanitizers. Some oxidizing agents, including chlorine and hydrogen peroxide, are useful and are effective against a broad range of microorganisms. Bacteriocins, such as those used as biopreservatives, including nisin, pediocin, reuterin, etc., are considered effective against microorganisms, including *Listeria monocytogenes* in a dairy processing plant. The antagonistic effects can be enhanced by acidic environment. Bacteriophages are viruses that infect bacteria, are considered natural and highly specific, and have great potential in controlling biofilms. A bacteriophage binds to the bacterial host irreversibly, and infects and lyses the bacteria. A bacteriophage engineered to express a biofilm-degrading enzyme can also be effective against both bacterial cells and the EPS. Use of quorum sensing antagonists, such as Furanones, Azithromycin, garlic extract, and 4-NPO (4-nitro-pyridine-N-oxide), is an intriguing approach to control biofilms.[9] In addition, processing equipment needs to be designed in such a way that would prevent formation of biofilm or have access to sanitizers to control and remove biofilms.

To reduce attachment of microorganisms to solid food surfaces, suitable control measures need to be adopted. Use of suitable preservatives, low $A_W$, low pH, and low storage temperature can be used judiciously in combination to reduce the rate of biofilm formation in food. Furthermore, to reduce pathogen loads on fruits and vegetables, thorough washing with sanitizers (Chapter 31) is effective. In addition, care should be taken to prevent the formation of a pathogen colonization niche on tissues by avoiding the induction of injury to fruits and vegetables during handling, processing, transportation, or storage.[6]

## Conclusion

Microbial attachment and biofilm formation are important strategies for microbial survival on food or food-contact surfaces under adverse conditions, such as limited nutrients, moisture, and oxygen. In the biofilm, quorum sensing facilitates bacterial cell-to-cell communication and persistence through production of EPS, which becomes the source of nutrients and helps resist sanitizers and physical removal from the surface.

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**QUESTIONS**

1. What is a biofilm? What surfaces are conducive for formation of biofilms?
2. What is the composition of a biofilm?
3. What methods are used for detection of bacterial biofilms?
4. Describe the mechanism or steps involved during biofilm formation.
5. List factors that contribute to biofilm formation.
6. Discuss various strategies that are used in the food industry to control and remove biofilms.

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# Chapter 8

# Microbial Metabolism of Food Components

## Introduction

Bacterial growth in food occurs though the metabolism of food components or nutrients mainly in the cytoplasm and cytoplasmic membrane (also in periplasmic space in Gram-negative bacteria) of cells.[1] The complete process involves the transport of nutrients from the environment (for macromolecules after their enzymatic breakdown) inside the cell through the cell wall and cell membrane, the breakdown of nutrients to generate energy and active building blocks, the synthesis of cell components from the building blocks (mainly macromolecules and structural and functional components), and the release of unusable end products into the environment. In addition, the recycling of cellular materials, cell differentiation, the repair of injured structures, and adaptation to environmental stresses are also mediated through metabolic processes. Some of these aspects are important in food microbiology and are briefly discussed in several sections in this book: sporulation in Chapter 9, sublethal injury and repair and stress adaptation in Chapter 10, and nutrient transport in Chapter 12. Several energy-generation and energy-degradation processes that are important in food microbiology are briefly discussed here (and also in Chapter 12).

## Respiration and Fermentation During Growth

During growth in a food, microorganisms synthesize energy and cellular materials. A large portion of the energy is used to synthesize cellular components. This is achieved through linking the energy-producing reactions with the reactions involved in the synthesis of cell materials. The energy-generating reactions are of the nature of oxidation, and in microorganisms, they are organized in sequences (metabolic pathways) for gradual liberation of energy from an organic substrate (in a reduced state). The energy is then either used directly in an endergonic reaction or stored for release during a later reaction. The energy can be stored by forming energy-rich intermediates



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capable of conserving the free energy as biochemical energy. Some of these are derivatives of phosphoric acid (nucleotide triphosphates, acylphosphates, and inorganic polyphosphates) and derivatives of carboxylic acids (acetyl-coenzyme A). The most important of these is ATP (adenosine triphosphate), which is formed from ADP (adenosine diphosphate) in coupled reactions through either oxidative phosphorylation or substrate-level phosphorylation.

The energy-liberating oxidation reactions of the substrates generate electrons ($H_2 \rightarrow 2H^+ + 2e^-$), which are then accepted by the oxidizing agents. This aspect is briefly discussed under redox potential in Chapter 6. In a food system, the substrates are mainly the metabolizable carbohydrates, proteins, and lipids. Microorganisms important in foods are heterotrophs (i.e., they require organic carbon sources, substances more reduced than $CO_2$) and chemoorganotrophs (i.e., they use organic compounds as electron donors to generate energy). On the basis of the nature of the terminal electron acceptors, there are different energy-generating reactions: aerobic respiration, which requires molecular oxygen as the electron acceptor; anaerobic respiration, in which inorganic compounds act as the electron acceptors; and fermentation, which uses organic compounds as electron acceptors.[1,2] The methods used for electron transfer by different groups of microorganisms important in food can be illustrated by the following scheme:

| Organic compounds Inorganic compounds ($NO_3$, $SO_4$) |
|---|
| 1 ↑5 2 ↑6 3 4<br>Substrates → (2H:NAD/FD) → cytochrome(s) → cyt.a → O2<br>↓7<br>$H_2O_2$ or $O_2$ |
| Aerobes (some *Bacillus* spp., *Pseudomonas*, molds, and yeasts): 1, 2, 3, 4 |
| Anaerobes |
| a. Cyt. independent, for example, some *Clostridium* spp.: 1, 5 |
| b. Cyt. dependent, for example, *Desulfotomaculum* spp.: 1, 2, 6 |
| Facultative anaerobes |
| a. Cyt. independent, for example, lactic acid bacteria, some yeasts |
| i. Aerobic: 1, 7 |
| ii. Anaerobic: 1, 5 |
| b. Cyt. dependent, for example, *Enterobacteriaceae* group |
| i. Aerobic: 1, 2, 3, 4, or 1, 7 |
| ii. Anaerobic: 1, 5 or 1, 2, 6 |

The energy-generating metabolic pathways also produce (from the substrates) many metabolic products that the microbial cells either use for the synthesis of cellular components or release into the environment. The nature of these metabolites differs greatly and depends on the nature of the substrates, the type of microorganisms with respect to their aerobiosis nature, and the oxygen availability (more correctly, redox potential) of the environment. The metabolism and growth of

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microorganisms in food are important for several reasons. Microbial spoilage of foods with the loss of acceptance qualities (e.g., flavor, texture, color, and appearance) is directly related to microbial growth and metabolism. Toxin production in food by food-poisoning microorganisms also results from their growth in a food. Many microbial metabolites are also important for their ability to produce desirable characteristics in fermented foods, such as texture, flavor, and long shelf life. Microbial metabolic products are also used in foods for processing (enzymes), preservation (bacteriocins and acids), and improving texture (dextran), and flavor (diacetyl).

Among the food components, the microbial metabolism of carbohydrates, proteins, and lipids are of major importance. Some of these metabolic pathways are briefly presented in this chapter. Foods, depending on the type, can contain many types of carbohydrates, proteins, and lipids. This has been discussed briefly previously. Depending on the type and source, foods also differ greatly in the amounts of the three groups of nutrients. Plant foods are, in general, rich in carbohydrates although some (e.g., nuts, lentils, and beans) are also rich in protein, and some others (e.g., oilseeds) are rich in lipids. Foods of animal origin are rich in proteins and lipids, whereas some (e.g., meat and fish) are low in carbohydrates. Others, such as milk, organ meats (liver), and mollusks (oysters), are rich in proteins as well as carbohydrates. Fabricated or formulated foods can have all the nutrients in sufficient quantities to support microbial growth. In general, microorganisms preferentially metabolize carbohydrates as an energy source over proteins and lipids. Thus, microorganisms growing in a food rich in metabolizable carbohydrates utilize carbohydrates, but in a food low in metabolizable carbohydrates and rich in metabolizable proteins, they metabolize proteins (after metabolizing the carbohydrates). In a food rich in both carbohydrates and proteins, microorganisms usually utilize the carbohydrates first, then produce acids and reduce the pH. Subsequent microbial degradation of proteins can be prevented at low pH, causing nondegradation of proteins or a protein-sparing effect. In the formulation of processed meat products, added carbohydrates can provide this benefit.

## Metabolism of Food Carbohydrates

Food carbohydrates comprise a large group of chemical compounds that include monosaccharides (tetroses, pentoses, and hexoses), disaccharides, oligosaccharides, and polysaccharides. Although carbohydrates are the most preferred source of energy production, microorganisms differ greatly in their ability to degrade individual carbohydrates.[1–5] Carbohydrates that are degraded at the cellular level as monosaccharides, disaccharides, and trisaccharides can be transported inside the cell and hydrolyzed to monosaccharide units before further degradation. Polysaccharides are broken down to monosaccharides and disaccharides by extracellular microbial enzymes (e.g., α-amylase) secreted in the environment before they can be transported and metabolized.

## Degradation of Polysaccharides

Molds, some *Bacillus* spp. and *Clostridium* spp., and several other bacterial species can degrade starch, glycogen, cellulose, pectin, and other polysaccharides by extracellular enzymes. The monosaccharides and disaccharides are then transported in the cell and metabolized. Breakdown of these polysaccharides, especially pectins and cellulose, in fruits and vegetables by microorganisms can affect the texture and reduce the acceptance quality of the products.

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## Degradation of Disaccharides

Disaccharides of foods, either present in food (lactose, sucrose) or produced through microbial degradation (maltose from starch), are hydrolyzed to monosaccharides inside the cell by specific enzymes: lactose by lactase to galactose and glucose, sucrose by sucrase to glucose and fructose, and maltose by maltase to glucose. Many microbial species cannot metabolize one or more disaccharides.[1–5]

## Degradation of Monosaccharides

Monosaccharides are degraded (catabolized) by aerobic, anaerobic, and facultative anaerobic microorganisms via several pathways that generate many types of intermediate and end products. The metabolic pathways are dependent on the type and amount of monosaccharides, the type of microorganisms, and the redox potential of the system. Although all microorganisms important in foods can metabolize glucose, they differ greatly in their ability to utilize fructose, galactose, tetroses, and pentoses. Fermentable monosaccharides are metabolized by five major pathways, and many microbial species have more than one pathway. They are the Embden-Meyerhoff-Parnas (EMP) pathway, the hexose monophosphate shunt (HMP) pathway, the Entner-Doudroff (ED) pathway, and two phosphoketolase (PK) pathways (pentose phosphoketolase and hexose phosphoketolase). Pyruvic acid produced via these pathways is subsequently metabolized by microorganisms in several different pathways through fermentation, anaerobic respiration, and aerobic respiration.[1–5]

## Fermentation

Monosaccharides are fermented by anaerobic and facultative microorganisms by the five major pathways mentioned previously.[1–3] In addition, several other pathways are used, especially for the metabolism of pyruvate, by some specific microbial species and groups. In general, the terminal electron acceptors are organic compounds, and energy is produced at the substrate level. The overall reactions of the five main pathways and the end products are briefly listed in Table 8.1 (also in Table 11.1).[1–3] The metabolic pathways are discussed in more detail in Chapter 12.

**Table 8.1 End Products of Carbohydrate Metabolism by Some Microorganisms**

| *Microbial Type* | *Fermentation Pattern* | *Major End Products* |
|---|---|---|
| Yeasts | Alcohol | Ethanol, $CO_2$ |
| Lactic acid bacteria | a. Homofermentative | Lactate |
| | b. Heterofermentative | Lactate, acetate, ethanol, $CO_2$, diacetyl, acetoin |
| Bifidobacteria | Bifidus (hexose ketolate) | Lactate, acetate |
| Propionibacteria | Propionic acid | Propionate, acetate, $CO_2$ |
| *Enterobacteriaceae* | Mixed acid | Lactate, acetate, formate, $CO_2$, $H_2$, succinate |
| *Bacillus, Pseudomonas* | Butanediol | Lactate, acetate, formate, 2,3-butanediol, $CO_2$, $H_2$ |
| *Clostridium* | Butyric acid | Butyrate, acetate, $H_2$, $CO_2$, butanol, ethanol, acetone, isopropanol |

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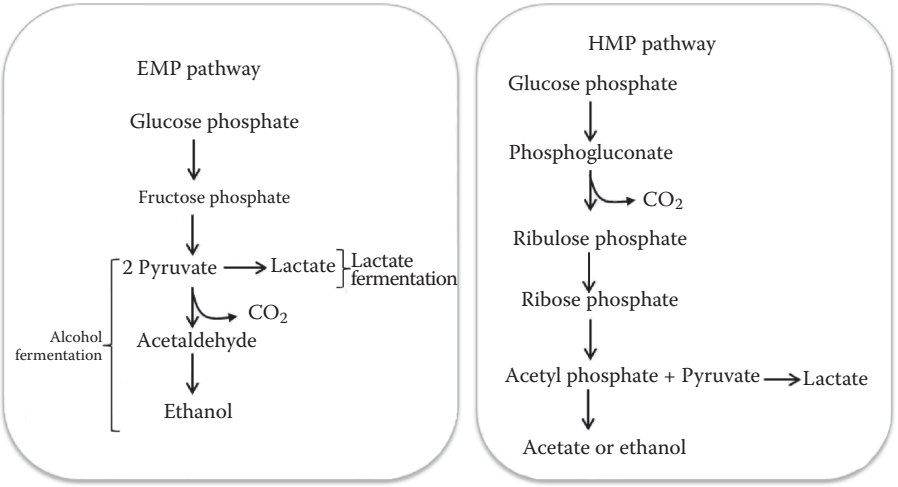



**Figure 8.1 The Embden-Meyerhoff-Parnas (EMP) pathway and the hexose monophosphate shunt (HMP) pathway.**

### *EMP Pathway*

The EMP pathway is used by homofermentative lactic acid bacteria, *Enterococcus faecalis*, *Bacillus* spp., and yeasts (Figure 8.1). Other hexose monophosphates enter the EMP pathway at different steps, mostly before fructose diphosphate.

### *HMP Pathway*

The HMP pathway is also called the HMP shunt, pentose cycle, or Warburg–Dickens–Horecker pathway (Figure 8.1). It is used by heterofermentative lactic acid bacteria, *Bacillus* spp., and *Pseudomonas* spp. Ribose phosphate can be used for the synthesis of ribose and deoxyribose moieties in nucleic acids.

### *Entner-Doudroff (ED) Pathway*

The ED pathway is used by *Pseudomonas* spp.

$$\text{Glucose phosphate} \rightarrow \text{Phosphogluconate} \rightarrow \text{Pyruvate} + \text{Glyceraldehyde phosphate}$$

$$\text{Pyruvate} \rightarrow \text{Acetaldehyde} \rightarrow \text{Ethanol}$$

$$\downarrow$$

$$CO_2$$

$$\text{Glyceraldehyde phosphate} \rightarrow \text{Pyruvate} \rightarrow \text{Acetaldehyde} \rightarrow \text{Ethanol}$$

$$\downarrow$$

$$CO_2$$

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### *Pentose Phosphoketolase Pathway*

This pathway is used by *Escherichia coli*, *Enterobacter aerogenes*, *Bacillus* spp., and some lactic acid bacteria.

$$\text{Ribose phosphate} \rightarrow \text{Xylulose phosphate} \rightarrow \text{Acetyl-Phosphate} + \text{Pyruvate} \rightarrow \text{Acetate or Ethanol} + \text{Lactate}$$

### *Hexose Phosphoketolase Pathway*

This pathway is also called the Bifidus pathway and is used by *Bifidobacterium* spp.

$$2\ \text{Glucose phosphate} \rightarrow \text{Acetyl phosphate} + 2\ \text{Xylulose phosphate} \rightarrow 3\ \text{Acetate} + 2\ \text{Pyruvate} \rightarrow 3\ \text{Acetate} + 2\ \text{Lactate}$$

### *Some Specific Pathways*

#### Mixed Acid Fermentation

Mixed acid fermentation is characteristic of members of *Enterobacteriaceae*. Pyruvate produced from monosaccharide fermentation can be used to produce different end products.

$$\text{Pyruvate} \rightarrow \text{Lactate}$$

$$\text{Pyruvate} \rightarrow \text{Formate} + \text{Acetyl} \sim \text{CoA}$$

$$\text{Formate} \rightarrow H_2 + CO_2$$

$$\text{Acetyl} \sim \text{CoA} \rightarrow \text{Acetate and Ethanol}$$

$$\text{Pyruvate} \rightarrow \text{Succinate}$$

#### Propionic Acid Fermentation

This type of fermentation is carried out by propionic bacteria and *Clostridium* spp.

$$\text{Lactate} \rightarrow \text{Pyruvate} \rightarrow \text{Propionyl} \sim \text{CoA} \rightarrow \text{Propionate (propionibacteria)}$$

$$\text{Lactate} \rightarrow \text{Acrylyl} \sim \text{CoA} \rightarrow \text{Propionyl} \sim \text{CoA} \rightarrow \text{Propionate (}\textit{Clostridium}\text{ spp).}$$

#### Butyrate, Butanol, and Acetone Fermentation

This type of fermentation is carried out by *Clostridium* spp.

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$$\text{Pyruvate} \rightarrow H_2 + CO_2 + \text{Acetyl} \sim \text{CoA}$$

$$\text{Acetyl} \sim \text{CoA} \rightarrow \text{Acetate, Butyrate, Butanol, and Acetone}$$

$$\text{Acetyl} \sim \text{CoA is used to produce different compounds}$$

### Diaetyl, Acetoin, and Butanediol Fermentation

This type of fermentation is carried out by some lactic acid bacteria and some members of Enterobacteriaceae.

$$2\ \text{Pyruvate} \rightarrow [\text{Pyruvate} + \text{Acetaldehyde} \sim \text{TPP}] \rightarrow \alpha\text{-Acetolactate}$$
$$\downarrow$$
$$CO_2$$

$$CO_2 \quad 2H^+ \qquad 2H^+$$
$$\downarrow \quad \downarrow \qquad \downarrow$$
$$\rightarrow \text{Diacetyl} \rightarrow \text{Acetoin} \rightarrow \text{2, 3-Butanediol}$$

## *Anaerobic Respiration*

Sulfate-reducing *Desulfotomaculum nigrificans* metabolizes glucose as an energy source, primarily through the EMP pathway, to produce pyruvate, which is then decarboxylated to generate acetate (or ethanol) and $CO_2$. Sulfate, acting as an electron acceptor, is reduced to generate $H_2S$. $NO_3$-reducing bacteria, containing nitrate reductase (such as the species in the family Enterobacteriaceae, some *Bacillus* spp., and *Staphylococcus* spp.), degrade metabolizable carbohydrates through EMP, HMS, and ED (also mixed-acid fermentation) pathways. Pyruvate produced through these pathways can act as an effective electron donor and, depending on the species, may be converted to lactate, acetate, ethanol, formate, $CO_2$, $H_2$, butanediol, acetoin, and succinate.

## *Aerobic Respiration*

Aerobes (*Bacillus* spp., *Pseudomonas* spp., molds, and yeasts) and many facultative anaerobes (*Enterobacteriaceae, Staphylococcus* spp.) under aerobic conditions can use molecular oxygen as the terminal electron acceptor during metabolism of carbohydrates to produce pyruvate by one or more of the major pathways mentioned earlier. Pyruvate, as well as other carboxylic acids, can be oxidized completely through oxidative decarboxylation to generate $CO_2$, $H_2O$, and large quantities of ATP. The pathway (designed as the Krebs cycle, the tricarboxylic acid cycle, or the citric acid cycle) also generates large numbers of intermediates that are utilized to synthesize cell materials. Initially, pyruvate is decarboxylated to generate acetyl~CoA and $CO_2$. Acetyl~CoA then combines with oxaloacetate (4C compound) to produce citrate (6C compound). Through successive reactions, citrate is metabolized to a 5C compound (and $CO_2$) and a 4C compound (and

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$CO_2$). The 4C succinate is then, through several steps, converted to oxaloacetate for reuse. During these reactions, reducing compounds are generated that, in turn, enter the electron transport system, thereby generating $2H^+$ and $2e^-$. The terminal cytochrome, cytochrome oxidase (cyt.a), releases the electron for its acceptance by oxygen. If the cyt.a transfers only two pairs of electrons to molecular oxygen, the end product is $H_2O$; if one pair of electrons is transferred, the product is $H_2O_2$, which is subsequently hydrolyzed by microbial catalase or peroxidase to $H_2O$ and $O_2$. Each pyruvate can potentially generate 15 ATP molecules.

### *Synthesis of Polymers*

*Leuconostoc mesenteroides* cells growing on sucrose hydrolyze the molecules and predominantly metabolize fructose for energy production. Glucose molecules are polymerized to form dextran (a polymer of glucose). Polymers are also formed from carbohydrates by some *Lactococcus lactis* and *Lactobacillus* strains, *Alcaligenes faecalis* strains, and *Xanthomonas* spp. Some of these polymers are useful as food stabilizers and to give viscosity in some fermented foods; they can also cause quality loss in some foods.

Metabolism of food carbohydrates by microorganisms is undesirable when it is associated with spoilage. On the other hand, fermentation of carbohydrates is desirable in food bioprocessing and production of metabolites for use in foods (such as lactate and diacetyl). Several end products are also used to identify microorganisms; for example, 2,3-butanediol production by *Enterobacter* spp. helps to differentiate them from the nonproducer *Esc. coli* strains (Voges Proskauer test). The microbial ability to metabolize different polysaccharides, disaccharides, and monosaccharides is also used to identify unknown isolates.

## Metabolism of Food Proteins

Proteinaceous compounds present in foods include different types of simple proteins (e.g., albumin, globulin, zein, keratin, and collagen), conjugated proteins (e.g., myoglobin, hemoglobin, and casein), and peptides containing two or more amino acids. Amino acids, urea, creatinine, trimethyl amine, and others form the nonprotein nitrogenous (NPN) group. In general, microorganisms can transport amino acids and small peptides (ca. 8–10 amino acids long) in the cells. Proteins and large peptides in a food are hydrolyzed to amino acids and small peptides by microbial extracellular proteinases and peptidases. Species from genera *Alcaligenes*, *Bacillus*, *Clostridium*, *Enterococcus*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Lactococcus*, *Micrococcus*, *Pseudomonas*, and *Serratia* are among those capable of producing extracellular proteinases and peptidases. Small peptides are transported in the cell and converted to amino acids before being metabolized further.[1,4,5]

### *Aerobic Respiration (Decay)*

Many aerobic and facultative anaerobic bacteria can oxidize amino acids and use them as their sole source of carbon, nitrogen, and energy. L-Amino acids generally undergo either oxidative deamination or transamination to produce respective keto acids, which are then utilized through different pathways. Several amino acids can also be oxidized in different pathways by many bacterial species. Some examples are conversion of L-threonine to acetaldehyde and glycine, L-tryptophan to anthranilic acid, L-lysine to glutaric acid, L-valine to ketoisovalerate, L-leucine to ketoisocaproate, L-arginine to citrulline, and L-histidine to urocanic acid.

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### *Fermentation (Putrefaction)*

Degradation of L-amino acids by anaerobic and facultative anaerobic bacteria is carried out either with single amino acids or two amino acids in pairs. Metabolism of single amino acids is carried out through different types of deamination (producing the C-skeletones and $NH_3$), decarboxylation (producing amines and $CO_2$), and hydrolysis (producing the C-skeletones, $CO_2$, $NH_3$, and $H_2$). The C-skeletones (fatty acids, α-keto acids, and unsaturated acids) are then used to supply energy and other metabolic products. The metabolism of amino acids in pairs involves simultaneous oxidation-reduction reactions between suitable pairs in which one acts as the hydrogen donor (oxidized) and the other as the hydrogen acceptor (reduced). Alanine, leucine, and valine can be oxidized, whereas glycine, proline, and arginine can be reduced by this type of reaction (Stickland reaction). The products in this reaction are fatty acids, $NH_3$, and $CO_2$. Figure 8.2 shows an example of fermented fish (proteinaceous) products from South Korea.

The products of microbial degradation of amino acids vary greatly with the types of microorganisms and amino acids and the redox potential of the food. Some of the products are keto acids, fatty acids, $H_2$, $CO_2$, $NH_3$, $H_2S$, and amines. Metabolic products of several amino acids are of special significance in food because many of them are associated with spoilage (foul smell) and health hazards. They include indole and skatole from tryptophan, putrescine and cadaverine from lysine and arginine, histamine from histidine, tyramine from tyrosine, and sulfur-containing compounds ($H_2S$, mercaptans, and sulfides) from cysteine and methionine. Some of these sulfur compounds as well as proteolytic products of proteinases and peptidases (both extracellular and endocellular) of starter-culture microorganisms are important for desirable and undesirable (bitter) flavor and texture in several cheeses. The breakdown of threonine to acetaldehyde by *Lactobacillus acidophilus* is used to produce the desirable flavor in acidophilus yogurt. Indole production from tryptophan is used to differentiate *Esc. coli* from other coliforms. Also, the amino acid metabolism profile is used in species identification of unknown bacterial isolates. In addition to degradation (catabolism) of proteinaceous compounds of foods, the synthesis (anabolism) of several proteins by some



**Figure 8.2 (See color insert.) Ethnic fermented fish products from South Korea.**

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foodborne pathogens while growing in foods is important because of its ability to produce proteins that are toxins. They include thermostable toxins of *Staphylococcus aureus*, thermolabile toxins of *Clostridium botulinum*, and toxins produced by some bacteria associated with foodborne infections (such as Shiga toxin). The ability of some microbial species to synthesize essential amino acids (such as L-lysine), antibacterial peptides (such as nisin and pediocin), and enzymes (such as amylases and proteinases) in relatively large amounts has been used for beneficial purposes in foods.

## Metabolism of Food Lipids

The main lipids in food are monoglycerides, diglycerides, and triglycerides; free saturated and unsaturated fatty acids; phospholipids; sterols; and waxes, with the glycerides being the major lipids. Microorganisms have a low preference for metabolizing lipids. Being hydrophobic, lipids are difficult to degrade when present in a large mass. In emulsion, they can be metabolized by the microorganisms at the oil-water interphase. Glycerides are hydrolyzed by extracellular lipases to release glycerol and fatty acids. The fatty acids then can be transported inside the cells and metabolized by β-oxidation to initially generate acetyl-CoA units before being utilized further. Fatty acids, if produced at a rapid rate, accumulate in the food. Unsaturated fatty acids can be oxidized by microbial oxidases to initially produce hydroperoxides and then carbonyl compounds (aldehydes and ketones).

Some of the microorganisms that are important in food and release lipases (hydrolytic enzymes) are found in the following genera: *Alcaligenes*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Aspergillus*, *Geotrichum*, and *Penicillium*. Oxidative enzymes are produced mainly by the molds. Both groups of enzymes are associated with food spoilage, but oxidative enzymes are also important for desirable flavor in mold-ripened cheeses.

## Conclusion

Microbial growth in food is accomplished through the metabolism of food nutrients, principally the metabolizable carbohydrates, proteins, and lipids. Many metabolic pathways can be used, which are determined by the nature and concentration of a nutrient, the microbial type, and the oxidation-reduction potential of the food environment. Many types of end products are produced during metabolism of the nutrients, which, depending on the chemical nature, are associated with food spoilage, food poisoning, or production of fermented food. Some are also used to improve texture and flavor of foods. The metabolic end products are also useful to identify unknown microbial isolates from a food. As long as the physical and nutritional environments are maintained, bacterial cells continue to multiply, generating energy and cellular materials through the metabolic processes. However, a change in the environment can cause some species to shut down the cell multiplication cycle and trigger the sporulation cycle, in which a cell is differentiated as an endospore. This survival strategy under unfavorable conditions and its importance in food microbiology are discussed in Chapter 9.

### QUESTIONS

1. Discuss the major differences among aerobic respiration, anaerobic respiration, and fermentation of food nutrients by microorganisms.
2. Discuss how aerobes, anaerobes, and facultative anaerobes differ from each other in their ability to transfer electrons by different acceptors.

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3. A facultative bacterial species is growing anaerobically in the following foods: one rich in carbohydrates but low in proteins, the second rich in proteins but low in carbohydrates, and the third rich in both. Also, the carbohydrates and proteins in all three foods can be metabolized by the bacterial species. Suggest how the bacterial species will metabolize the two nutrients in these foods.
4. How are the polysaccharides and disaccharides metabolized by microorganisms?
5. List the five major pathways microorganisms use to metabolize monosaccharides found in foods.
6. List some metabolites produced by *Esc. coli* and *Enterobacter* spp. by mixed acid fermentation and *Clostridium* spp. by butyric acid fermentation.
7. Discuss briefly the significance of microbial metabolism of carbohydrates by aerobic and anaerobic respiration.
8. How are food proteins metabolized by bacteria? What is the importance of protein synthesis during growth of microorganisms in food?
9. Discuss the differences and importance of amino acid degradation by microorganisms via fermentation and aerobic respiration in food.
10. What is the protein sparing effect and how can it be used advantageously in the production of low-carbohydrate, high-protein foods?
11. List three examples of metabolism of amino acids that can be used for laboratory identification of an unknown bacterial isolate from food.
12. What is the significance of lipid metabolism by microorganisms in food?

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# Chapter 9

# Microbial Sporulation and Germination

## Introduction

Microorganisms that are important in food normally divide by binary fission (or elongation as in nonseptate molds). In addition, molds, some yeasts, and some bacteria can form spores. In molds and yeasts, sporulation is associated with reproduction (and multiplication), whereas in bacteria it is a process of survival in an unfavorable environment. In molds and yeasts, sporulation can occur by sexual and asexual reproduction, and sexual reproduction provides a basis for strain improvement for those that are used industrially. In bacteria, sporulation occurs through differentiation, and it provides a means to retain viability in a harsh environment. Among the spores, bacterial spores have special significance in foods because of their resistance to many processing and preservation treatments used in food. Compared with bacterial spores, mold and yeast spores are less resistant to such treatments. Spore formation in molds, yeasts, and bacteria is briefly discussed here.

## Mold Spores

Molds form spores by both asexual and sexual reproduction and, on this basis, are classified as perfect or imperfect molds, respectively.[1] Molds form large numbers of asexual spores and, depending on the type, can form conidia, sporangiospores, and arthrospores. Conidia are produced on special fertile hyphae called conidiophores (Figure 9.1). Among the important molds in food, *Aspergillus* and *Penicillium* species form conidia (see Figure 28.1, Chapter 28). Sporangiospores are formed in a sack (sporangium) at the tip of a fertile hypha (sporangiospores). *Mucor* and *Rhizopus* species are examples of molds that form sporangiospores. Arthrospores, formed by the segmentation of a hypha, are produced by *Geotrichum*. An asexual spore in a suitable environment germinates to form a hypha and resumes growth to produce the thallus. Sexual spores form from the union of the tips of two hyphae, two gametes, or two cells. However, among the molds important in food, sexual reproduction is rarely observed. Some examples include *Mucor* and *Neurospora*.



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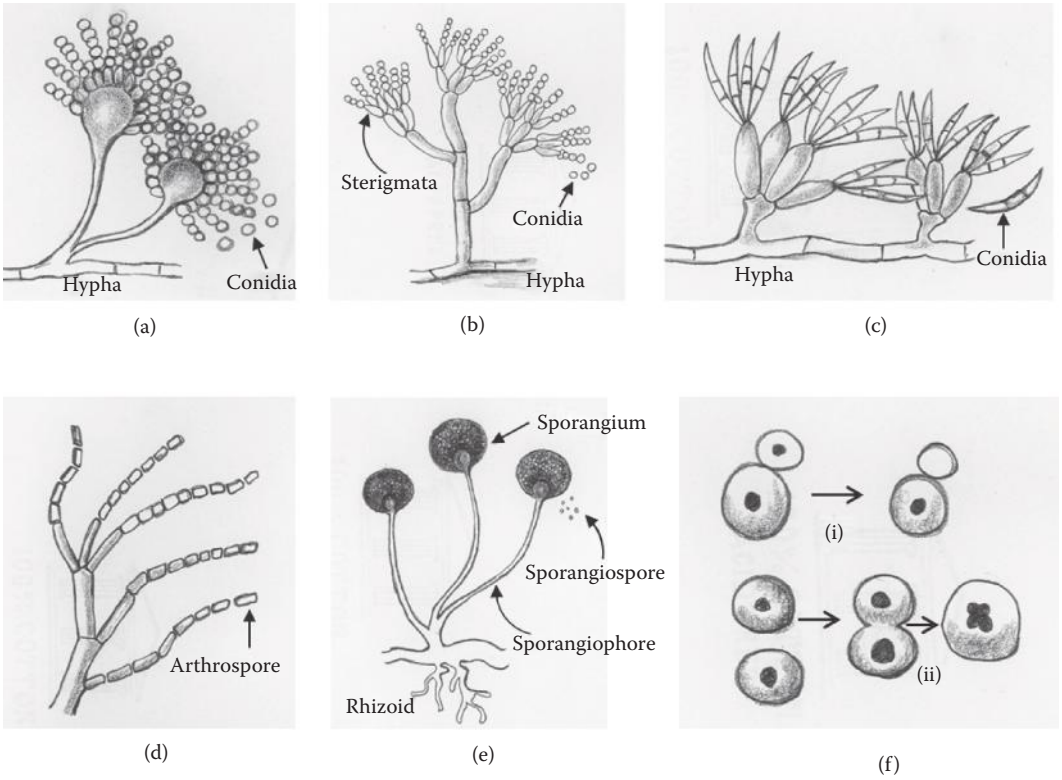



**Figure 9.1 Schematics of spores of molds and yeasts. Conidiophore with condial head and conidia of (a) *Aspergillus* sp., (b) *Penicillium* sp., (c) *Fusarium* sp., (d) arthrospore in *Geotrichum* sp., (e) sporangiophore with sporangium containing sporangiospores of *Rhizopus* sp., (f) ascospore formation in yeasts by conjugation of (i) mother–daughter and (ii) two separate cells.**

## Yeast Spores

On the basis of spore-forming ability, yeasts important in food are divided into two groups: Those that can produce sexual ascospores are designated as *Ascomycetes* (true yeasts), and those that do not form spores are called false yeasts. Examples of some yeasts important in food that form ascospores are *Saccharomyces*, *Kluyveromyces*, *Pichia*, and *Hansenula*. Species in the genera *Candida*, *Torulopsis*, and *Rhodotorula* do not form spores. Ascospores form by the conjugation of two yeast cells; in some cases, this can result from the union of the mother cell and a bud (daughter cell) (Figure 9.1). The number of spores developed in an ascus varies by species. In a suitable environment, each spore develops into a yeast cell.

## Bacterial Spores

The ability to form spores is confined to only a few bacterial genera, namely the Gram-positive *Bacillus*, *Alicyclobacillus*, *Clostridium*, *Sporolactobacillus*, and *Sporosarcina* and the

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Gram-negative *Desulfotomaculum* species. Among these, *Bacillus*, *Alicyclobacillus*, *Clostridium*, and *Desulfotomaculum* are of considerable interest in food because they include species implicated in food spoilage and foodborne diseases.[2–4] Several *Bacillus* and *Clostridium* species are used to produce enzymes important in food bioprocessing.

In contrast to mold and yeast spores, bacterial cells produce endospores (inside a cell and one spore per cell). During sporulation and until a spore emerges following cell lysis, a spore can be located terminally, centrally, or off-center, causing bulging of the cell. Under a phase-contrast microscope, spores appear as refractile spheroid or oval structures. The surface of a spore is negatively charged and hydrophobic. Spores, as compared with vegetative cells, are much more resistant to physical and chemical antimicrobial treatments, many of which are employed in the processing and preservation of food. This is because the specific structure of bacterial spores is quite different from that of the vegetative cells from which they are formed. From inside to outside, a spore has the following structures (Figure 9.2): a protoplasmic core, containing important cellular components, such as DNA, RNA, enzymes, dipicolinic acid (DPN), divalent cations, and very little water; an inner membrane, which is the forerunner of the cell cytoplasmic membrane; the germ cell wall, which surrounds this membrane and is the forerunner of the cell wall in the emerging vegetative cell; the cortex, around the cell wall, composed of peptides, glycan, and an outer forespore membrane; and the spore coats, outside the cortex and membrane, composed of layers of proteins that provide resistance to the spores. Spores of some species can have a structure called exosporium outside the coat. During germination and outgrowth, the cortex is hydrolyzed and the outer forespore membrane and spore coats are removed by the emerging cell.

The spores are metabolically inactive or dormant and can remain in dormant form for years but are capable of emerging as vegetative cells (one cell per spore) in a suitable environment. As opposed to non-spore-forming bacteria, the life cycle of spore-forming bacteria has a vegetative cycle (by binary fission) and a spore cycle (Figure 9.3). The spore cycle also goes through several stages in sequence, during which a cell sporulates and a vegetative cell emerges from a spore. These stages are genetically controlled and influenced by different environmental parameters and biochemical events, which are briefly discussed here.

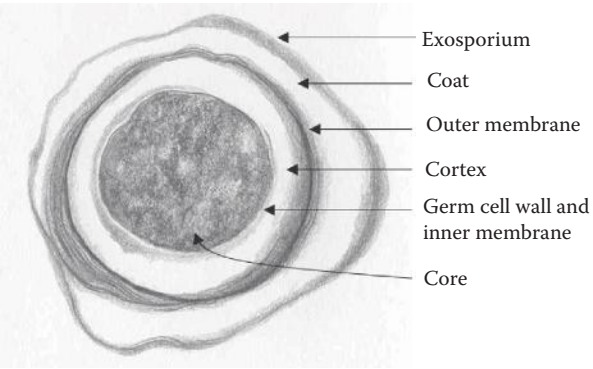



**Figure 9.2 Schematic section of a bacterial spore from *Clostridium botulinum*.**

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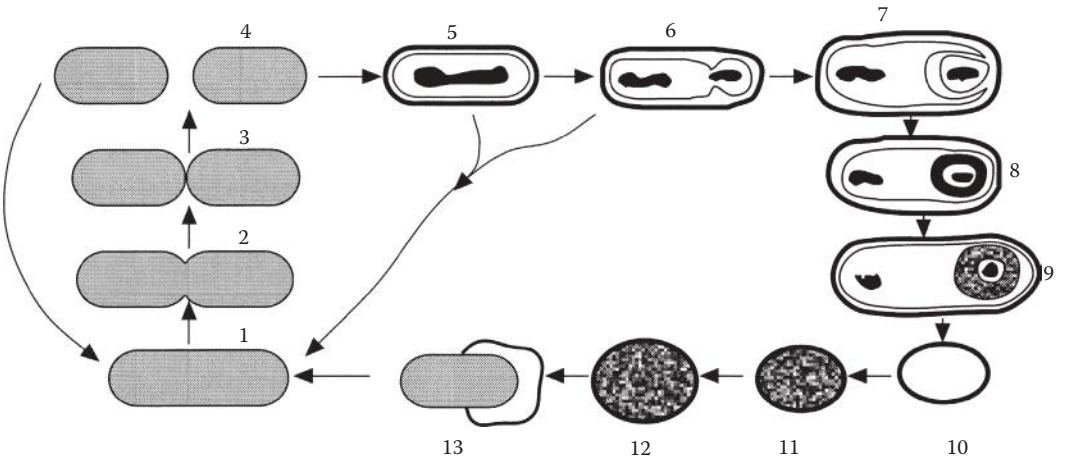



**Figure 9.3 Schematic presentation of the cycles of cell multiplication (1–4) and endospore formation, germination, and outgrowth of spore-forming bacteria (5–13). Different steps are (5) formation of axil filament, (6) septation, (7) prespore formation, (8) cortex formation, (9) coat formation, (10) free spore, (11) germination following activation, (12) swelling of spore, (13) outgrowing cell. Cells from step 4 can either divide (1–4) or sporulate. Cells from steps 5 and 6 can reverse back to cell division; from step 7, the process is irreversible.**

## *Sporulation*

The transition from a normal vegetative cell cycle to sporulation in spore-forming bacteria is triggered by the changes in the environmental parameters in which the cells are growing as well as a high cell number existing in the environment. The environmental factors include reduction in nutrient availability (particularly carbon, nitrogen, and phosphorous sources) and changes in the optimum growth temperature, $A_W$, and pH.[5] Transition from cell division cycle to sporulation is genetically controlled, involving many genes. A cell initiates sporulation only at the end of completion of DNA replication. A triggering compound may be involved at that time for a cell to decide to either go through normal cell division or to initiate steps for sporulation. The triggering compound is probably synthesized when nutrition depletion and other unfavorable conditions occur. Adenosine bistriphosphate (Abt) could be one of the triggering compounds as it is synthesized by spore formers under carbon or phosphorous depletion.

Sporulation events can be divided into approximately seven stages[2,3,6] (Figure 9.3):

1. Termination of DNA replication, alignment of chromosome in axial filament, and formation of mesosome.
2. Invagination of cell membrane near one end and completion of septum.
3. Engulfment of prespore or forespore.
4. Formation of germ cell wall and cortex, accumulation of $Ca_2^+$, and synthesis of DPN.
5. Deposition of spore coats.
6. Maturation of spore: dehydration of protoplast, resistance to heat, and refractile appearance.
7. Enzymatic lysis of wall and liberation of spore.
8. The process is reversible before stage 3. However, once the process has entered stage 3, a cell is committed to sporulation.[2,3]

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## *Dormancy*

Spores are formed in such a manner as to remain viable in unfavorable conditions. This is achieved by increasing their resistance to extreme environments and reducing metabolic activity to dormancy. Dehydration of the core and reduced molecular movement have been attributed to dormancy.

In a suitable environment, the dormancy of a spore can be ended through a series of biochemical reactions involved in spore activation, germination, outgrowth, and growth. Some spores may need a long time before they go through the sequences of germination and are called superdormant spores. They are quite common in *Bacillus* and *Clostridium*. Superdormancy is thought to be the consequence of the inherent nature of a spore, spore injury, and environmental factors. Some spores have stringent germination needs and do not germinate with other spores. Injured spores need to repair their injury before they can germinate and outgrow. Some components in the media can prevent germination of some spores. In food, superdormant spores could cause problems. Following processing, they may not be detected in a food by conventional testing methods. But during storage, they can germinate and outgrow and subsequently cause spoilage of a food, or if it's a pathogen, a spore can make a food unsafe for consumption.[2,3]

## *Activation*

Spore activation before germination is accompanied by reorganization of macromolecules in the spores. Spores can be activated in different ways, such as sublethal heat treatment, radiation, high-pressure treatment with oxidizing or reducing agents, exposure to extreme pH, treatment with high pressure, and sonication. These treatments probably accelerate the germination process by increasing the permeability of spore structures to germinating agents for macromolecular reorganization. This process is reversible, that is, a spore does not have to germinate after activation if the environment is not suitable.[2,3]

## *Germination*

Several structural and functional events occur during germination. Once the germination process starts, the dormant stage is irreversibly terminated. Structural changes involve hydration of core, excretion of $Ca_2^+$ and DPN, and loss of resistance and refractile property. Functional changes include initiation of metabolic activity, activation of specific proteases and cortex-lytic enzymes, and release of cortex-lytic products. Generally, germination is a metabolically degradative process.

Germination can be initiated (triggered) by low pH, high temperature, high pressure, lysozyme, nutrients (amino acids, carbohydrates), calcium-DPN, and other factors. The process can be inhibited by D-alanine, ethanol, EDTA, NaCl (high concentrations), $NO_2$, and sorbate.[2,3]

## *Outgrowth*

Outgrowth constitutes the biosynthetic and repair processes between the periods following germination of a spore and before the growth of a vegetative cell. The events during this phase include swelling of the spore as a result of hydration and nutrient uptake; repair and synthesis of RNA, proteins, and materials for the membrane and cell wall; dissolution of coats; cell elongation; and DNA replication. The factors that can enhance the process include favorable nutrients, pH, and temperature. With the termination of the outgrowth stage, vegetative cells emerge from spores and enter the vegetative cell cycle of growth by binary fission.[2,3,6]

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The sporulation process, as discussed previously, involves irreversible cellular differentiation processes in an unfavorable environment and is regulated and expressed by a large number of genes. The functions of many genes have been studied with mutants of *Bacillus subtilis* (*spo* mutants) that block formation of different components during the sporulation process. Expression of these functional genes is regulated by the genes, coding for specific sigma factors (δ). In a favorable environment, germination and outgrowth are also mediated through other genes that enable an endospore to enter into the cell division cycle.[2]

## Importance of Spores in Food

Spore formation, especially by molds and some bacterial species, enables them to survive for a long time and provides a basis for the continuation of the species. It also provides a means of their easy dissemination by dust and air in the environment.[1,2] In this manner, foods can be contaminated by their spores rather easily from various sources. In a suitable food environment, spores germinate, grow, and produce undesirable (or desirable) effects. Mold and yeast spores are relatively sensitive to heat, and their growth can also be prevented by storing foods in the absence of air.

Many species of *Bacillus*, *Clostridium*, and *Desulfotomaculum* are associated with food spoilage and foodborne diseases. As a result of high heat resistance, the spores are of special interest and importance in food processing. Special attention must be given to processing and preserving the foods so that the spores are either destroyed or prevented from undergoing germination and outgrowth because ungerminated spores cannot cause spoilage or foodborne disease. Another possibility is to induce the spores to germinate and outgrow and then expose them to an antibacterial treatment to destroy them. Superdormant spores of the spoilage and pathogenic species pose another problem. As they are not detected normally with the other spores, a processing condition could be wrongly adopted with the idea that it will eliminate all spores. Subsequently, these surviving superdormant spores can germinate, outgrow, and grow, and can either cause the food to spoil or make it unsafe. As it is impossible to destroy all spores in many foods, several specific methods or a combination of processing and preservation methods have been developed to overcome problems of spores in food.[1–3]

In the canning of low-acid foods, very high heat treatment is employed to achieve commercial sterility that kills spores of all pathogenic bacteria and most spoilage bacteria (except some thermophilic spoilage bacteria). To prevent germination of spores, depending on the food type, nitrites (in processed meat), low pH (acid products), low $A_W$, or high salt are used. High hydrostatic pressure is currently being studied to determine its spore destruction potential. Although spores of molds are destroyed at relatively low pressure (<400 MPa), spores of many pathogenic and spoilage bacteria need a combination of very high pressure (≥700 MPa) and high temperature (≥90°C) to obtain commercial sterility.[7] It has been known for long that spores of many foodborne bacteria can be activated to germination and outgrowth at a lower pressure range. Following such a pressure treatment, another antibacterial treatment, such as another pressure cycle, heat, or antimicrobial preservative can be given to destroy the germinated and outgrown spores before cell growth starts. The influence of several parameters on germination, such as pressure range, time of pressurization, pressurization temperature, and holding time following pressurization, is currently being studied.[8] Table 9.1 presents the results of a study. It can be seen that under any given condition, germination induction varies greatly with species. In general, germination increases with pressure and temperature within the range studied. More such studies with many strains of the important species will provide more meaningful data to determine the potential of such treatment to control bacterial spores in food.

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**Table 9.1 Germination Induction of Bacterial Endospores by Hydrostatic Pressure at 25°C and 50°C**

| *Bacterial Strain* | *Pressurization for Five Minutes at Temperature (°C)* | *% Germination Induction[a] at MPa[b]* | | |
|---|---|---|---|---|
| | | *138* | *345* | *483* |
| *Bacillus cereus* ATCC10876 | 25 | 99 | 99 | 99 |
| | 50 | 99 | 99 | 99 |
| *Bacillus stearothermophilus* ATCC 12980 | 25 | 45 | 88 | 88 |
| | 50 | 99 | 98 | 98 |
| *Clostridium sporogenes* PA 3679 | 25 | 0 | 0 | 4 |
| | 50 | 40 | 50 | 82 |
| *Clostridium perfringens* ATCC 1027 | 25 | 17 | 21 | 29 |
| | 50 | 12 | 40 | 44 |

[a] The spore suspensions were pressurized for five minutes either at 25°C or 50°C, then stored at 4°C for one hour and heated at 75°C for 15 minutes to destroy the germinated spores. Germination induction was determined by enumerating the surviving spores and subtracting the number from the unpressurized (control) spore suspensions.

[b] MPa: megapascal (1 MPa = 145 psi).

## Conclusion

Spore formation by certain yeasts, molds, and bacterial species is a means of survival and continuation of the life process. In yeasts and molds, sporulation occurs by asexual and sexual processes; in bacteria, it occurs through differentiation, regulated and expressed by many genes.

Spore formation enables the dissemination of the species widely in the environment as well as contamination of foods. Their growth in food can be undesirable when they cause spoilage and produce toxins (except yeasts) in food and can be desirable in the processing of some foods. Methods of destruction of spores and inhibition of germination of spores (of bacteria) by different means are used to control their growth in food.

Some bacterial species sporulate as a means of survival strategy under conditions of physical, chemical, or environmental stresses by genetically regulated processes. Cells of many bacterial species, when exposed to conditions suboptimal for growth or sublethal, manifest different characteristics, the control mechanisms of which are not properly understood currently. These conditions are observed with many foodborne bacteria and have important implications in food microbiology, which are discussed in Chapter 10.

### QUESTIONS

1. List the differences among mold, yeast, and bacterial spores.
2. List five genera of foodborne bacteria that form spores.
3. Draw and label the structure of a bacterial spore and discuss the functions or characteristics of each structural component.

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4. List the stages between the formation of a bacterial spore and its emergence as a vegetative cell. Also, list the major events that occur in each stage.
5. Discuss the triggering mechanisms in sporulation and spore germination in bacteria.
6. Discuss the importance of bacterial spores in food.
7. Briefly discuss the methods used to control problems associated with bacterial spores in food.
8. Explain how low-range hydrostatic pressure can be combined with other antibacterial treatment to destroy bacterial spores in food.

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# Chapter 10

# Microbial Stress Response in the Food Environment

## Introduction

Foodborne bacterial cells (and other microbes) are usually exposed to different physical and chemical environments during production, processing, preservation, storage, transportation, and consumption of foods as well as during microbiological quality evaluation of foods and food ingredients by the recommended procedures. As a consequence, bacterial cells may become stressed and manifest several types of altered characteristics. A stressed environment can be both in the suboptimal growth range and beyond the growth range (Figure 10.1). Depending on the nature and level of a stress, cells in a population can develop a higher level of resistance to the same as well as several other types of stresses or suffer reversible sublethal injury or even apparently lose culturability (the ability to multiply) in some recommended bacteriological media and methods or lose viability (or the ability to multiply) permanently. Researchers have used many different terminologies for these altered states of bacterial cells, some of which are not well defined or are for the same condition or even scientifically contradictory. This has generated confusion among interested individuals and controversy among researchers. Currently, there is a move among some scientists researching in the area of microbial stress to conduct direct basic studies to understand the response of bacterial cells under different levels and the nature of stresses and to develop consensus scientific opinion for suitable definitions and specific terminologies.

In the last 50 years, three different broad terminologies have appeared in microbiology literature to describe altered characteristics of bacterial cells following exposure to some physical and chemical stresses: sublethal injury (in the 1960s–1980s),[1–3] a viable but nonculturable (VBNC) state (in the 1980s and 1990s),[4] and stress adaptation (in the 1990s).[5] In most publications, each aspect has been treated as a separate and unique phenomenon. In this chapter, the three are described separately, and then it is suggested that all three are probably related, differing only in the degree of bacterial response following exposure to different levels of a stress.[6,7]



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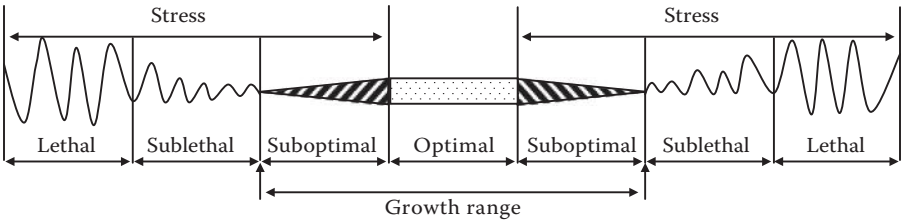



**Figure 10.1 Different levels or degrees of environmental stresses to which bacterial cells can be exposed during processing and preservation of food. Bacterial cells exposed to a suboptimal growth condition show stress adaptation. Beyond the growth range, the cells are usually either sublethally or lethally stressed. See text for further explanations.**

## Stress Adaptation

### *Definition and Observations*

Stress adaptation or stress response has been explained as a situation whereby a brief exposure of a bacterial population to a suboptimal physical or chemical (growth) environment enables the cells to resist subsequent exposure to the same or other types of harsher treatment to which the species is normally susceptible. This phenomenon has been observed among many foodborne pathogens and spoilage bacteria following exposure of cells to various suboptimal physical and chemical environments, such as cold and warm temperatures, low $A_W$, low hydrostatic pressure, UV light, high salt concentrations, bacteriocins, preservatives, detergents, several dyes, and antibiotics. It is assumed that a brief exposure to a suboptimal environment triggers some cellular mechanisms that enables them to resist subsequent exposure to harsher treatment.[5] However, once the cells are removed and allowed to grow for several generations in the optimum conditions, they do not remain resistant; rather, they revert to the original state. Several confusing and ambiguous terms have been used by different research groups to describe the stress adaptation phenomenon in bacteria. Some terms for suboptimal pH are included here.

- *Acid resistance or acid adaptation*. An exposure of cells for an extended period to a mild acidic environment (e.g., pH 5.0–5.8) enables them to develop resistance to subsequent exposure to pH ≤ 2.5.
- *Acid tolerance or acid tolerance response (ATR)*. A brief exposure of cells to a mild acidic environment enables them to survive subsequent exposure to pH 2.4–4.0.
- *Acid shock response (ASR)*. The response of bacterial cells to a low pH without previous adaptation to a mild pH.

Many studies have been conducted to determine the stress adaptation of foodborne bacteria. *Escherichia coli* cells exposed for one or two generations at pH 5.0 survive better subsequently at pH 3–4 (but not pH < 2). Similarly, a brief exposure of *Esc. coli* cells to 50°C enables the cells to survive better at 60°C (but not >72°C). Similarly, acid-adapted *Listeria monocytogenes* cells survive well when exposed to pH 3.5. Acid-adapted *Lis. monocytogenes* cells also develop resistance to nisin. *Lis. monocytogenes* cells briefly exposed to 0.1% $H_2O_2$ also developed cross-resistance against subsequent exposure to 0.5% $H_2O_2$, 5% ethanol, 7% NaCl, pH 5.0, or 45°C as compared with unadapted control cells. Heat resistance (increase in D value) of *Lis. monocytogenes*, *Salmonella* serovars, and *Esc. coli* O157:H7 suspended in low-pH fruit juices has been reported.

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The cells of the pathogens were initially acid adapted by exposing them to pH 5.0. They were then suspended in orange, apple, or grape juice (pH 3.5–3.9) and heated to 56°C. In all instances, the D values of acid-adapted cells as compared with those of control cells increased significantly. For *Lis. monocytogenes*, D values for control and acid-adapted cells were 2.1 and 3.8 minutes in orange juice, 1.6 and 5.0 minutes in apple juice, and 2.3 and 4.6 minutes in grape juice, respectively. These results suggest that stress adaptation of microorganisms can occur both in culture broth and in food systems. In food processing and preservation methods, results of studies developed with normal cells (not stress adapted) may not be effective to control or kill stress-adapted foodborne pathogens and spoilage bacteria. To overcome this problem, it is necessary to understand the underlying mechanisms that confer resistance to stress-adapted cells and develop methods to control them.

## *Mechanisms of Stress Adaptation*[8–10]

An earlier theory was that bacterial cells cope with stress (such as temperature stress) by changing the lipid composition of the cytoplasmic or inner membrane so that the fluid state is maintained. Thus, at lower or higher ranges of growth temperature, the membrane lipid accumulates more low-molecular weight and unsaturated fatty acids, but at optimum growth temperature, it accumulates more high-molecular-weight saturated fatty acids. In recent years, stress adaptation by bacterial cells (and other microorganisms) is viewed to be mediated through the synthesis of many types of shock proteins or stress proteins, some of which are specific for specific stress, whereas others are nonspecific and expressed against more than one stress. Stress proteins provide protection to structures that could be otherwise adversely affected by the stress, such as DNA and many enzymes. Synthesis of stress proteins in large quantities is mediated through the expression of stress-related gene systems, some of which are inducible, whereas others are constitutive but expressed at a low level when cells are not under stress. As some of the gene systems are global, gene expression by one stress can also help cells to adapt to other related stresses.

Expression of stress-related genes is initiated by specific polypeptides or sigma factor (σ) synthesized by specific genes. Some of these, such as $\sigma^B$ or $\sigma^{37}$ (encoded by gene sig B), help cope with general stress in Gram-positive bacteria; $\sigma^{32}$ (encoded by the *rpoH* gene) and $\sigma^{24}$ (encoded by the rpoE gene) help cope with heat response; and $\sigma^{38}$ or $\sigma^s$ (encoded by the *rpoS* gene) helps cope with general stress and starvation in Gram-negative bacteria. Under a specific stress (such as under heat-shock condition), *rpoH* is turned on to affect the synthesis of *rpoH* or $\sigma^{32}$ protein in high amounts (Figure 10.2). This sigma factor (also called a regulon) then combines with the core RNA polymerase (consisting of four subunits, $\alpha\alpha\beta\beta$) to form the complete RNA polymerase enzyme or holoenzyme. This holoenzyme then binds to the promoter of a heat-shock gene family, leading to synthesis of heat-shock proteins (e.g., in *Esc. coli*), which then protects the structural and functional units of stressed cells susceptible to heat damage (e.g., DNA and proteins). They can also protect against other stresses. Involvement of different sigma factors in protecting against stresses (such as cold, heat, low pH, UV) has been studied with several species of foodborne bacteria.

## *Importance of Stress-Adapted Microorganisms in Food*[11–13]

As indicated previously, during the handling of food and food ingredients from the farm to the table, foodborne bacteria are exposed to different suboptimal physical and chemical environments. This can enable foodborne pathogens and spoilage bacteria as well as beneficial bacteria to develop

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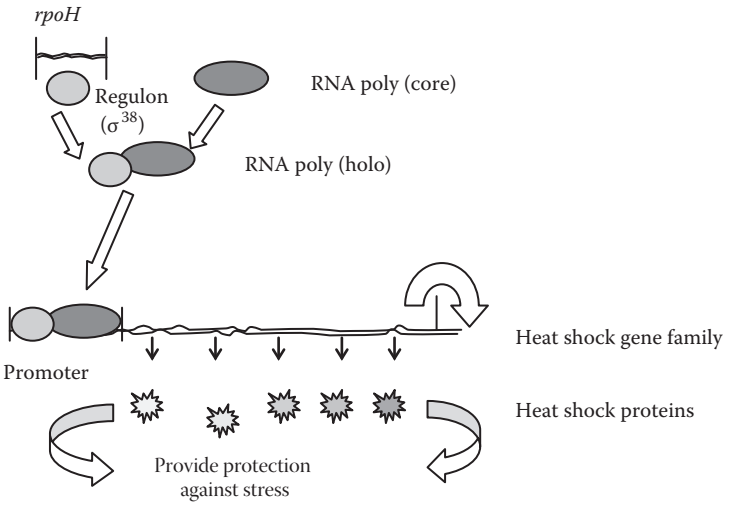



**Figure 10.2 Kinetic basis of coping stress with sigma factors by bacterial cells. See text for explanations.**

characteristics that are different from those of normal cells. This situation can be both disadvantageous and advantageous, as described in the following sections.

## *Pathogens and Spoilage Bacteria Surviving in Low-pH Foods*

Most foodborne pathogens (especially the enteric pathogens) and spoilage bacteria (especially Gram-negative) are susceptible to low pH and die off rapidly in high-acid foods (pH ≤ 4.5) during storage. Also, at low pH, normal cells are susceptible to other antimicrobial treatments (such as hydrostatic pressure, pasteurization temperature, or preservatives) at a much lower level. However, if they are first acid-adapted, they become relatively resistant to lower pH and other treatments at minimal levels and survive in food. Recent occurrence of foodborne diseases from the consumption of fruit juices, fermented sausages, and acidified foods containing viable *Salmonella*, *Esc. coli* O157:H7, and *Lis. monocytogenes* are thought to be a result of acid-adapted pathogenic strains surviving low pH and low heat treatment.

To overcome the problem, it is necessary to avoid exposure of the food (containing the target cells) to mild treatments; instead, several mild treatments can be used simultaneously to destroy the strains (hurdle concept, see Chapter 41).

## *Stress-Adapted Pathogens Surviving Stomach pH*

Cells of most enteric pathogens associated with foodborne infections (see Chapter 26) are highly susceptible to low pH of the stomach and die off. This is probably one of the reasons why dose level is high (≥$10^6$ viable cells) for many enteric pathogens to cause infection in the gastrointestinal (GI) tract. Many of the cells ingested are killed in the stomach, but a few survive, enter the GI tract, and set up infection. However, if a pathogenic strain in a food is stress-adapted, even consumption of a much lower number will enable it to survive in the stomach and cause infection in the GI tract.[14] An attempt should be made to eliminate or reduce the presence of stress-adapted pathogens in a ready-to-eat food.

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### *Enhancing Viability of Starter Cultures and Probiotic Bacteria*[7]

Commercial starter cultures are normally frozen or freeze-dried before their use by food processors with the intention of having high levels of survivors. Viability of these cultures, especially freeze-dried cultures, is generally low. Similarly, many probiotic bacteria are normally susceptible to stomach pH and low-pH food products (e.g., yogurt containing *Lactobacillus acidophilus*). However, first exposing the cultures to a mild stress to release stress proteins may enable the cells to survive subsequent freezing, freeze-drying, or exposure to low pH in the stomach or in food products. Genetic engineering techniques can also be used to develop new strains capable of producing cryoproteins and different stress proteins and surviving better.

## Sublethal Stress and Injury[1–3,6]

### *Definition and Observations*

Sublethal injury occurs following exposure of bacterial cells to unfavorable physical and chemical environments (beyond the growth range but not in the lethal range) that cause reversible alterations in the functional and structural organizations of the cells. As early as 1932, Rahn[15] suggested that death of microbes exposed to sublethal stresses is a gradual process that can be reversed under the proper conditions if the reactions have not progressed too far. In 1959, Strake and Stokes[16] showed that bacterial cells exposed to cold temperatures developed characteristics that were different from those of the normal population; the cells also temporarily lost the ability to multiply. However, in an appropriate environment, they repaired their injury and initiated multiplication. Later studies revealed that cells of yeasts and molds and spores of bacteria also incur reversible injury following exposure to sublethal stresses. Stress has been shown to alter morphology of cells because of down-regulation of the gene products that are responsible for cell division, such as muramidase, autolysin amidase, and so forth. In *Lis. monocytogenes*, exposure to a high temperature (45°C) or 5.5% NaCl caused cells to elongate or appear in chains (Figure 10.3).[17]

Many of the treatments include those used directly during food processing and storage as well as microbial detection from foods. Treatments include low heat (such as pasteurization), low temperature (freezing, refrigeration, and chilling), low $A_W$ (different types of drying or adding high solutes, such as sugar or salt), radiation (UV or X-ray), high hydrostatic pressure, electric pulse, low pH (both organic and inorganic acids), preservatives (sorbates or benzoates), sanitizers (chlorine or quaternary ammonium compounds), hot microbiological media (especially selective agar media above 48°C), and different selective enumeration and detection methods. This phenomenon is observed in many species of bacterial cells and spores, yeasts, and molds that are important in foodborne diseases, food spoilage, and food bioprocessing, and as indicators of sanitation (Table 10.1). From this list, it becomes apparent that other microorganisms that have not been studied will most likely also be injured by sublethal stresses. In general, Gram-negative bacteria are more susceptible to injury than Gram-positive bacteria, and bacterial spores are much more resistant than vegetative cells to a particular stress.

Microbial injury and growth of injured cells have been studied with both bacterial cells and spores. The material discussed here mainly covers bacterial cell and spore injury.

### *Manifestation of Bacterial Sublethal Injury*

A bacterial population, following exposure to a sublethal stress, contains three physiologically different subpopulations: the uninjured (normal) cells, reversibly injured (injured) cells, and

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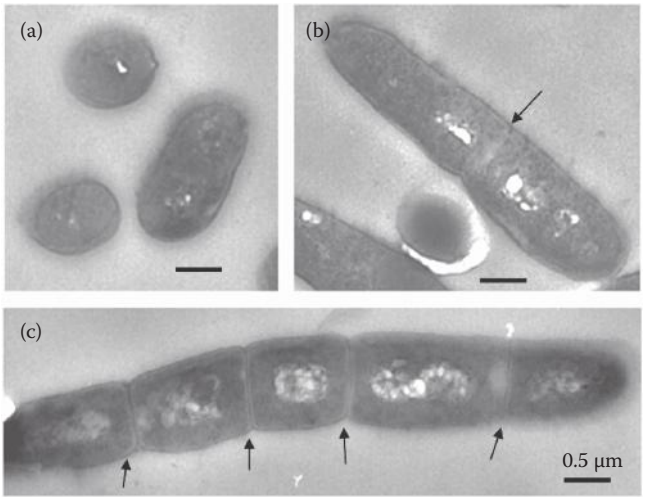



**Figure 10.3 Transmission electron microscopic photographs showing the alteration of morphology of *Lis. monocytogenes* cells following exposure to stress conditions of (b) high temperature (45°C for 16 hours) or (c) 5.5% NaCl. Control cells (a) appeared normal. (Adapted from Geng, T. et al., *J. Appl. Microbiol.*, 95, 4, 2003. With permission.)**

irreversibly injured (dead) cells. Their relative percentages vary greatly and are dependent on the species and strains, nature of suspending media, nature and duration of stress, and methods of detection. Injured cells differ in the level of injury and in several characteristics from their normal counterparts. One of these is increased sensitivity to many compounds to which normal cells are resistant, such as surface-active compounds (bile salts, deoxycholate, or SDS), NaCl,

**Table 10.1 Cells and Spores of Some Microorganisms Important in Food in Which Sublethal Injury Is Detected**

| |
|---|
| Gram-positive pathogenic bacteria: *Sta. aureus, Clo. botulinum, Lis. monocytogenes, Clo. perfringens, Bac. cereus* |
| Gram-negative pathogenic bacteria: *Salmonella, Shigella* spp., enteropathogenic *Esc. coli, Esc. coli O157:H7, Vib. parahaemolyticus, Cam. jejuni, Yer. enterocolitica, Aer. hydrophila* |
| Gram-positive spoilage bacteria: *Clo. sporogenes, Clo. bifermentum, Bac. subtilis, Bac. stearothermophilus, Bac. megaterium, Bac. coagulans* |
| Gram-negative spoilage bacteria: *Pseudomonas* spp., *Serratia* spp. |
| Gram-positive bacteria used in food bioprocessing: *Lac. lactis, Lab. delbrueckii* subsp. *bulgaricus, Lab. acidophilus* |
| Gram-positive indicator bacteria: *Ent. faecalis* |
| Gram-negative indicator bacteria: *Esc. coli, Enterobacter aerogenes, Klebsiella* spp. |
| Bacterial spores: *Clo. botulinum, Clo. perfringens, Clo. bifermentum, Clo. sporogenes, Bac. cereus, Bac. subtilis, Bac. stearothermophilus, Des. nigrificans* |
| Yeasts and molds: *Sac. cerevisiae, Candida* spp., *Asp. flavus* |

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**Table 10.2 Effect of Freezing and Thawing on Viability Loss and Injury of *Esc. coli* NCSM**

| Enumeration Media[a] | Media | CFUs/mL | | Subpopulations |
|---|---|---|---|---|
| | | Before Freezing | After Freezing | |
| TS agar | Nonselective | $276 \times 10^6$ | $17.5 \times 10^6$ | Original population-dead: $276 \times 10^6 - 17.5 \times 10^6$ (93.7%) |
| TSD agar | Selective | $267 \times 106^6$ | $3.5 \times 10^6$ | Among survivors-uninjured: $3.5 \times 10^6$ (19.9%); injured: $17.6 \times 10^6 - 3.5 \times 10^6$ (80.1%) |

[a] *Esc. coli* cells in water suspension were enumerated before and after freezing (–20°C for 16 hours) and thawing simultaneously in TS (tryptic soy) agar and TSD (TS + 0.075% deoxycholate) agar media by pour plating followed by incubation at 37°C for 24 hours.

some chemicals (LiCl, selenite, bismuth sulfite, or tetrathionate), hydrolytic enzymes (lysozyme or RNase), antibiotics, dyes (crystal violet or brilliant green), and low-pH and undissociated acids. They also lose cellular materials, such as $K^+$, peptides, amino acids, and RNA. When exposed to or maintained in an unfavorable environment, the injured cells die progressively. Also, the injured cells do not multiply unless the injury has been repaired. In a nonselective and preferably nutritionally balanced medium, the cells are able to repair their injury, which can extend 1–6 hours, depending on the nature of the stress and degree of injury. After an extended repair phase, the cells regain their normal characteristics and initiate multiplication. The injured cells in a population can be detected by a suitable nonselective enumeration technique. The results in Table 10.2 show that normal *Esc. coli* cells grow almost equally in both the nonselective and selective media, indicating that the cells are not sensitive to the surface-active agent, deoxycholate, in the medium. Following freezing and thawing, 93.7% of cells die. The survivors form colonies in the nonselective tryptic soy (TS) agar (they repair and multiply), but 80.1% of the survivors fail to form colonies in the selective TS-containing deoxycholate (TSD) agar because of their injury and developed sensitivity to deoxycholate (bile salt). Among the survivors, however, 19.9% are normal or uninjured cells, inasmuch as they grow equally well both in the TS and TSD agar media and are no longer sensitive to deoxycholate. Many food systems can reduce both the cell death and injury incurred from a specific stress.

Bacterial spore injury has been observed following heating, UV and ionizing radiation, treatment by hydrostatic pressure, and some chemicals (e.g., hypochlorite, $H_2O_2$, ethylene oxide, and probably nitrite) that are important in foods. The injured spores develop sensitivity to NaCl, low pH, $NO_2$, antibiotics, redox potential, gaseous atmosphere, and temperature of incubation. They also have delayed germination and longer lag for outgrowth, and they develop a need for some specific nutrients. These manifestations also vary with the nature of stress to which the spores are exposed.

## *Sites and Nature of Injury*

Altered physiological characteristics of the injured bacterial cells have been used to determine directly the site of damage in the cellular structural and functional components. There is evidence

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that some cell components are damaged by almost all types of stresses studied. In addition, specific components can be damaged by specific stresses. The structural and functional components known to be damaged by sublethal stresses are the cell wall (or outer membrane, OM), cytoplasmic membrane (or inner membrane, IM), ribosomal RNA (rRNA) and DNA, and some enzymes (see Figure 2.2). Damages in the cell wall (or OM) and cytoplasmic membrane (or IM) are more evident in injury caused by freezing and drying, whereas damage to rRNA is more extensive in sublethal heating and DNA damage following radiation of cells.

In Gram-positive and -negative bacteria, freezing and drying cause changes in cell surface hydrophobicity and the inability to form compact pellets and to adsorb some phages. In Gram-positive bacteria, surface layer proteins are also lost. In sublethally stressed Gram-negative bacteria, the lipopolysaccharide (LPS) layer undergoes conformational alteration and loses its barrier property to many chemicals (such as SDS, bile salts, antibiotics, lysozyme, and RNase), which can easily enter the injured cells and kill them. Very little LPS is lost from the cells into the environment. The alteration in conformation of LPS is a result of loss of divalent cations, which are necessary for the normal stability of LPS. In both Gram-positive and -negative cells, the cytoplasmic membrane (or IM) remains intact in injured cells, but it loses its permeability barrier function. The cells become sensitive to NaCl and also lose different cellular materials. It is suggested that protein molecules in this structure probably undergo conformational changes in the injured cells. rRNA is extensively degraded by the activated RNase. DNA can undergo single- and double-strand breaks. In some strains, autolytic enzymes can be activated by stress, causing lysis of the cells.

In bacterial spores, depending on the type of sublethal stress, different structural and functional components can be injured. High heat causes damage to the lytic enzymes, necessary for the lysis of the cortex before spore germination and to the spore membrane structures, causing loss of permeability barrier functions. Damages by irradiation (UV and γ) are mainly confined to DNA in the form of single-strand breaks and, by some chemicals ($H_2O_2$, antibiotics, or chlorine), to the lytic enzymes of the germination system. Hydrostatic pressure, in combination with heat, damages the cortex, whereas γ irradiation damages both the cortex and DNA. Mild to strong acid treatments can cause the spores to become dormant by removing $Ca_2^+$ from the spores and making them sensitive to heat.

## *Repair of Reversible Injury*

One of the most important characteristics of injured bacterial cells is the ability to repair the injury in a suitable environment and become similar to normal cells. The repair process and the rate of repair can be measured by specific methods. One of them is to measure regain in resistance of injured cells to the surface-active agents by repair in the cell wall or the OM. Suspending a sublethally stressed population in a repair medium and simultaneously enumerating the colony-forming units (CFUs) during incubation in nonselective and selective plating media help determine the rate of repair (Figure 10.4). Initially, the injured survivors fail to form colonies in the selective, but not in the nonselective, media. However, as they repair and regain resistance to the selective agent, they form colonies on both media, as indicated by the increase in counts in the selective media only. Injured cells differ in the levels of injury (from low to high) as manifested from the differences in recovery time in a suitable medium.

The injured cells can repair in a medium devoid of selective compounds but containing the necessary nutrients during incubation at optimum pH and temperature. In general, the cells repair well in a medium rich in metabolizable carbon and nitrogen sources and several vitamins.

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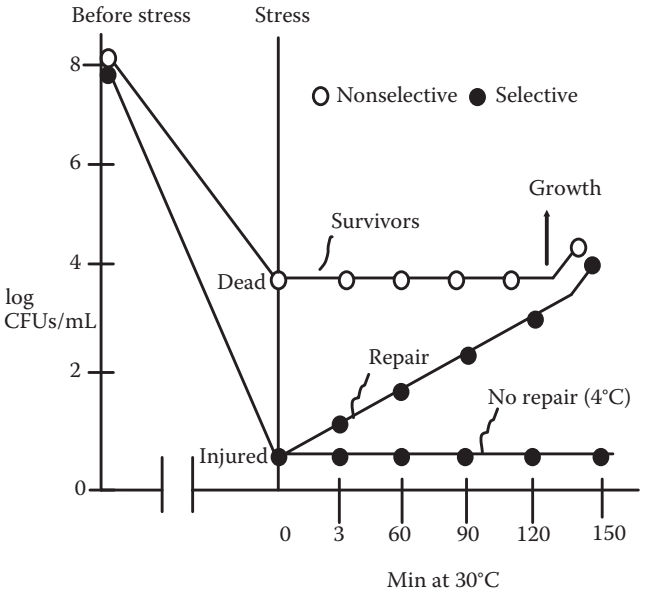



**Figure 10.4 A hypothetical repair curve of injured bacteria. Repair is indicated by an increase in counts only in selective agar media during incubation in nonselective repair broth. Cell multiplication is indicated by a simultaneous increase in counts in both selective and nonselective agar media.**

Supplementation with catalase and pyruvate (to destroy $H_2O_2$ produced by the cells) also enhances repair and increases the number of repaired cells. It has been suggested that during the rapid repair process, many cells generate $H_2O_2$ but fail to hydrolyze it because of an injured peroxidase system. Accumulated $H_2O_2$ then causes cell death. A simple medium with a suitable energy source can also enable some cells with low levels of injury to repair damage in the OM (or cell wall). Depending on the sublethal stress, complete repair can be achieved in 1–6 hours at 25°C–37°C. For freezing and drying injuries, the rate is very rapid; for heat injuries, the rate can be slow. Specific studies show that the metabolic processes during repair vary with the nature of a stress and involve synthesis of ATP, RNA, DNA, and mucopeptide. Reorganization of the existing macromolecules can also be an important event during the repair process. The cell wall (or OM) regains the ability to prevent entrance of many chemicals to the cells; the cytoplasmic membrane (or IM) regains its permeability barrier function as well as the enzymes; and RNA and DNA regain their original characteristics. Finally, the cells regain their ability to multiply. Direct or indirect studies have not been conducted to show that the repair process is associated with activation or inactivation of genetic material.

Repair conditions for injured spores vary with the type (aerobic or anaerobic species) of spores. The composition of media is very important; in addition to good carbon and nitrogen sources, addition of special compounds, such as starch, a reducing compound (such as cysteine), lysozyme, and divalent cations, may be necessary. Time, temperature, and gaseous environment also have to be optimal for the species. Generally, a longer repair time is required by spores damaged by heat, radiation, and chemicals than by low temperatures.

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## Injury in Yeasts and Molds

Very limited studies on injury and repair in yeasts and molds have been conducted. Freezing, low heat treatment, and irradiation have been reported to cause injury in vegetative cells of *Saccharomyces*, *Kluyveromyces*, *Candida*, *Aspergillus*, *Penicillium*, and *Rhizopus* spp. Spores of the molds have been found to be damaged by irradiation. The main characteristic of the injured cells and spores is their increased sensitivity to many selective environments. The cell membrane seems to be the major structure implicated in injury. Repair in nutritionally rich, nonselective media occurs before multiplication.

## Importance of Sublethally Injured Microorganisms in Food

Many of the physical and chemical treatments that are able to induce sublethal injury in microbial cells and spores are used in the processing, storage, and preservation of foods and in the sanitation of facilities. Thus, it is quite likely that the foods and the facilities will harbor injured microorganisms. Microbial injury is important in food microbiology for several reasons.

### Detection of Undesirable Microorganisms

Injured microorganisms are potentially capable of multiplying. Thus, injured pathogens can cause foodborne disease, and injured spoilage microorganisms can reduce the shelf life of a product. It is important that they be detected if present in a food. For detection of many microorganisms in food, several types of selective liquid and solid media are used (Chapter 42). Injured microorganisms may not be detected in these media. As a result, foods containing viable but injured pathogens and indicators above the regulatory or acceptable limits and high numbers of spoilage microorganisms can be sold. These products can be hazardous and have a short shelf life despite meeting the regulatory standards and specifications. To overcome these problems, a short repair phase has been incorporated before the selective detection procedures of important microorganisms in foods. This information is also important in designing processing parameters to obtain proper reduction of undesirable microorganisms in finished products (such as heating temperature and time).

### Enhancing Shelf Life of Foods

Injured cells are susceptible to many physical and chemical environments. These conditions, where possible, can be used in the preservation of foods (such as low temperature, lower pH, or preservatives for low-heat-processed foods) to kill injured cells and spores. In this manner, they will be unable to repair, and their potential ability to grow and cause product spoilage can be reduced.

### Enhancing Viability of Starter Cultures

In the bioprocessing of foods, starter cultures are used, such as frozen concentrates or freeze-dried preparations. However, both these conditions are known to cause death and injury to the cells. By studying the mechanisms responsible for cell death or cell injury, it may be possible to stop the events and reduce cell death and injury. This will help produce starter

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concentrates that can be stored for a long time without a reduction in their viability or desirable characteristics.

## Viable-but-Nonculturable[4,18]

### *Definition and Terminologies*

Under unfavorable environments, some cells in a bacterial population remain viable but are not able to multiply in many recommended bacteriological media unless they are subjected to a prior resuscitation treatment. This phenomenon has been reported with many microorganisms, such as *Vibrio vulnificus*, *Vib. cholerae*, *Vib. parahaemolyticus*, *Aeromonas hydrophila, Salmonella* serovars, *Esccherichia coli* O157:H7, *Campylobacter jejuni*, *Enterococcus faecalis*, *Shigella dysenterie*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas fluorescens*, and others (the list is growing), exposed to low temperatures, seawater, water, saliva, phosphate buffer, heavy metals, nutrient starvation, osmotic stress, food preservatives, and oxygen levels. Because this phenomenon is observed with many foodborne pathogens, a concern was raised that the pathogens can be present in a food in a VBNC state but could not be detected by the recommended methods. However, these cells can resuscitate under a favorable environment, multiply, and, following consumption of the food, cause foodborne diseases.

In recent years, the existence of the VBNC state of bacterial cells has been questioned because of the study methods used by researchers. In addition, the scientific validity of the VBNC terminology, especially in food microbiology, has been questioned by others. The operational definitions of some terminologies related to this phenomenon are listed here:

- *Viability*. Cells that are metabolically active and able to multiply in an appropriate environment. In bacteriology, this term is analogous with the term culturability.
- *Metabolically active*. Cells capable of carrying out at least some metabolic activity but may not necessarily multiply.
- *Nonculturability*. Inability to multiply under any conditions.
- *Dead cells*. Cells unable to divide in an appropriate environment.
- *Resuscitation.* Metabolically active cells changed from a state of inability to multiply in one environment to a state of ability to multiply in another environment.

These definitions suggest that the term VBNC may be contradictory. The cells included under this term could fail to multiply in one selective environment but multiply in another environment. Also, following resuscitation, they can multiply in the selective environment (having some similarities with sublethally injured cells).

Because of current controversy among researchers studying the VBNC phenomenon of bacterial cells, the views and observations of the groups are summarized in this chapter.

### *Proponent Views*

The existence of the VBNC phenomenon was determined initially in starving *Vib. vulnificus* cells kept in artificial seawater at 5°C and simultaneously determining, during storage, the total microscopic cell counts (TMCs) following staining with acridine orange; direct viable cell counts

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(TVCs) microscopically following exposure either to an inhibitor that enables cells to elongate but prevents cell division or to a tetrazolium salt that is metabolized to formazan (a fluorescence compound) by the metabolically active cells; and enumerating CFUs in a selective agar media. Now, VBNC cells are counted by using SYTO-9 and propidium iodide (also in the commercially available LIVE/DEAD BacLight™ kit) staining followed by microscopic analysis. Figure 10.5 presents the results of a typical study.[19] In such a study, the difference between TMC and TVC is regarded as dead cells and that between TVCs and CFUs is considered as VBNC cells. Researchers hypothesize that under unfavorable environmental conditions, the VBNC cells (in the populations) enter a dormant state as a survival strategy. As in sporulation in bacterial cells, this is an inducible, programmed strategy and part of the life cycle of the cells. The cells remain metabolically active but stop multiplication. There are also suggestions that the formation of the VBNC state occurs in two phases. In the initial state, the cells lose culturability but retain cellular integrity and nucleic acid structures. Gradually, the integrity of the cells is lost, and degradation of nucleic acid starts, leading to cell death.

Cells can be triggered to multiply by removing the VBNC-inducing factor or by supplying a resuscitation-inducing factor in the culture media. Also, heat shock, inoculation in the egg embryo, exposure to HeLa cells, animal passage (for the pathogens), and other treatments can initiate multiplication (and restore disease-producing capability). Once resuscitated in an appropriate environment, the cells multiply rapidly like the cells in a normal population. Other indirect physiological studies have suggested that VBNC cells have increased cell volume and reduced internal $K^+$, membrane potential, membrane permeability, and adenylate energy (ATP). Structural DNA remains intact, respiratory activity is maintained, and expression of some genes is retained.

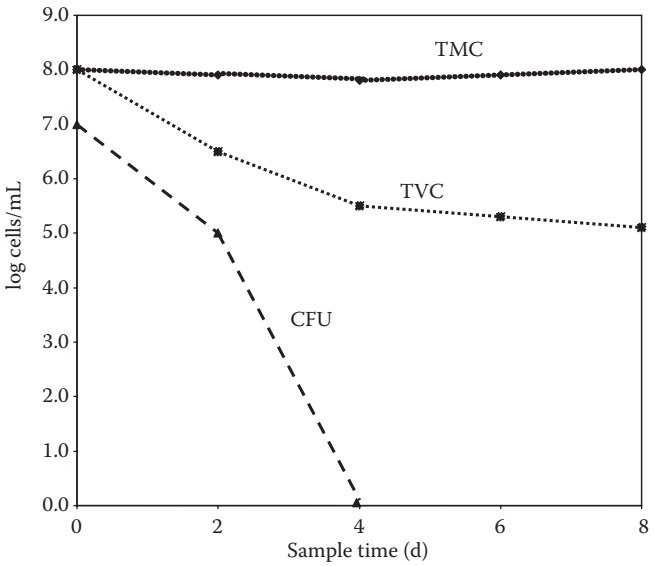



**Figure 10.5 Total cell counts (TMCs), direct viable counts (TVCs), and colony-forming unit (CFU) counts of a bacterial cell suspension in artificial seawater at 5°C. TMCs and TVCs are obtained microscopically, and CFUs are obtained by enumeration on an agar medium. See text for other explanations. (Adapted from Colwell, R.R., *Zentralbl. Bakteriol.*, 279, 154, 1993. With permission.)**

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## Opponent Views

Several questions have been raised by some researchers regarding the existence of the bacterial VBNC phenomenon. One is based on the hypothesis of the proponent group that the VBNC state is a survival strategy programmed into the life cycle, induced under unfavorable conditions (as in the sporulation process), and thus genetically determined. Although the genetic basis of bacterial sporulation is well understood, there is no information to prove directly that the VBNC state in bacteria is programmed and has a genetic basis. This information is necessary to resolve the hypothesis. Another question has been raised on the study methods used to determine the VBNC phenomenon. In the direct method, VBNC cells in a population are determined from the differences in TVCs, determined microscopically, and CFUs, determined by agar media plating methods. Vital staining and optical methods used in TVC studies are not accepted by many in the scientific community as effective methods to differentiate between dead cells and viable cells. In the indirect methods used to study cell volume, membrane permeability, membrane potential, the total population consisting of a mixture of dead cells (in large numbers), VBNC cells, and cells capable of forming CFUs were used instead of using only the VBNC cells. Currently, no method is available to separate the three cell groups in a population and conduct studies on the cellular changes only in the VBNC cells. Therefore, the validity of these indirect studies is open to question. Finally, some have questioned the validity of the resuscitation method used by proponent groups to prove the existence of the VBNC phenomenon. An increase in CFUs, following incubation of a total population of stressed cells in a suitable environment for one or more days, was suggested because of the regain of culturability of the VBNC cells. Some researchers have suggested that this large increase in CFUs (as much as six or more log cycles) can be a result of the multiplication of a few viable cells during prolonged incubation. Determining simultaneously the TMCs, TVCs, and CFUs during a resuscitation period, as was conducted during the determination of VBNC cells (Figure 10.5), would have proven that the increase in CFUs during resuscitation was not from growth of a few viable cells but from the transformation of VBNC cells to the culturable state. Similarly, VBNC cells regain virulence following resuscitation, as determined by the resuscitated cells attaching to HeLa cells, colonizing egg embryo, and causing intestinal infection, resulted from the growth of a few viable cells in the original population.

## Current Views

The results of several recent studies have provided a better insight on bacterial cells exposed to an unfavorable environment for a prolonged period. These results show that the so-called VBNC cells are really viable as well as culturable but are not recovered unless they are exposed to appropriate culture conditions. Many of the starved or stressed cells have imbalanced metabolic pathways. When these cells are exposed to a nutritionally rich medium, they start metabolism and generate large quantities of superoxides and free radicals. However, because of defective metabolic processes, they cannot detoxify these products and are killed. This death can be prevented or greatly reduced by exposing the cells in a medium that either reduces the production of the superoxides and free radicals or increases their degradation. Supplementation of pyruvate or catalase to resuscitation media was thus found to transform the cells to a culturable state very rapidly, as determined by an almost immediate increase in CFUs without any change in TMCs. Several studies in the 1970s and 1980s on bacterial sublethal injury have shown that following environmental stress of a bacterial population, the recovery of the survivors can be increased either by incubating the cells in a minimal medium or in a nutritionally rich medium supplemented with pyruvate or catalase.

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It was suggested at that time that in a repair medium, especially in a nutritionally rich medium, the cells generate $H_2O_2$. The cells with greater levels or degrees of injury (see Figure 10.4) have defective peroxidase or catalase systems and fail to degrade $H_2O_2$; supplementation of a medium with pyruvate or catalase helps degrade the $H_2O_2$ and prevent cell death.

It appears from the similarities of these observations that there are many similarities between the VBNC phenomenon and sublethal injury of bacterial cells. The differences are more in the study methods used and interpretation of the results.

### *Physiological Properties of VBNC Cells*

The VBNC state is considered a survival strategy for many pathogens; thus bacteria undergo genetic and physiologic changes. As a result, there are several noticeable changes in the appearance and physiology of cells under the VBNC state: (1) Bacteria become significantly smaller with reduced amounts of macromolecular synthesis; (2) Bacteria lose virulence during the VBNC state, however, they regain virulence immediately after resuscitation; (3) Cells are resistant to antibiotics. Antibiotics are effective primarily against growing cells, and VBNC cells are metabolically less active; thus the antibiotics are less effective.

### *Importance of VBNC Microorganisms in Food*

The existence of bacterial cells that cannot be cultured by many currently recommended bacteriological methods cannot be disputed. They can be present in food and the food environment and can potentially cause foodborne diseases and food spoilage. Food microbiologists have to recognize this and develop proper resuscitation and detection procedures for effective identification of their presence in food to safeguard the health of consumers and reduce waste of food from spoilage.

## Conclusion

Bacterial cells exposed to environmental stresses respond differently, depending on the level or degree of a stress (Figure 10.6). In the suboptimal growth range, away from the optimal growth range, the cells can have a stress adaptation as manifested by an increased resistance to the same and several other stresses at higher levels. An exposure beyond the growth range will cause injury in different structural and functional components of some cells without affecting some others. The levels or degrees of injury in the cells differ with the nature and duration of a stress, and under any stress the level of injury increases as the level of stress increases. For some cells, the injury can be repaired rapidly in a nonselective or a nutritionally rich medium; these cells, usually designated as sublethally injured cells, probably have a low level of injury. Some other cells, probably because of high level of injury, require a more exact resuscitation environment for repair; these cells probably include VBNC cells (alternatively designated as not normally culturable or NNC cells). Finally, some stressed cells fail to multiply under any resuscitation conditions and are designated as lethally injured (or dead) cells.

Foodborne pathogens and spoilage bacteria can be stress adapted, sublethally injured, or VBNC (or NNC) by the many processing, preservation, transportation, and storage conditions used in food production. They may not be detected from a contaminated food by many of the recommended methods used for their detection. Effective methods have to be developed and used

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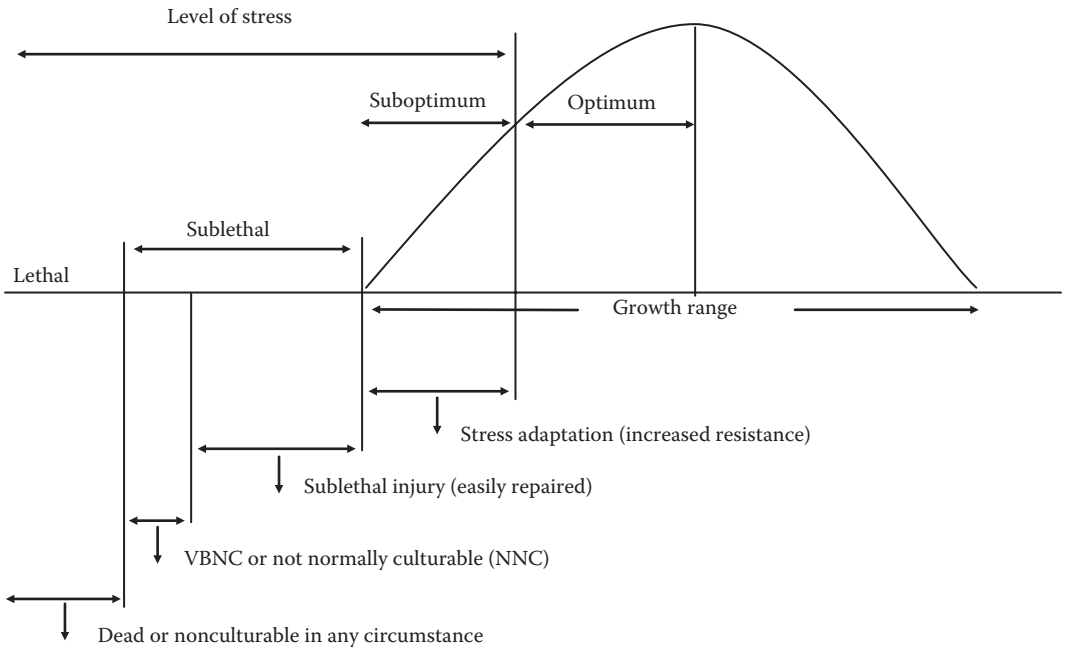



**Figure 10.6 Schematic representation of bacterial stress response when exposed to a physical or chemical environment beyond optimum growth range. See text for explanations.**

for their detection in food to safeguard the health of the consumers and to prevent loss of food from spoilage.

## QUESTIONS

1. Define stress adaptation of foodborne bacterial cells and list six types of environments where bacterial cells can encounter stress during the handling of foods.
2. Describe the possible mechanisms by which bacterial cells can adapt to a suboptimal stress. Also explain how exposure to one stress can provide protection against multiple stresses.
3. List the importance of bacterial stress adaptation in food microbiology.
4. Explain the phenomenon of bacterial sublethal injury and list six conditions of food-handling procedures that can inflict sublethal injury to bacterial cells, bacterial spores, and yeast and molds.
5. List six altered characteristics (manifestations) of sublethally injured bacterial cells.
6. List two structural and two functional components of bacterial cells that show sublethal injury and indicate the nature of changes of the cell components.
7. Discuss the mechanism of repair of sublethal injury and the nature of the repair environment.
8. A suspension of *Salmonella* cells was subjected to a sublethal stress. Enumeration of cell suspensions before and after stress and after repair in a nonselective broth for one hour at 30°C provided the following data (CFUs/mL; NSA, nonselective agar; SA, selective agar media):

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Controlled cells: NSA: $3 \times 10^7$ and SA: $2.8 \times 10^7$
Stressed cells: NSA: $0.5 \times 10^7$ and SA: $6 \times 10^5$
Repaired cells: NSA: $0.5 \times 10^7$ and SA: $3 \times 10^6$

Calculate the percentage of dead, injured, normal, repaired, and unrepaired cells.

9. List the importance of sublethal injury in food microbiology and explain how the phenomenon can be used in problem solving and for beneficial purposes.
10. Define the phenomenon of VBNC of bacterial cells. Explain the importance of the phenomenon.
11. Briefly list the findings and suggestions of the proponent groups on the VBNC phenomenon.
12. List the views of the opponent groups against the existence of the VBNC phenomenon.
13. Explain briefly the suggestions that could be associated with the so-called VBNC phenomenon in light of some recent findings.
14. How justified is the assumption that stress adaptation, sublethal injury, and the VBNC state can be grouped together as different manifestations of bacterial cells under different levels or degrees of stress?
15. Define or explain the importance of the following terms (in food microbiology): stress protein; sigma factors; holoenzyme (RNA polymerase); loss of divalent cations of LPS layer; conformational alteration of structural and functional components under stress; spore injury; survival strategy of VBNC cells; culturability and nonculturability.

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# III BENEFICIAL USES OF MICROORGANISMS IN FOOD

The major concern of microbial presence in food is because of its undesirable properties. Most are able to spoil foods, and several are associated with foodborne health hazards. However, there are other microorganisms that have beneficial properties in food production, maintaining normal health of the gastrointestinal tract of humans and controlling the undesirable spoilage and pathogenic bacteria in food. The beneficial attributes of the desirable microorganisms are briefly discussed in this section through the following topics:

Chapter 11: Microorganisms Used in Food Fermentation
Chapter 12: Biochemistry of Some Beneficial Traits
Chapter 13: Genetics of Some Beneficial Traits
Chapter 14: Starter Cultures and Bacteriophages
Chapter 15: Microbiology of Fermented Food Production
Chapter 16: Intestinal Bacteria and Probiotics
Chapter 17: Food Biopreservatives of Microbial Origin, Bacteriocin, and Nanotechnology
Chapter 18: Food Ingredients and Enzymes of Microbial Origin

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# Chapter 11

# Microorganisms Used in Food Fermentation

## Introduction

Beneficial microorganisms are used in foods in several ways. These include actively growing microbial cells, nongrowing microbial cells, and metabolic byproducts and cellular components of microorganisms. An example of the use of growing microbial cells is the conversion of milk to yogurt by bacteria. Nongrowing cells of some bacteria are used to increase the shelf life of refrigerated raw milk or raw meat. Many byproducts, such as lactic acid, acetic acid, some essential amino acids, and bacteriocins produced by different microorganisms, are used in many foods. Finally, microbial cellular components, such as single-cell proteins (SCPs), dextran, cellulose, and many enzymes, are used in food for different purposes. These microorganisms or their byproducts or cellular components have to be safe, food grade, and approved by regulatory agencies. When the microbial cells are used in such a way that they are consumed live with the food (as in yogurt), it is very important that they and their metabolites have no detrimental effect on the health of the consumers. When a byproduct (such as an amino acid) or a cellular component (such as an enzyme) is used in a food, the microorganisms producing it have to be regulated and approved, and the byproduct and cellular component have to be safe. If a food-grade microorganism is genetically modified, its use in food has to be approved, especially if the genetic material used is obtained from a different source or is synthesized. Thus, the microorganisms used for these purposes have to meet some commercial and regulatory criteria. In this chapter, characteristics of some microorganisms used in the processing of foods (designated as fermented foods) are discussed. Many of these microorganisms are used to produce several byproducts and cellular components used in foods.

## Microbiology of Fermented Foods

Food fermentation involves a process in which raw materials are converted to fermented foods by the growth and metabolic activities of the desirable microorganisms. The microorganisms



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utilize some components present in the raw materials as substrates to generate energy and cellular components, to increase in population, and to produce many usable byproducts (also called end products) that are excreted in the environment. The unused components of the raw materials and the microbial byproducts (and sometimes microbial cells) together constitute fermented foods. The raw materials can be milk, meat, fish, vegetables, fruits, cereal grains, seeds, and beans, fermented individually or in combination. Worldwide, more than 3500 types of fermented foods are produced. Many ethnic types are produced and used in small localities by small groups of people. Many of the fermented foods consumed currently have been produced and consumed by humans for thousands of years. The old city civilizations, dating as far back as 3000 B.C.–5000 B.C. in the Indus Valley, Mesopotamia, and Egypt, developed exceptional skills in the production of fermented foods from milk, fruits, cereal grains, and vegetables. The process not only produced new foods but also helped preserve the excess of raw materials both of plant and animal origins.[1]

The basic principles developed by these ancient civilizations are used even today to produce many types of fermented foods by a process known as natural fermentation. In this method, either the desirable microbial population naturally present in the raw materials or some products containing the desirable microbes from a previous fermentation (called back slopping), are added to the raw materials. Then the fermentation conditions are set so as to favor growth of the desirable types but prevent or retard growth of undesirable types that could be present in the raw materials. In another type of fermentation, called controlled or pure culture fermentation, the microorganisms associated with fermentation of a food are first purified from the food, identified, and maintained in the laboratory. When required for the fermentation of a specific food, the microbial species associated with this fermentation are grown in large volume in the laboratory and then added to the raw materials in very high numbers. Then the fermentation conditions are set such that these microorganisms grow preferentially to produce a desired product. Characteristics of some of the microorganisms used in fermentations are discussed here. These microbial species, when used in controlled fermentation, are also referred to as starter cultures. Many of these microbial species are present in raw materials that are naturally fermented along with other associated microorganisms, some of which may contribute to the desirable characteristics of the products.

## Lactic Starter Cultures[2,3]

At present, bacterial species from 12 genera are included in a group designated as lactic acid bacteria because of their ability to metabolize relatively large amounts of lactic acids from carbohydrates.[4,5] The genera include *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Aerococcus*, *Vagococcus*, *Tetragenococcus*, *Carnobacterium*, *Weissella*, and *Oenococcus*. Many of the genera have been created recently from previously existing genera and include one or a few species. For example, *Lactococcus* and *Enterococcus* were previously classified as *Streptococcus* Group N and Group D, respectively. *Vagococcus* is indistinguishable from *Lactococcus*, except that these bacteria are motile. *Weissella* and *Oenococcus* are separated from *Leuconostoc*. *Tetragenococcus* includes a single species that was previously included with *Pediococcus* (*Pediococcus halophilus*). *Carnobacterium* was created to include a few species that were previously in genus *Lactobacillus* and were obligatory heterofermentative. However, species from the first five genera, that is, *Lactococcus, Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Lactobacillus*, are used as starter cultures in food fermentation and are discussed here. The status of others, except *Tetragenococcus halophilus* and *Oenococcus oeni*, with respect to use in food, is not clear at present.

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## Lactococcus

This genus includes several species, but only one species, *Lactococcus lactic*, has been widely used in dairy fermentation. It has three subspecies (ssp.); *lactis*, *cremoris*, and *hordniae*, but only the first two are used in dairy fermentation. The biovar *Lac. lactis* ssp. *lactis* biovar *diacetylactis* is also used in dairy fermentation.

The cells are ovoid, approximately 0.5–1.0 μm in diameter, present in pairs or short chains, nonmotile, nonsporulating, and facultative anaerobic to microaerophilic (Figure 11.1). In general, they grow well between 20°C and 30°C but do not grow in 6.5% NaCl or at pH 9.6. In a suitable broth, they can produce approximately 1% L(+)-lactic acid and reduce the pH to approximately 4.5. Subsp. *cremoris* can be differentiated from subsp. *lactis* by its inability to grow at 40°C, in 4% NaCl, ferment ribose, and hydrolyze arginine to produce $NH_3$. Biovar diacetylactis, as compared with others, produces larger amounts of $CO_2$ and diacetyl from citrate. They are generally capable of hydrolyzing lactose and casein. They also ferment galactose, sucrose, and maltose. Natural habitats are green vegetation, silage, the dairy environment, and raw milk.

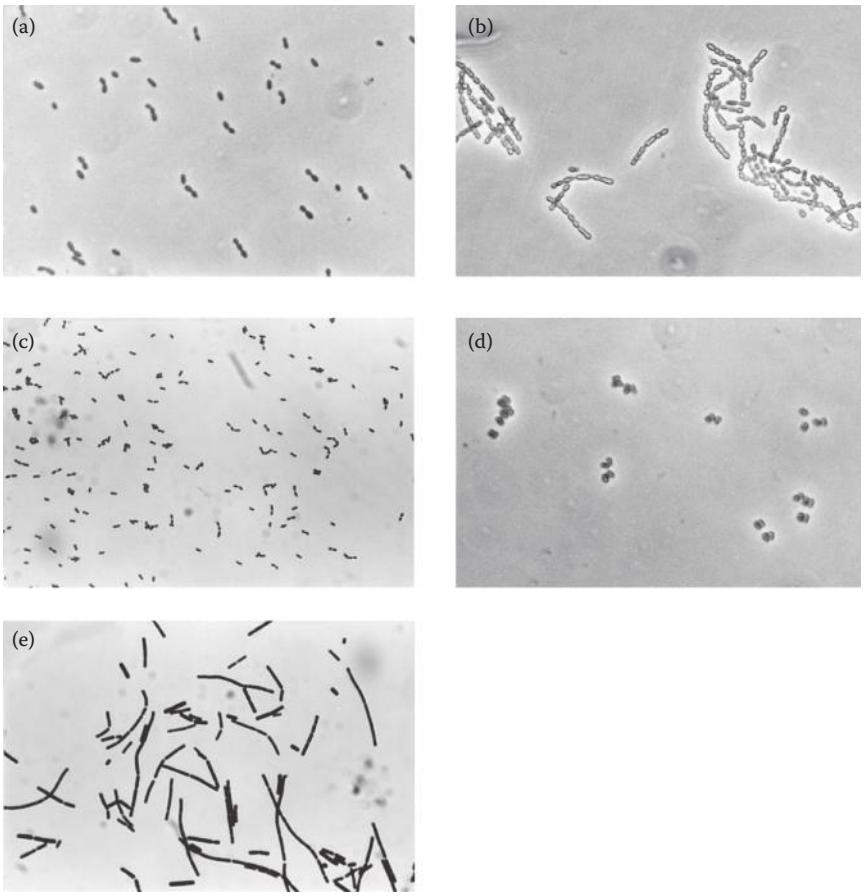



**Figure 11.1 Photograph of lactic acid bacteria: (a) *Lactococcus lactis*, (b) *Streptococcus thermophilus*, (c) *Leuconostoc mesenteroides*, (d) *Pediococcus acidilactici*, and (e) *Lactobacillus acidophilus*.**

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## Streptococcus

Only one species, *Streptococcus thermophilus*, has been used in dairy fermentation. A change in designation to *Str. salivarius* ssp. *thermophilus* was suggested but not made. They are used in dairy fermentation. The Gram-positive cells are spherical to ovoid, 0.7–0.9 µm in diameter, and exist in pairs to long chains (Figure 11.1). The cells grow well at 37°C–40°C but can also grow at 52°C. They are facultative anaerobes and, in glucose broth, can reduce the pH to 4.0 and produce L(+)-lactic acid. They ferment fructose, mannose, and lactose, but generally not galactose and sucrose. Cells can survive at 60°C for 30 minutes. Their natural habitat is unknown although they are found in milk.

## Leuconostoc

The Gram-positive cells are spherical to lenticular, arranged in pairs or in chains, nonmotile, nonsporulating, catalase negative, and facultative anaerobes (Figure 11.1). The species grow well between 20°C and 30°C with a range of 1°C–37°C. Glucose is fermented to D(–)-lactic acid, $CO_2$, ethanol, or acetic acid, with the pH reduced to 4.5–5.0. The species grow in milk but may not curdle. Also, arginine is not hydrolyzed. Many produce dextran while growing on sucrose. Citrate is utilized to produce diacetyl and $CO_2$. Some species can survive at 60°C for 30 minutes. *Leuconostoc* species are found in plants, vegetables, silage, milk and some milk products, and raw and processed meats.

At present, five species are known: *Leuconostoc mesenteroides*, *Leu. paramesenteroides*, *Leu. lactis*, *Leu. carnosum*, and *Leu. gelidum*. *Leu. mesenteroides* has three subspecies: subsp. *mesenteroides*, ssp. *dextranicum*, and ssp. *cremoris*. *Leu. mesenteroides* ssp. *cremoris* and *Leu. lactis* are used in some dairy and vegetable fermentations. Many of these species, particularly *Leu. carnosum* and *Leu. gelidum*, have been associated with spoilage of refrigerated vacuum-packaged meat products.

Leuconostocs are morphologically heterogenous and may contain genetically diverse groups of bacteria. Recently, two new genera have been created from it: *Weisella* and *Oenococcus*. *Oen. oeni* is found in wine and related habitats and is used for malolactic fermentation in wine.

## Pediococcus

These cells are spherical and form tetrads, but they can be present in pairs. Single cells or chains are absent (Figure 11.1). They are Gram-positive, nonmotile, nonsporulating, facultative anaerobes. They grow well between 25°C and 40°C; some species grow at 50°C. They ferment glucose to L(+)- or DL-lactic acid, some species reducing the pH to 3.6. Depending on the species, they can ferment sucrose, arabinose, ribose, and xylose. Lactose is not generally fermented, especially in milk, and milk is not curdled. Some strains may have weak lactose-hydrolyzing capability, especially in broth containing lactose as a carbohydrate source.

Depending on the species, they are found in plants, vegetables, silage, beer, milk, and fermented vegetables, meats, and fish. The genus has seven to eight species, of which *Pediococcus pentosaceus* and *Pediococcus acidilactici* are used in vegetables, meat, cereal, and other types of fermented foods. They have also been implicated in ripening and flavor production of some cheeses as secondary cultures. These two species are difficult to differentiate, but compared with *Ped. acidilactici*, *Ped. pentosaceus* ferments maltose, does not grow at 50°C, and is killed at 70°C in 5 minutes. *Ped. halophilus*, used in fermentation of high-salt products, is now classified as *Tet. halophilus*.

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## Lactobacillus

The genus *Lactobacillus* includes a heterogenous group of Gram-positive, rod-shaped, usually nonmotile, nonsporulating, facultative anaerobic species that vary widely morphologically and in growth and metabolic characteristics (Figure 11.1). Cells vary from very short (almost coccoid) to very long rods, slender or moderately thick, often bent, and can be present as single cells or in short to long chains. While growing on glucose, depending on the species, they produce either only lactic acid [L(+), D(–), or DL] or a mixture of lactic acid, ethanol, acetic acid, and $CO_2$. Some also produce diacetyl. Many species utilize lactose, sucrose, fructose, or galactose, and some species can ferment pentoses. Growth temperature can vary from 1°C to 50°C, but most that are used as starter cultures in controlled fermentation of foods grow well from 25°C to 40°C. Several species involved in natural fermentation of some foods at low temperatures can grow well from 10°C to 25°C. While growing in a metabolizable carbohydrate, depending on the species, the pH can be reduced to between 3.5 and 5.0.

They are distributed widely and can be found in plants; vegetables; grains; seeds; raw and processed milk and milk products; raw, processed, and fermented meat products; and fermented vegetables; some are found in the digestive tract of humans, animals, and birds. Many have been associated with spoilage of foods.

Among the large number of species, some have been used in controlled fermentation (dairy, meat, vegetables, and cereal), some are known to be associated with natural fermentation of foods, a few are consumed live for their beneficial effect on intestinal health, and some others have an undesirable effect on foods. On the basis of their metabolic patterns of hexoses and pentoses (discussed in Chapter 12), the species have been divided into three groups (Table 11.1). Those in Group I ferment hexoses (and disaccharides, such as lactose and sucrose) to produce mainly lactic acids and do not ferment pentoses (such as ribose, xylose, or arabinose). Those in Group II, depending on the carbohydrates and the amounts available, either produce mainly lactic acid, or a mixture of lactic, acetic, and formic acids; ethanol; and $CO_2$. Group III species ferment carbohydrates to a mixture of lactate, acetate, ethanol, and $CO_2$.

The three *Lactobacillus delbrueckii* subspecies are used in the fermentation of dairy products, such as some cheeses and yogurt. They grow well at 45°C and ferment lactose to produce large amounts of D(–) lactic acid. β-Galactosidase in these subspecies is constitutive. *Lactobacillus acidophilus* and *Lab. reuteri* are considered to be beneficial intestinal microbes (probiotic) and are present in the small intestine. *Lab. acidophilus* is used to produce fermented dairy products and also either added to pasteurized milk or made into tablets and capsules for consumption as probiotics. It metabolizes lactose and produces large amounts of D(–)-lactic acid. However, in *Lab. acidophilus*, β-galactosidase is generally inducible. *Lab. helveticus* is used to make some cheeses and ferment lactose to lactic acid (DL). *Lab. casei* ssp. *casei* is used in some fermented dairy products. It ferments lactose and produces L(+)-lactic acid. Some strains are also used as probiotic bacteria. Strains of *Lab. casei* ssp. *rhamnosus* (also called *Lab. rhamnosus*) are now used as a probiotic bacterium. Some strains of *Lab. johnsonii* are also used in probiotics. *Lab. plantarum* is used in vegetable and meat fermentation. It produces DL-lactic acid. *Lab. curvatus* and *Lab. sake* can grow at low temperatures (2°C–4°C) and ferment vegetable and meat products. *Lab. sake* is used to ferment sake wine. *Lab. kefir* is important in the fermentation of kefir, an ethnic fermented sour milk. *Lab. sanfrancisco* is associated with other microorganisms in the fermentation of San Francisco sourdough bread. *Lab. viridescens*, *Lab. curvatus*, and *Lab sake* are associated with spoilage of refrigerated meat products.

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**Table 11.1 Division of *Lactobacillus* Species into Groups**

| *Characteristics* | *Group I* | *Group II* | *Group III* |
|---|---|---|---|
| Previous designation | Thermobacterium | Streptobacterium | Betabacterium |
| Carbohydrate fermentation patterns[a] | Obligately homofermentative | Facultatively heterofermentative | Obligately heterofermentative |
| End products of carbohydrate fermentation | Lactate | Lactate or lactate, acetate, ethanol, $CO_2$, formate | Lactate, acetate, ethanol, $CO_2$ |
| Ferment pentoses | – | + | + |
| Representative species | *Lab. delbrueckii* ssp. *delbrueckii, bulgaricus, lactis*<br>*Lab. leichmannii*<br>*Lab. acidophilus*<br>*Lab. helveticus* | *Lab. casei* ssp. *casei, rhamnosus, pseudoplanatarum*<br>*Lab. plantarum*<br>*Lab. curvatus*<br>*Lab. sake* | *Lab. fermentum*<br>*Lab. divergens*<br>*Lab. kefir*<br>*Lab. confuses*<br>*Lab. brevis*<br>*Lab. sanfrancisco*<br>*Lab. reuteri* |

[a] Homofermentatives (produce mainly lactic acid) and heterofermentatives (produce lactic acid as well as large amounts of other products).

### Oenococcus

*Oen. oeni*, previously designated as *Leu. oeni*, has the general characteristics of *Leuconostoc* spp. It is found in the winery environment. It is sometimes used to accelerate malolactic fermentation in wine.[6] The cells transport malate in wine and metabolize it to lactic acid and $CO_2$. This process reduces the acidity of wine.

## Other Starter Cultures

### Bifidobacterium

These are morphologically similar to some *Lactobacillus* spp. and were previously included in the genus *Lactobacillus*. The cells are Gram-positive rods of various shapes and sizes, present as single cells or in chains of different sizes. They are non-spore-forming, nonmotile, and anaerobic although some can tolerate $O_2$ in the presence of $CO_2$. The species grow optimally at 37°C–41°C with a growth temperature range of 25°C–45°C. They usually do not grow at a pH above 8.0 or below 4.5. They ferment glucose to produce lactic and acetic acids in a 2:3 molar ratio without producing $CO_2$ and also ferment lactose, galactose, and some pentoses.

They have been isolated from the feces of humans, animals, and birds and are considered beneficial for the normal health of the digestive tract. They are present in large numbers in the feces of

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infants within 2–3 days after birth and are usually present in high numbers in breast-fed babies. They are usually found in the large intestine.

Many species of this genus have been isolated from the feces of humans and animals. Some of these include *Bifidobacterium bifidum*, *Bif. animalis*, *Bif. longum*, *Bif. breve*, *Bif. infantis*, *Bif. angulatum*, and *Bif. adolescentis*.[7] The genome sequence of many of the species is now completed. Many also produce bacteriocins and may play an important role in controlling pathogens during intestinal colonization in the gut. All have been isolated in humans; however, some species are more prevalent in infants than in adults. Some of these species have been added to dairy products to supply live cells in high numbers to restore and maintain intestinal health in humans.

### Propionibacterium

This genus includes species in the classical or dairy propionibacterium group and the cutaneous or acne propionibacterium group. Here, only the dairy group is discussed.

The cells are Gram-positive, pleomorphic thick rods 1–1.5 μm in length and occur in single cells, pairs, or short chains with different configurations. They are nonmotile, nonsporulating, anaerobic (can also tolerate air), and catalase positive, and they ferment glucose to produce large amounts of propionic acid and acetic acid. They also, depending on the species, ferment lactose, sucrose, fructose, galactose, and some pentoses. They grow optimally at 30°C–37°C. Some species form pigments. They have been isolated from raw milk, some types of cheeses, dairy products, and silage.

At present, four species of dairy propionibacterium are included in the genus: *Propionibacterium freudenreichii*, *Pro. jensenii*, *Pro. thoenii*, and *Pro. acidipropionici*. All four are associated with natural fermentation of Swiss-type cheeses, but *Pro. freudenreichii* has been used as a starter culture in controlled fermentation.

### Brevibacterium

The genus contains a mixture of coryniform bacterial species, some of which have important applications in cheese production and other industrial fermentations. *Brevibacterium linens* is used in cheese ripening as it has extracellular proteases. The cells are nonmotile, Gram-positive, and capable of growing in high salt and wide pH ranges.

### Acetobacter

A species in this genus, *Acetobacter aceti*, is used to produce acetic acid from alcohol. The cells are Gram-negative, aerobic, rods (0.5–1.5 μm); occurring as single cells, pairs, or chains; and can be motile or nonmotile. They are obligate aerobes, catalase positive, and oxidize ethanol to acetic acid and lactic acid to $CO_2$ and $H_2O$. They grow well from 25°C to 30°C. They are found naturally in fruits, sake, palm wine, cider, beer, sugarcane juice, tea fungus, and soil. Some species synthesize large amounts of cellulose.

## Yeasts and Molds

Many yeasts and molds are important in food, but most are involved with the spoilage of food and mycotoxin production (by molds). Several are, however, used in food bioprocessing. At present,

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genetic improvements are being made to improve their desirable characteristics. A brief discussion is provided here for some that are used in food fermentation.

## Yeasts

Among the many types of yeasts, only a few have been associated with fermentation of foods and alcohol, production of enzymes for use in food, production of single cell proteins (SCPs), and as additives to impart desirable flavor in some foods. The most important genus and species used is *Saccharomyces cerevisiae*.[8] It has been used to leaven bread[9] and produce beer, wine, distilled liquors, and industrial alcohol; produce invertase (enzyme); and flavor some foods (soups). However, many strains have been developed to suit specific needs, such as ethanol fermentation from biomass as biofuel.[10]

The cells are round, oval, or elongated. They multiply by multipolar budding or by conjugation and formation of ascospores. The strains are generally grouped as bottom yeasts or top yeasts. Top yeasts grow very rapidly at 20°C, producing alcohol and $CO_2$. They also form clumps that, because of rapid $CO_2$ production, float at the surface. In contrast, bottom yeasts grow better at 10°C–15°C, produce $CO_2$ slowly (they also grow slowly), do not clump, and thus settle at the bottom. Top yeasts and bottom yeasts are used according to the need of a particular fermentation process.

*Candida utilis* has been used to produce SCPs. It is a false yeast (*Fungi imperfecti*) and reproduces by budding (not by conjugation). The cells are oval to elongated and form hyphae with large numbers of budding cells. They are also involved in food spoilage.

*Kluyveromyces marxianus* and *Klu. marxianus* var. *lactis* can hydrolyze lactose and have been associated with natural fermentation, along with other yeasts and lactic acid bacteria, of alcoholic dairy products such as kefir. They have also been associated with spoilage of some dairy products.[6] At present, they are used to produce β-galactosidase (lactase) for commercial use to hydrolyze lactose. The enzyme is now used to produce low-lactose milk.

## Molds

Although most molds are associated with food spoilage and many form mycotoxins while growing in foods, other species and strains are used in processing of foods and to produce additives and enzymes for use in foods.[11]

In general, molds are multicellular, filamentous fungi. The filaments (hyphae) can be septate or nonseptate and have nuclei. They divide by elongation at the tip of a hypha (vegetative reproduction) or by forming sexual or asexual spores on a spore-bearing body.

Among many genera, several species from genera *Aspergillus* and *Penicillium* and a few from *Rhizopus* and *Mucor*, have been used for beneficial purposes in food. Strains to be used for such purposes should not produce mycotoxins. It is difficult to identify a nonmycotoxin-producer strain in the case of natural fermentation, but it should be an important consideration in the selection of strains for use in controlled fermentation.

*Aspergillus oryzae* is used in fermentation of several oriental foods, such as sake, soy sauce, and miso. It is also used as a source of some food enzymes. *Asp. niger* is used to produce citric acid and gluconic acid from sucrose. It is also used as a source of the enzymes pectinase and amylase. *Rhizopus oryza* is used to make tempe or temphe, murcha, chu, and nuruk. *Penicillium roquefortii* is used for ripening of Roquefort, Gorgonzola, and blue cheeses. Some strains can produce the neurotoxin roquefortin. In the selection and development of strains for use in

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cheese, this aspect needs careful consideration. *Pen. camembertii* is used in Camembert cheese and *Pen. caseicolum* is used in Brie cheese. They are also used to produce the enzyme glucose oxidase.

## Conclusion

Food-grade bacteria, yeasts, and molds are used in different combinations to produce several thousands of fermented foods worldwide by natural or controlled fermentation of milk, meat, fish, egg, fruits, vegetables and others. The species and strains used as starter cultures in controlled fermentation should not only be safe and regulated but also be able to produce desirable characteristics in the fermented foods. These characteristics are the result of metabolic breakdown of carbohydrates, proteins, and lipids present in the food. Some of these are discussed briefly in Chapter 12 (also see Chapter 8).

### QUESTIONS

1. Discuss the criteria used to select a microorganism for beneficial purposes in foods.
2. List the different ways microorganisms are used beneficially in foods.
3. List the genera that are now included in the group of lactic acid bacteria.
4. List the genera currently used as starter cultures in food fermentation.
5. List one species each from *Lactococcus* and *Streptococcus* and two each from *Leuconostoc*, *Pediococcus, Bifidobacterium*, and *Propionibacterium* used in food fermentation.
6. How are the species in genus *Lactobacillus* (the basis) divided into groups? List two species from each group.
7. When are the following terms used: lactococci and *Lactococcus*; streptococci and *Streptococcus*; leuconostocs and *Leuconostoc*; pediococci and *Pediococcus*; and lactobacilli and *Lactobacillus*?
8. Name three bacterial species that are now used as probiotic bacteria.
9. In yeast fermentation of different foods and beverages, only one species is used. Name the species, and discuss how one species can be effective in so many fermentation processes. Discuss the characteristics of bottom and top yeasts. Name a species that is now used commercially to produce β-galactosidase (lactase).
10. How are molds used in different ways in food? Name two species and list their uses. What precautions are needed while using a mold strain in food fermentation?

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# Chapter 12

# Biochemistry of Some Beneficial Traits

## Introduction

Beneficial microorganisms metabolize some of the components present in starting materials (i.e., food, such as milk or meat) to produce energy and cellular materials and to multiply. In this process, they produce some end products that are no longer necessary for the cells, and therefore, these byproducts are excreted into the environment. Some of these byproducts impart unique characteristics (mostly texture and flavor) to the remaining components of the starting materials. These are bioprocessed or fermented products and are considered desirable by consumers. Some of the byproducts of fermentation can also be purified and used as food additives. The production of several of these byproducts by some desirable microorganisms is discussed in this chapter (see also Chapter 8).

Before describing the metabolic pathways used by these microbes, it will be helpful to review the components of foods (substrates) used in fermentation. Before the substrates are metabolized inside microbial cells, they have to be transported from the outside environment. It will be beneficial to recognize, in brief, the cellular components involved in the transport of these substrates.

The important substrates available in the starting materials of fermentation include several carbohydrates, proteinaceous and nonprotein nitrogenous (NPN) compounds, and lipids. The important fermentable carbohydrates in foods are starch, glycogen (in meat), lactose (in dairy products), sucrose, maltose (from breakdown of starch), glucose, fructose, and pentoses (from plant sources). The proteinaceous and NPN components include mainly large proteins (both structural and functional), peptides of different sizes, and amino acids. Lipids can include triglycerides, phospholipids, fatty acids, and sterols. Microorganisms differ greatly in their ability to transport these components from the outside and metabolize them inside the cells.

## Mechanisms of Transport of Nutrients

Nutrient molecules have to pass through the cell barriers: the cell wall and the cell membrane. However, in most Gram-positive lactic acid bacteria, the main barrier is the cytoplasmic membrane.



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The cytoplasmic membrane is made up of two layers of lipids in which protein molecules are embedded, some of which span the lipid bilayer from the cytoplasmic side to the cell wall side (Figures 12.1; also see Figure 2.4). Many of them are transport proteins involved in carrying nutrient molecules from the outside into the cell (also removing many byproducts from the cell into the environment).[1,2]

In general, small molecules, such as monosaccharides and disaccharides, amino acids, and small peptides (up to 8–10 amino acids), are transported almost unchanged inside the cell by specific transport systems, either singly or in groups. Fatty acids (either free or hydrolyzed from glycerides) can dissolve and diffuse through the lipid bilayers. In contrast, large carbohydrates (polysaccharides, such as starch), large peptides, and proteins (such as casein and albumen) cannot be transported directly inside the cell. If a cell is capable of producing specific extracellular hydrolyzing enzymes that are either present on the surface of the cell wall or released into the environment, then large nutrient molecules can be broken down to small molecules and then transported by the appropriate transport systems.[3]

Monosaccharides and disaccharides, amino acids, and small peptides are transported through the membrane by different active transport systems, such as primary transport systems (e.g., ATP-binding cassette or ABC transporters), secondary transport systems (e.g., uniport, symport, and antiport systems that use proton motive force), and phosphoenolpyruvate-phosphotransferase (PEP-PTS) systems. A system can be specific for a type of molecule or for a group of similar molecules (group transfer) and can transport against the concentration gradient of a substrate, and the transport process requires energy. In the PEP-PTS system for PTS sugars, the energy is derived from PEP; in the permease system (for permease sugars, amino acids, and probably small peptides), energy is derived from the proton motive force.

## Transport and Metabolism of Carbohydrates

In lactic acid bacteria and other bacteria used in food fermentation, disaccharide and monosaccharide (both hexoses and pentoses) molecules can be transported by PEP-PTS as well as by permease

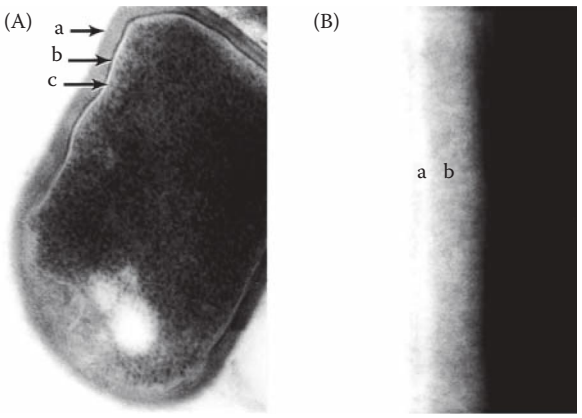



**Figure 12.1 (A) Transmission electron microscopy photograph of a thin section of *Lactobacillus acidophilus* cell showing the (a) anionic (teichoic, teichouronic, lipoteichoic acids) polysaccharide layer, (b) mucopeptide layer, and (c) cytoplasmic membrane. (B) Negatively stained electron microscopy photograph of *Lactobacillus acidophilus* showing (a) surface-layer protein and (b) cell wall. Cytoplasm is stained dark.**

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systems.[2–4] The same carbohydrate can be transported by the PEP-PTS system in one species and by the permease system in another species. Similarly, in a species, some carbohydrates are transported by the PEP-PTS system, whereas others are transported by the permease system.

## *PEP-PTS System for Lactose Transport in* Lactococcus lactis

The high-energy phosphate from PEP is transferred sequentially to EnzI, HPr (both in the cytoplasm and nonspecific for lactose), $FacIII_{Lac}$, and $EnzII_{Lac}$ (both on the membrane and specific for lactose), and finally to lactose. Lactose from the environment is transported in the cytoplasm as lactose-phosphate (galactose-6-phosphate-glucose) (Figure 12.2).

## *Permease System for Lactose in* Lactobacillus acidophilus

$$\text{Lactose} + [H^+]$$
$$\downarrow$$
$$\text{Permease}_{Lac} \rightarrow [\text{Permease} : \text{Lactose} : H^+] \rightarrow \text{Lactose} + [H^+]$$

One molecule of lactose carries one $H^+$ with it in the $Permease_{Lac}$ molecule (specific for lactose). Once inside, there is a conformation change in the permease molecule, which causes release of the lactose molecule and $H^+$ inside the cytoplasm. Release of lactose and $H^+$ causes permease to change to the original conformation. Lactose is transported as lactose (galactose–glucose).

## *Carbohydrates Available Inside the Cells for Metabolism*

Monosaccharides and disaccharides are transported from the environment inside the cells either by the permease or PEP-PTS system. In food fermentation, they generally include several pentoses, glucose, fructose, sucrose, maltose, and lactose. Pentoses and hexoses are metabolized by several different pathways as described later. Sucrose, maltose, and lactose, the three disaccharides, are hydrolyzed inside the cell by the enzymes sucrase, maltase, and lactase (β-galactosidase), respectively, to hexoses. Lactose-P (galactose-6-phosphate-glucose) is hydrolyzed by phospho-β-galactosidase to yield glucose and galactose-6-phosphate before further metabolism.

## *Homolactic Fermentation of Carbohydrates*

Hexoses in the cytoplasm, either transported as hexoses or derived from disaccharides, are fermented by homolactic species of lactic acid bacteria to produce mainly lactic acid. Theoretically, one hexose molecule will produce two molecules of lactate. The species include genera from *Lactococcus*, *Streptococcus*, *Pediococcus*, and Group I and Group II *Lactobacillus* (Table 12.1; also

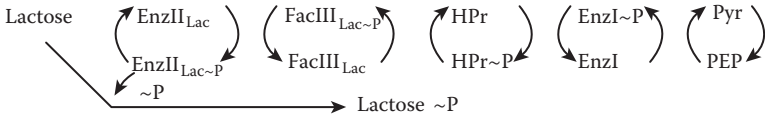



**Figure 12.2 PEP-PTS system for lactose transport.**

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**Table 12.1 Fermentation of Monosaccharides by Some Starter-Culture Bacteria to Produce Different Byproducts**

| *Genus* | *Monosaccharide* | *Fermentation* | *Pathway*[a] | *Main Products* |
|---|---|---|---|---|
| *Lactococcus* | Hexoses | Homolactic | EMP | Lactate |
| *Streptococcus* | Hexoses | Homolactic | EMP | Lactate |
| *Pediococcus* | Hexoses | Homolactic | EMP | Lactate |
| *Leuconostoc* | Hexoses | Heterolactic | HMS | Lactate, $CO_2$, acetate/ethanol (1:1:1) |
| | Pentoses | Heterolactic | PP | Lactate, acetate/ethanol (1:1) |
| *Lactobacillus* | | | | |
| Group I | Hexoses | Homolactic | EMP | Lactate |
| Group II | Hexoses | Homolactic | EMP | Lactate |
| | Pentoses | Heterolactic | PP | Lactate, acetate/ethanol (1:1) |
| Group III | Hexoses | Heterolactic | HMS | Lactate, $CO_2$, acetate/ethanol (1:1:1) |
| | Pentoses | Heterolactic | PP | Lactate, acetate/ethanol (1:1) |
| *Bifidobacterium* | Hexoses | Heterolactic | BP | Lactate, acetate (1:1.5) |

[a] EMP, Embden-Meyerhoff-Parnas; HMS, hexose monophosphate shunt (also called phosphogluconate-phosphoketolase); PP, pentose-phosphate; BP, bifidus (also called fructose ketolase).

see Table 11.1). Hexoses are metabolized through the Embden-Meyerhoff-Parnas (EMP) pathway (Figure 12.3). These species have fructose diphosphate (FDP) aldolase, which is necessary to hydrolyze a 6C hexose to yield two molecules of 3C compounds. They also lack a key enzyme, phosphoketolase (in HMS pathway), present in species that are heterolactic fermentors.[2,5]

In the EMP pathway, with glucose as the substrate, two ATP molecules are used to convert glucose to fructose-1,6-diphosphate (Figure 12.3). Hydrolysis of these molecules generates two molecules of 3C compounds. Subsequent dehydrogenation (to produce NADH + $H^+$ from NAD), phosphorylation, and generation of two ATP molecules lead to production of PEP (can be used in PEP-PTS sugar transport). Through the generation of substrate-level ATP, PEP is converted to pyruvate, which, by the action of lactate dehydrogenase, is converted to lactic acid. The ability of a lactic acid bacterial species to produce L(+)-, D(–)-, or DL-lactic acid is determined by the type of lactate dehydrogenase (L, D, or a mixture of both, respectively) it contains. The overall reaction involves the production of two molecules each of lactic acid and ATP from one molecule of hexose. The lactic acid is excreted into the environment.

Other hexoses, such as fructose (transported as fructose or from hydrolysis of sucrose), galactose (from hydrolysis of lactose), and galactose-6-phosphate (from hydrolysis of lactose-phosphate following transport of lactose by the PEP-PTS system), undergo different molecular conversion

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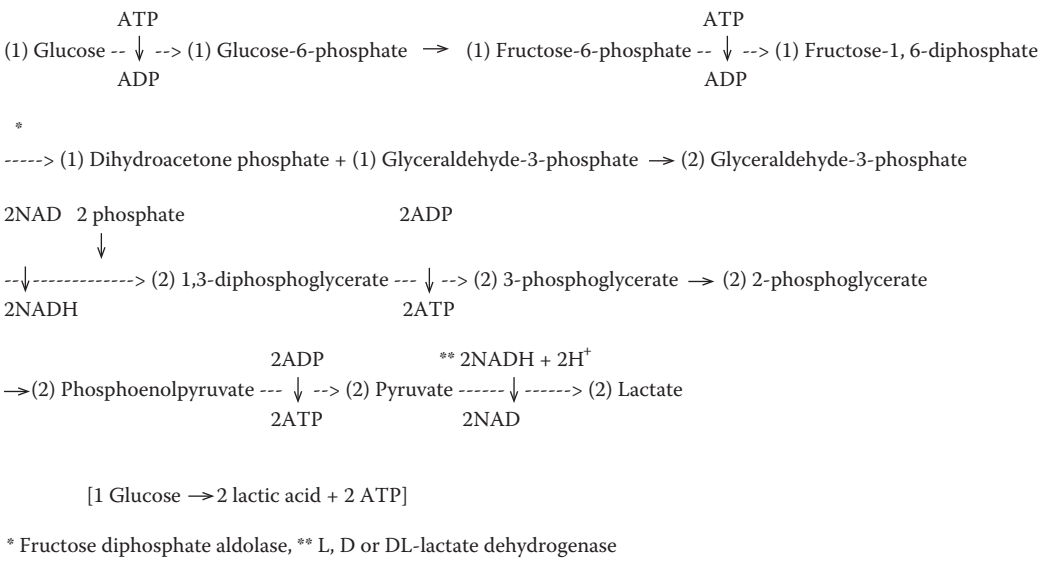



**Figure 12.3 Homolactic fermentation of hexoses through EMP pathway.**

before they can be metabolized in the EMP pathway. Thus, fructose is phosphorylated by ATP to fructose-6-phosphate before being used in the EMP pathway. Galactose is converted first to galactose-1-phosphate, then to glucose-1-phosphate, and finally to glucose-6-phosphate through the Leloir pathway before entering the EMP pathway. Galactose-6-phosphate is first converted to tagatose-6-phosphate, then to tagatose-1,6-diphosphate, and then hydrolyzed to dihydroacetone phosphate and glyceraldehyde-3-phosphate by the tagatose pathway before entering the EMP pathway.

Besides being an important component in the production of fermented foods (e.g., yogurt, cheeses, and fermented sausage and vegetables), lactic acid is used as an ingredient in many foods (e.g., processed meat products). For this purpose, L(+)-lactic acid is preferred and approved by regulatory agencies as a food additive (as it is also produced by the muscle). Lactic acid bacteria capable of producing L(+)-lactic acid in large amounts (mainly some *Lactobacillus* spp.) are commercially used for this purpose. At present, genetic studies are being conducted to develop strains by inactivating the D-lactate dehydrogenase system in species that have both the L and D systems and produce large amounts of a mixture of L(+)- and D(–)-lactic acids. Some of the species that produce L(+)-lactic acid (>90% or more) are *Lactococcus lactis* ssp. *lactis* and *cremoris*, *Streptococcus thermophilus*, *Lactobacillus amylovorus*, *Lab. amylophilus*, *Lab. casei* spp. *casei*, *Lab. casei* spp. *rhamnosus*, and *Lab. divergens*. Some of the common species used in food fermentation, namely *Pediococcus acidilactici*, *Ped. pentosaceus*, *Lab. delbrueckii* spp. *bulgaricus* and *helveticus*, *Lab. acidophilus, Lab. reuteri*, and *Lab. plantarum*, produce a mixture of D(–)- and L(+)-lactic acids with 20%–70% being L(+)-lactic acid.[2]

## *Heterolactic Fermentation of Carbohydrates*

Hexoses are metabolized to produce a mixture of lactic acid, $CO_2$, and acetate or ethanol by heterofermentative lactic acid bacteria (Table 12.1). Species from genera *Leuconostoc* and Group III *Lactobacillus* lack fructose diphosphoaldolase (of the EMP pathway) but have glucose phosphate

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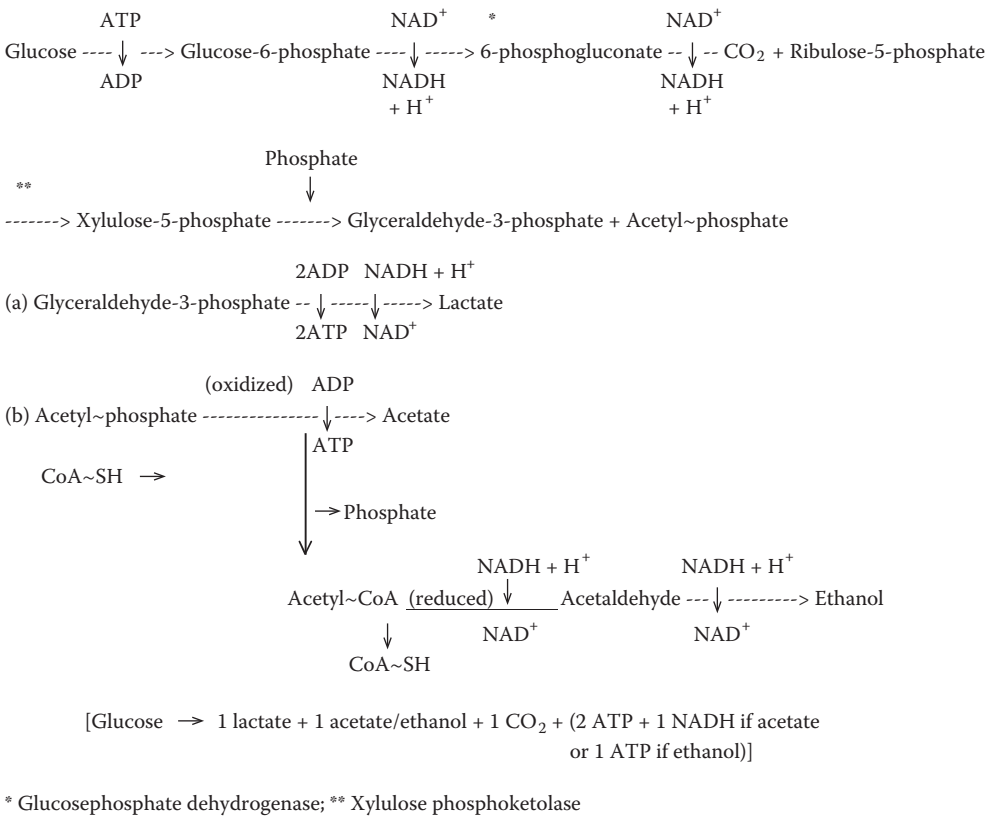



**Figure 12.4 Heterolactic fermentation of hexoses through HMS.**

dehydrogenase and xylulose phosphoketolase enzymes, which enable them to metabolize hexoses through the phosphogluconate-phosphoketolase pathway (or hexose monophosphate shunt) to generate energy.

This pathway has an initial oxidative phase followed by a nonoxidative phase (Figure 12.4). In the oxidative phase, glucose following phosphorylation is oxidized to 6-phosphogluconate by glucose phosphate dehydrogenase and then decarboxylated to produce one $CO_2$ molecule and a 5C compound, ribulose-5-phosphate. In the nonoxidative phase, the 5C compound is converted to xylulose-5-phosphate, which, through hydrolysis, produces one glyceraldehyde-3-phosphate and one acetyl phosphate. Glyceraldehyde-3-phosphate is subsequently converted to lactate. Acetyl phosphate can be oxidized to yield acetate or reduced to yield ethanol (depending on the O-R potential of the environment). Species differ in their abilities to produce ethanol, acetate, or a mixture of both. The end products are excreted into the environment.

## *Metabolism of Pentoses*

The species in genera *Leuconostoc* and Group III *Lactobacillus* can ferment different pentose sugars by the pentose-phosphate pathway to produce ATP, lactate, and acetate because they have the phosphoketolase enzyme. In Group II *Lactobacillus*, this enzyme is inducible and is produced only

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when a pentose is present in the environment. Although *Ped. pentosaceus*, *Ped. acidilactici*, and *Lac. lactis* can metabolize some pentoses, the pathways are not clearly known.[2,6]

The metabolizable pentoses by the *Leuconostoc* and *Lactobacillus* (Group II and Group III) are first converted to xylulose-5-phosphate in several different ways. Xylulose-5-phosphate is then metabolized to produce lactate and acetate or ethanol by the mechanisms described in the nonoxidizing portion of metabolism of hexoses by heterofermentative lactic acid bacteria (Figure 12.4). No $CO_2$ is produced from the metabolism of pentoses through this pathway.

## *Hexose Fermentation by* Bifidobacterium

*Bifidobacterium* species metabolize hexoses to produce lactate and acetate by the fructose-phosphate shunt or bifidus pathway. For every two molecules of hexoses, two molecules of lactate and three molecules of acetate are produced without generation of any $CO_2$ (Figure 12.5). From two molecules of fructose-6-phosphate, generated from two molecules of glucose, one molecule is converted to produce one 4C erythrose-4-phosphate and one acetyl-phosphate (which is then converted to acetate). Another molecule of fructose-6-phosphate combines with erythrose-4-phosphate to generate two molecules of the 5C xylulose-5-phosphate through several intermediate steps. Xylulose-5-phosphates are then metabolized to produce lactates and acetates by the method described in the nonoxidizing part of heterolactic fermentation (also in pentose fermentation, Figure 12.4).

## *Diacetyl Production from Citrate*

Diacetyl, a 4C compound, is important in many fermented dairy products for its pleasing aroma or flavor (butter flavor). It is also used separately in many foods to impart a butter flavor. Many lactic acid bacteria can produce it in small amounts from pyruvate, which is generated from

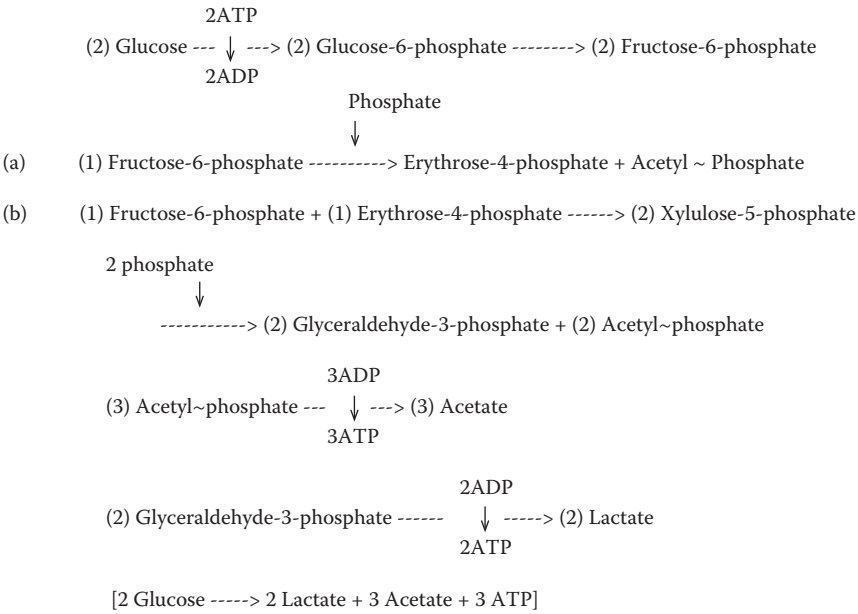



**Figure 12.5 Hexose fermentation by *Bifidobacterium*.**

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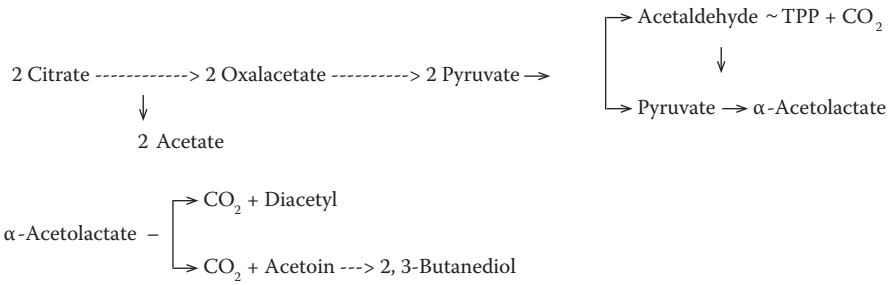



**Figure 12.6 Diacetyl production from citrate.**

carbohydrate metabolism (Chapter 8). However, *Lac. lactis* ssp. *lactis* biovar diacetylactis and *Leuconostoc* species can produce large amounts of diacetyl from citrate (Figure 12.6). Citrate, a 6C compound, is transported from the outside into the cells by the citrate-permease system. It is then metabolized through pyruvate to acetaldehyde ~ TPP (thiamin pyrophosphate). It then combines with pyruvate to form alpha-acetolactate, which is converted to diacetyl with the generation of $CO_2$. Under a reduced condition, diacetyl can be converted to acetoin, with a loss of desirable flavor. A producer strain can also be genetically modified to produce excess diacetyl (metabolic engineering).[2]

### *Propionic Acid Production by* Propionibacterium

The desirable flavor of some cheeses (such as Swiss) in which dairy *Propionibacterium* is used as one of the starter cultures is from propionic acid. *Propionibacterium* generates pyruvate from hexoses through the EMP pathway, and the pyruvate is used to generate propionic acid (Figure 12.7). Pyruvate and methylmalonyl ~ CoA produce propionyl ~ CoA and oxalacetate. Propionyl ~ CoA is then converted to propionate. Oxalacetate is recycled to generate methylmalonyl ~ CoA through succinyl ~ CoA. *Propionibacterium* also generates acetate and $CO_2$ ($CO_2$ contributes to eye formation in Swiss cheese) from pyruvate.

## Transport and Metabolism of Proteinaceous Compounds and Amino Acids

Many lactic acid bacteria used in food fermentation have active transport systems for transporting amino acids and small peptides (ca. 8–10 amino acids long).[7] Large peptides and metabolizable

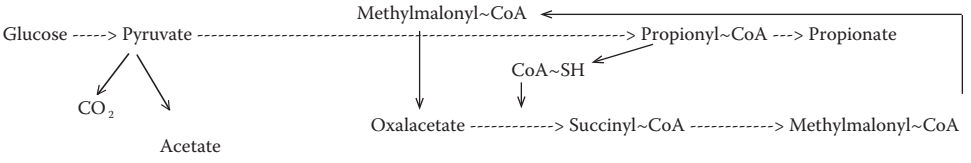



**Figure 12.7 Propionic acid metabolism in *Propionibacterium*.**

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proteins in the environment are hydrolyzed by the proteinase and peptidase enzymes, located mainly on the cell wall of these species, to small peptides and amino acids and then transported, mainly by active group transport systems, inside the cells. Inside the cells, peptides are converted to amino acids, and the amino acids are then metabolized differently to produce many different end products, which are excreted into the environment. Some of the metabolic processes involve decarboxylation (generates amines, some of which are biologically active, such as histamine from histidine), deamination, oxidative reduction, and anaerobic reduction. Amino acid metabolism by lactic acid bacteria and different associative bacteria during fermentation can produce diverse products, many of which have specific flavor characteristics. Some of these include ammonia, hydrogen sulfide, amines, mercaptans, and disulfides. Many of them, in low concentrations, contribute to the desirable flavor of different fermented foods. Proper hydrolysis of food proteins by the proteolytic enzymes of starter microorganisms is important for the desirable texture of many fermented foods (such as in cheeses). Rapid hydrolysis of proteins can result in production and accumulation of some specific hydrophobic peptides that impart a bitter taste to the product (such as in some sharp Cheddar cheeses).

## Transport and Metabolism of Lipid Compounds

Many starter-culture bacteria metabolize lipids poorly. However, molds have better lipid metabolism systems. Triglycerides and phospholipids are hydrolyzed outside of the cells by lipases, produced by the microorganisms, releasing fatty acids and glycerol and glycerides (monoglycerides or diglycerides). Fatty acids can diffuse through the membrane into the cells and be metabolized. Some fatty acids are incorporated in the membrane. Hydrolysis of glycerides, especially those with small fatty acids, such as butyric acid, can cause hydrolytic rancidity of the products. Oxidation of unsaturated fatty acids by microorganisms, especially molds, can produce many flavor compounds, either desirable or undesirable.

## Conclusion

Many safe and food-grade microbial species are used to produce fermented foods and food additives. They metabolize the carbohydrates, proteins, and lipids in the food by specific pathways and produce metabolites that, in turn, bring about the desirable acceptance characteristics of the fermented foods and food additives. The metabolic pathways are regulated by many enzymes, the genetic information of which is coded in the genes located in the chromosome and plasmid in a cell. The nature of genes and the mechanisms by which the genetic codes are translated into enzymes in lactic acid bacteria are discussed in Chapter 13.

### QUESTIONS

1. Name two species of yeasts and briefly discuss the use of each in the production of fermented foods and food additives.
2. List three mold species and indicate their specific uses in food bioprocessing.
3. List the main carbohydrates, proteinaceous and NPN compounds, and lipids in food systems that starter-culture bacteria have at their disposal to metabolize.
4. Discuss how lactic acid bacteria, while growing in milk, are able to transport lactose.

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5. What are the different types of food carbohydrates transported inside the lactic acid bacterial cells? Explain the functions of β-galactosidase and phospho-β-galactosidase.
6. Define homolactic and heterolactic fermentation of carbohydrates by lactic acid bacteria. Give three examples of lactic acid bacteria in each fermentation group.
7. List the end products (including energy generation) of homolactic and heterolactic fermentation when glucose is used as a substrate. Which enzymes play crucial roles in each pathway?
8. How are galactose, galactose-6-phosphate, and fructose metabolized by the EMP pathway by homolactic fermentors? List three species from three genera that produce only L(+)-lactic acid from glucose.
9. Why is the bifidus pathway of the metabolism of hexose also called the fructose-phosphate shunt? What are the end products?
10. How is citrate transported and metabolized to produce diacetyl by some lactic acid bacteria? List two species from two genera that produce diacetyl from citrate. How can other lactic acid bacteria produce diacetyl from carbohydrate metabolism?
11. Pyruvate can be used to produce products other than lactic acid. List four such products and show the reactions involved in their production.
12. Briefly discuss how food proteins and food lipids are transported and metabolized by starter-culture bacteria.

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# Chapter 13

# Genetics of Some Beneficial Traits

## Introduction

Since the 1930s, it has been recognized that many important characteristics (traits or phenotypes) in dairy starter-culture bacteria are unstable. For example, a *Lactococcus lactis* strain, while growing in milk, once able to ferment lactose and coagulate the milk, was found to no longer ferment lactose and became useless commercially. Similar losses of other commercially important traits of starter cultures used in dairy and nondairy fermentations, such as the ability to hydrolyze proteins (necessary for some cheese production), the ability to utilize citrate (for diacetyl production), resistance to bacteriophages, and hydrolysis of sucrose, were observed. However, the specific mechanisms involved in the instability of these important phenotypes were not understood. In the 1960s, the genetic basis of instability of different microbial phenotypes started unfolding. Similar studies, when extended to dairy starter-culture bacteria, revealed the genetic basis of the instability of the important traits. In those days, only a few laboratories were conducting research on the genetics of lactic acid bacteria (notably, Dr. Larry McKay's laboratory at the University of Minnesota). Since the late 1970s, many other laboratories have started working in this area, and, at present, genetic research of starter-culture bacteria has generated a major interest in many laboratories worldwide. The genetic basis of some of the commercially important phenotypes in some lactic acid bacteria; methods of transfer of desirable traits from one bacterial strain to another to develop a better strain for use in food fermentation; and current advances in genetic studies, such as metabolic engineering and genome sequencing in lactic acid bacteria, are discussed briefly in this chapter.

## Plasmids and Plasmid-Linked Traits in Starter-Culture Bacteria

Starter-culture bacteria, like other bacteria, carry genetic information (a genetic code) in the circular chromosomal DNA, circular plasmids, and transposons. Chromosomal DNA carries



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genetic codes for vital functions of a cell (such as a key enzyme in the EMP or HMS pathway in lactic acid bacteria). Although both plasmid DNA and transposons can carry genetic codes, they are only for nonvital functions, that is, characteristics that are not absolutely necessary for the survival of a cell (such as the ability to hydrolyze a large protein). However, having such a genetic code gives a strain a competitive advantage over other strains that lack it but share the same environment. Initial research in the early 1970s revealed that many industrially important phenotypes in different lactic acid bacteria are plasmid linked. Since then, because specific techniques are available, the genetic basis of many plasmid-encoded important phenotypes in many starter-culture bacteria, particularly in *Lactococcus lactis*, some *Leuconostoc* spp., and some *Lactobacillus* spp. has been studied. These studies have not only helped identify the locations of many genes, their structure, and the control systems involved in their expression, but also enabled researchers to transfer genes into a cell lacking a specific phenotype and to create a new desirable strain. Characteristics of plasmids and some plasmid-linked traits in starter-culture bacteria are discussed here.[1]

## *Important Characteristics of Bacterial Plasmids*

- Plasmids are double-stranded, circular, self-replicating DNA that can vary in size (<1 to >100 kb).
- Plasmids may not be present in all species or all strains in a species.
- A strain can have more than one type of plasmid that differs in size and the genetic code it carries.
- A plasmid can be present in more than one copy in a cell (copy number; this is in contrast to a single copy of chromosome that a cell can carry).
- For some plasmids, copy numbers can be reduced (depressed) or increased (amplified) by manipulating the control systems.
- Plasmids can differ in their stability in a cell. A plasmid from a cell can be lost spontaneously or by manipulation.
- Two types of plasmids in a cell may be incompatible, resulting in the loss of one.
- Plasmids can be transferred from one cell (donor) to another compatible cell (recipient) spontaneously or through manipulation.
- Plasmid transfer can occur either only between closely related strains (narrow host range) or between widely related strains from different species or genera (broad host range).
- A plasmid can be cryptic (i.e., not known to carry the genetic code for a known trait).
- Effective techniques for the isolation, purification, molecular weight determination, and nucleotide sequence determination of bacterial plasmids have been developed.
- Genetic codes from different sources (different prokaryotes and eukaryotes) can be introduced into a plasmid, which then can be transferred into the cell of an unrelated bacterial species in which the phenotype may be expressed.

## *Some Characteristics of Small (ca. 10 kb) and Large (over 10 to ca. 150 kb) Plasmids*

- *Copy number*. Small plasmids generally occur in multiple copies (10–40 per cell); large plasmids are generally present in lesser copies (for a very large plasmid even one copy per cell).
- *Amplification*. Many small plasmids can be amplified to a very high copy number; large plasmids, especially very large ones, cannot be amplified.

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- *Conjugal transfer*. Small plasmids are nonconjugative (but can be transferred along with a conjugative plasmid); large plasmids are generally conjugative.
- *Stability*. Small plasmids are usually unstable; large plasmids are usually stable.
- *Genetic code*. A large plasmid can encode many phenotypes; a small plasmid can encode only one or a few phenotypes. Plasmids can also have the operon system.

## Presence of Plasmids in Some Starter-Culture Bacteria

- *Lactococcus*. Many strains from both subspecies and the biovar have been analyzed. Most strains have 2–10 or more types of plasmids, both small and large.
- *Streptococcus thermophilus*. Among the strains examined, a few strains carry plasmids, generally only one to three types, mostly small and not very large.
- *Leuconostoc*. Many species and strains carry plasmids of different sizes, ranging from 1 to 10 or more types.
- *Pediococcus*. Limited studies show that the strains either have none or have two to three plasmids, both small and large types.
- *Lactobacillus*. Only a limited number of species and strains have been tested. Some species rarely carry plasmids, such as *Lactobacillus acidophilus*. Some carry usually a few, such as *Lactobacillus casei*. Some carry a large number (two to seven) of various sizes, such as *Lactobacillus plantarum*.
- *Bifidobacterium*. Limited studies reveal that the species harbor two to five different types of plasmids.
- *Propionibacterium*. Limited studies show that some species contain only a few plasmids.

## Phenotype Assignment to a Plasmid

Following the understanding of plasmid characteristics and the revelation that many starter-culture bacteria carry plasmids, studies were conducted to determine whether a particular plasmid in a strain carries genetic codes necessary for the expression of a specific phenotype or to determine whether a particular phenotype is linked to a specific plasmid in a strain. The loss of lactose-fermenting ability ($Lac^+$ phenotype) among *Lac. lactis* strains was suspected to be because of the loss of a plasmid that encodes genes necessary for lactose hydrolysis (also for lactose transport). The possible linkage of the $Lac^+$ phenotype to a plasmid in a *Lac. lactis* strain was first studied according to the following protocol:

A $Lac^+$ strain can become $Lac^-$ (with the inability to hydrolyze lactose) spontaneously or when grown in the presence of a chemical curing agent (e.g., acriflavin) or a physical curing agent (e.g., high temperature).

Analysis of the plasmid profile, that is, types of plasmids present as determined from their molecular size (kb), showed that a 53 kb plasmid present in the $Lac^+$ wild strain was missing in the $Lac^-$ cured variant. Thus, loss of this plasmid was correlated with the loss of the $Lac^+$ phenotype in this strain.

To determine further that the 53 kb plasmid actually encoded the $Lac^+$ phenotype, the wild $Lac^+$ strain was conjugally mated with a plasmidless $Lac^-$ strain, and several $Lac^+$ transconjugants were obtained. (This aspect is discussed later in mechanisms of DNA transfer.) When these transconjugants were analyzed, all were found to contain the 53 kb plasmid (Figure 13.1). Following curing, the transconjugants were converted to $Lac^-$, and an analysis showed that these variants no longer had the 53 kb plasmid.

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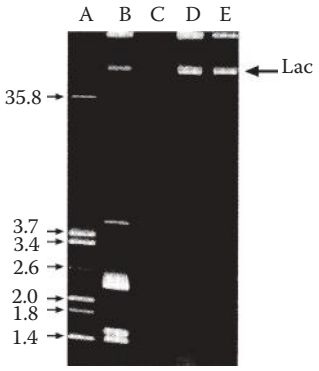



**Figure 13.1 Plasmid profiles of *Lactococcus lactis* ssp. *lactis*. Lane A: Plasmid standards of different molecular weights (Mda); lane B: donor strain with lac-plasmid (35.5 MDa or 53 kb; Lac$^+$ Sm$^s$); lane C: plasmidless recipient strain (Lac$^-$ Sm$^r$); lanes D and E: transconjugants showing lac-plasmid (Lac$^+$ Sm$^r$).**

From this series of experiments, it was determined that the 53 kb plasmid in the specific *Lac. lactis* strain used encodes Lac$^+$ phenotype.

Similar studies were conducted to determine plasmid linkage of several other traits in starter-culture bacteria.

## Plasmid-Linked Traits in Starter-Culture Bacteria

Many starter-culture bacteria, especially *Lactococcus lactis* strains, have been examined for the plasmid linkage of different phenotypes. The studies have revealed that in these bacteria, many commercially important traits are plasmid-encoded. The following are some traits:

- *Lac. lactis*
  Lac$^+$, lactose hydrolysis (also the lactose transport trait)
  Pro$^+$, proteinase activity
  Cit$^+$, citrate hydrolysis (also the citrate transport trait)
  Bac$^+$, production of several bacteriocins (also their respective immunity, processing, and translocation traits; bacteriocin, like nisin, is encoded in a transposon)
  Phage$^r$, resistance to specific bacteriophages
  R/M system, restriction/modification
  Resistance to several antibiotics (such as Km$^r$, resistance to kanamycin)
  Metabolism of several carbohydrates (such as Gal$^+$, galactose utilization)
  Muc$^+$, mucin production
- *Str. thermophilus*. Plasmid linkage of a phenotype not conclusively known
- *Leuconostoc* species. Bac$^+$, production of different bacteriocins (also immunity to them)
- *Pediococcus* species.
  Suc$^+$, sucrose hydrolysis
  Bac$^+$, production of different bacteriocins (also immunity to them and translocation)
- *Lactobacillus* species.
  Lac$^+$, lactose hydrolysis
  Mal$^+$, maltose hydrolysis

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Bac$^+$, production of some bacteriocins (also immunity to them)
Muc$^+$, ability to produce mucin
Resistance to some antibiotics (such as erythromycin, Em$^r$)
R/M system, restriction/modification

The same phenotype in a species can be encoded in different-size plasmids.

## Cryptic Plasmids

Many plasmids of lactic acid bacteria carry genes that encode for specific phenotypes. An example is the plasmid pSMB74 in *Pediococcus acidilactici* H, which encodes genes associated with the production of a bacteriocin, pediocin AcH. However, there are other plasmids in lactic acid bacteria for which no specific phenotypes can be assigned, and they are designated as cryptic plasmids. Some of them are able to integrate in the host chromosome and others are able to replicate in different homologous and heterologous hosts. For this reason, some of them have been used to construct vectors for cloning and expression of genes in lactic acid bacteria and some Gram-positive and Gram-negative bacteria. Plasmids pWVO1 and pSH71 have been extensively used to develop a series of cloning and expression vectors.

## Plasmid Replication

Plasmids of lactic acid bacteria have a high degree of sequence homology in the origin of replication (*repA*) and the gene encoding the replication protein (*repB*). Depending on the size, a plasmid replicates either by a sigma-type or by a theta-type mechanism. In the sigma (or rolling circle) replication mode, replication is initiated by the binding of the plasmid encoding the Rep protein to the origin (plus-origin site). This is followed by a nick in one and a fixation of Rep at the 5′ end of the nicked strand. The other strand is replicated from the nick site, causing displacement of the nicked strand, thus forming the shape of a sigma. The single strand is then ligated, forming a single-stranded circle and a duplex plasmid. The replication of the single strand is then followed to produce a double-stranded plasmid. This process generally occurs in small and cryptic plasmids (ca. 12 kb or less), which are relatively unstable.

In the theta-type replication, initiation starts with the formation of replication forks (as in the chromosome) resulting from separation of the base pairs. Both strands then simultaneously replicate, either in the same or in the opposite direction, and two copies of the plasmids are formed at the end of the replication. The plasmids are more than 12 kb, stable with a limited host range, and encode for many economically important phenotypes.

## Plasmid Mapping and Sequencing

Initially, the cells of a strain are lysed and plasmids are isolated, purified, and separated on the basis of molecular weight by agarose gel electrophoresis. The plasmid of interest is purified from the gel and used for mapping and sequencing. The purified plasmid is subjected to single and double digestions with a set of restriction endonuclease (RE) enzymes, and the number and molecular weight of the fragments generated after each digestion are determined. These results are used to construct the RE map of the plasmid. Individual fragments are then sequenced to construct the complete nucleotide sequence of the plasmid and to locate the open reading frames (ORFs) and regulatory regions in the plasmid. In many studies, the complete nucleotide sequence of a plasmid, especially if it is

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large, is not determined. To locate the genes expressing a phenotype, different fragments are cloned in a suitable vector, and afterward, transformation host cells are examined for the expression of the phenotype. The fragment that expresses the phenotype is then sequenced to determine the locations of the genes and the regulatory mechanisms. This process has been simplified in recent years by first purifying the protein coded by a gene and sequencing five or more N-terminal amino acids. From this information, nucleotide probes are constructed, and by PCR techniques, the location of the genes in the plasmid is determined; this helps sequence the gene by the several procedures available.

## Gene Transfer Methods in Starter-Culture Bacteria

Once the genetic basis of a phenotype in bacteria was understood, studies were conducted to develop means to transfer the genetic materials from one bacterial cell to another. It is recognized that exchange of genetic materials occurs among bacteria naturally but at a slower pace. However, if a process of introducing genetic materials can be developed under laboratory conditions, the process of genetic exchange can not only be expedited but can also help develop desirable strains. In starter-culture bacteria, this will help to develop a strain for a specific fermentation process that carries many desirable phenotypes and the least number of undesirable phenotypes.[2]

Results of the studies conducted since the 1970s, initially in *Lac. lactis* subspecies and later in other lactic acid bacteria, revealed that genetic materials can be introduced into bacterial cells by several different mechanisms, some of which are discussed here.

### *Transduction*

In this process, a transducing bacteriophage mediates the DNA exchange from one bacterial cell (donor) to another cell (recipient). DNA of some phages (designated as temperate bacteriophages) following infection of a cell can integrate with bacterial DNA and remain dormant (see Chapter 14). When induced, the phage DNA separates out from the bacterial DNA and, on some occasions, also carries a portion of the bacterial DNA-encoding genes in it. When the phage-carrying portion of a bacterial DNA infects a bacterial cell and integrates its DNA with bacterial DNA, the phenotype of that gene is expressed by the recipient cell. Initially, the $Lac^+$ phenotype from a lactose-hydrolyzing *Lac. lactis* strain carrying a temperate phage was transduced to a $Lac^-$ *Lactococcus lactis* strain to obtain a $Lac^+$ transductant. This method has been successfully used to transduce $Lac^+$ phenotype and several other phenotypes (such as $Pro^+$) in different strains of *Lac. lactis* subspecies. Investigations show that both chromosomal- and plasmid-encoded genes from bacteria can be transduced. Transduction has been conducted successfully in some strains of *Str. thermophilus* and in strains of several *Lactobacillus* species.

The transduction process in starter-culture bacteria is important to determine the location of a gene on the DNA for genetic mapping and to study its characteristics. A temperate phage can be induced spontaneously, resulting in lysis of bacterial cells; thus, it is not very useful in commercial fermentation. Also, this method cannot be applied in species that do not have bacteriophages, such as some *Pediococcus* species.

### *Conjugation*

In this process, a donor bacterial cell transfers a replica of a portion of its DNA to a recipient cell. The two cells have to be in physical contact to affect this transfer. If the transferred DNA encodes

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a phenotype, the transconjugant will have the phenotype. To make DNA transfer possible, the donor cells should have several other genes, such as a clumping factor (for physical contact through clumping) and a mobilizing factor (to enable the DNA to move from a donor to a recipient). The process consists of selecting the right donor and recipient strains, mixing the two cell types in a donor:recipient ratio of 2:1 to 10:1 in several different ways for DNA transfer to occur, and then identifying the transconjugants by appropriate selection techniques.

This technique has been used successfully to transfer several plasmid-linked phenotypes in some lactic acid bacteria. The plasmid-linked $Lac^+$ phenotype was transferred conjugally between two *Lac. lactis* species. The transconjugant was $Lac^+$ and had the specific plasmid, the loss of which resulted in its phenotype becoming $Lac^-$. Subsequently, the $Lac^+$ phenotypes located in different plasmids in many *Lac. lactis* subspecies and strains were conjugally transferred to $Lac^-$ strains of the same species. Conjugal transfer of different plasmid-linked traits has also been reported in other lactic acid bacteria, such as the diacetyl production trait in *Lac. lactis* ssp. *lactis* biovar diacetilactis.

The method has several limitations, some of which have been listed previously with the characteristics of plasmids. They include plasmid size, plasmid incompatibility and instability in recipient strains, inability to express in hosts, inability to have proper donors and recipients, and, in some cases, inability to recognize the transconjugant. However, by using a broad-host-range plasmid (e.g., pAMβ1, a plasmid of *Enterococcus* spp. encoding an antibiotic gene), it was shown that plasmid transfer by conjugation is possible among lactic acid bacteria between the same species, between two different species in the same genus, or even between two different species from different genera.

## Transformation

This method involves extraction and purification of DNA from a donor bacterial strain and mixing the purified DNA with the recipient cells. Some DNA fragments encoding a phenotype are expected to pass through the cell barriers (wall and membrane) and become part of the host DNA expressing the new phenotype. In some Gram-positive bacteria (e.g., *Bacillus* spp.), this method has been effective to transfer certain traits. In lactic acid bacteria, limited studies have revealed that the technique was not very effective; however, a modified method was effective. First, *Lac. lactis* cells were treated with lysozyme or mutanolysin, or both, to remove the cell wall and to form protoplasts in a high-osmotic medium. The protoplasts were then exposed to purified DNA (chromosomal, plasmid, or phage DNA) in the presence of polyethylene glycol. The growth conditions were then changed for the protoplasts to regenerate the cell wall. The transformants were then detected in a selective medium. By this method, $Lac^+$ phenotype and $Em^r$ (erythromycin-resistance phenotype) and phage DNA (transfection) were transferred to recipient strains of *Lac. lactis* subspecies. Because of limitations in the success rate, this method is not widely studied in lactic acid bacteria.

## Protoplast Fusion

The technique involves preparation of protoplasts of cells from two different strains and allowing them to fuse together in a suitable high-osmotic environment. Fusion of cells of the two strains and recombination of the genetic materials may occur. By allowing the protoplasts to regenerate the cell wall and by using proper selection techniques, recombinants carrying genetic information from both strains can be obtained. The technique has been used successfully to produce recombinants of *Lac. lactis* subspecies for both $Lac^+$ and $Em^+$ phenotypes. However, because of the low success rate, this technique is not used much in lactic acid bacteria.

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### Electrotransformation

In this method, a suspension of recipient cells in high population levels ($10^8$ cells/200 mL) is mixed with purified DNA (1–2 μg) from a donor strain and then exposed to a high-voltage electric field for a few microseconds. This results in temporary formation of small holes in the cell barrier (membrane) through which purified DNA can pass. Subsequently, the cells are allowed to repair their damage and express the new phenotype to enable their isolation.

This method has been widely used in many lactic acid bacteria to introduce plasmids from different strains of the same species as well as from separate species and genera. In addition, vectors carrying cloned genes from diverse sources have been successfully introduced in several species of lactic acid bacteria. This is currently the most preferred method to transfer DNA from a source into recipient cells of lactic acid bacteria.

### Conjugative Transposons

Transposons or transposable elements are segments of DNA in chromosomes or plasmids of bacteria that can move from one site to another. Because of this, they can cause rearrangement in the sequence of chromosomal and plasmid DNA and loss or gain of phenotypes in the hosts. The simplest transposons are known as insertion sequences (IS). Each IS element is an autonomous unit, containing a coding region that has the gene encoding the transposase enzyme necessary for its transposition on the host DNA and an inverted nucleotide sequence repeated at each end. Transposition of an IS element occurs at target sites in host DNA, and after transposition, the host DNA contains short, direct repeats on either end of an IS element. Larger transposons (Tn) contain different genes, such as those that encode for an antibiotic-resistance phenotype in the central region and an IS element on each end. Transposons can be conjugative and can be transferred from a donor to a host by the same method used for a conjugative plasmid. When such a transfer occurs, the phenotypes encoded by the resident genes (or genes cloned into it) are also transferred and expressed in a recipient strain.

A few IS elements and Tn have been identified in some strains of lactic acid bacteria. *Lactobacillus casei* S1 contains the insertion element IS1, which has two ORFs and an inverted repeat at either end. It is chromosomally located, and, when inserted in a prophage, the lysogenic phage becomes a virulent phage. The nisin-producing *Lac. lactis* ssp. *lactis* ATCC 11454 contains a conjugative transposon, Tn5276, in the chromosome, which is approximately 70 kb and contains in the central region the genes necessary for nisin A production (*nis* operon) and sucrose utilization. In addition, some conjugative transposons from several *Streptococcus* and *Enterococcus* species that encode antibiotic-resistance markers, such as Tn916 (which encodes tetracycline resistance), have been transferred to different strains of lactic acid bacteria. They have also been used in genetic recombination studies in lactic acid bacteria. Besides, transposons (Tn916, Tn1545, Tn5) are also routinely used for gene disruption to study the role of various virulence factors in pathogenesis for foodborne pathogens.

## Gene Cloning

To transfer and express a gene from a donor into a recipient, the usual procedure is to clone the gene into a suitable plasmid or cloning vector and introduce the vector into the recipient cells. In the simplest form of this technique, a DNA segment carrying the genes is obtained by digesting the purified DNA of the donor with the suitable DNA restriction endonucleases and purifying it from the mixture. A suitable plasmid (cloning vector) that has one or several gene

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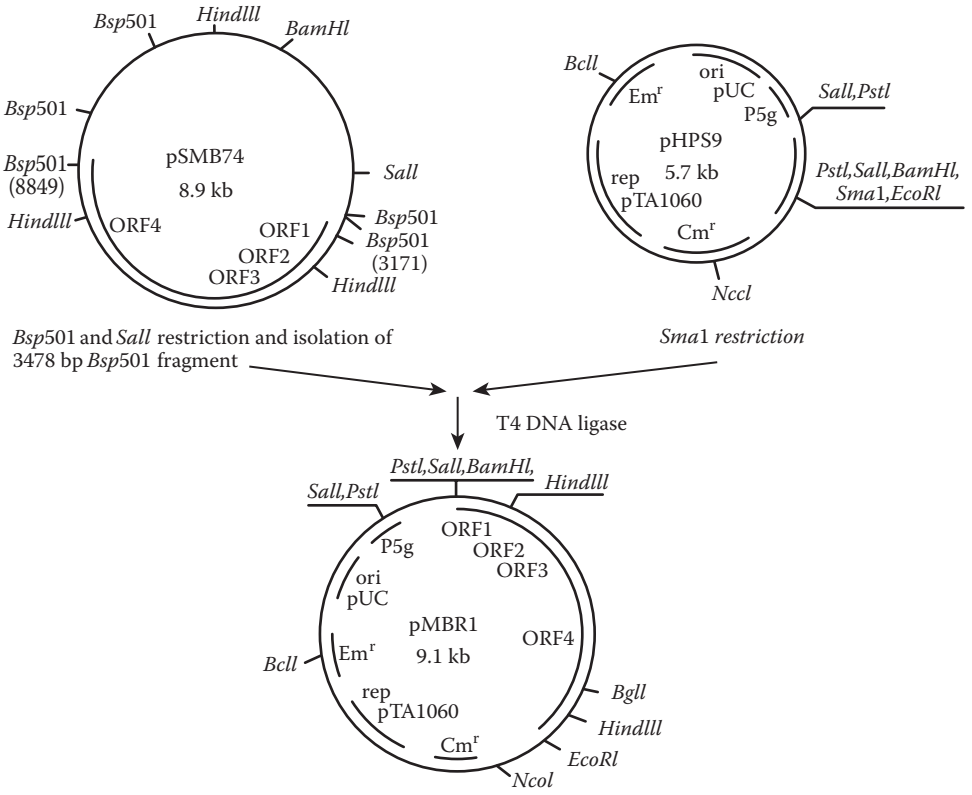



**Figure 13.2 Construction of recombinant plasmid. A 3.5-kb fragment carrying four open reading frames from pSMB74 was derived by digesting with *Bsp*501 and cloned in the *Sma*1 site of pHPS9 to produce the 9.1-kb pMBR1.0.**

markers (such as resistance to an antibiotic or metabolizing a carbohydrate) and a site that can be hydrolyzed, preferably with the same restriction enzymes, is selected. The plasmid is digested with this enzyme, mixed with the donor DNA fragment, and incubated for the fragment to align in the opening of the plasmid DNA (Figure 13.2). The open ends are then sealed by using suitable enzymes and nucleotide bases. This plasmid carrying the genes from the donor can then be introduced in a bacterial cell in several ways, most effectively by electroporation in lactic acid bacteria as described previously. This method is now being used to transfer genes from different sources into lactic acid bacteria.

## *Cloning Vectors*

Many vectors have been constructed by combining nucleotide segments encoding desirable characteristics from different sources, such as the origin of replication, highly efficient promoters, multiple cloning sites, selective markers, and inducible systems. A vector should be stable and able to replicate in host cells. Generally, vectors that replicate by the theta mechanism are more stable than those that replicate by the sigma mechanism. Many small vectors have been constructed from small cryptic plasmids of lactic acid bacteria, such as pGK12 from pWV01. They

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are very efficient for cloning, transforming, and expressing genes in different lactic acid bacteria. Several shuttle vectors have also been constructed, which can be used to transfer and express genes between heterologous donors and recipients (e.g., pHPS9 in Figure 13.2). Because there are restrictions on the use of vectors carrying antibiotic markers to develop lactic acid bacterial strains for use in food fermentation, several food-grade vectors have been constructed. They carry food-grade selective markers, such as resistance to nisin, sucrose hydrolysis, and ochre suppressor, and help in selecting out the transformed cells.

For the efficient expression of the cloned genes, a vector should have proper transcription and translation signals upstream of the coding region. Both constitutive and inducible promoters have been integrated in vectors, which greatly enhance the expression of the cloned genes (e.g., the inducible *lacA* promoter and *hisA* promoter). In addition, vectors that allow the isolation of secretion signals have been constructed. Their use has enabled the secretion of heterologous proteins as well as their anchorage to the cell wall of host cells.[3,4]

## Metabolic Engineering

Many lactic acid bacteria are used to produce fermented foods and desirable byproducts because they can metabolize several carbohydrates, proteins, and lipids present in the raw materials used in the fermentation process. In most strains, the ability to produce a desirable characteristic in a fermented food or a valuable byproduct is quite limited. Also, the possibility of producing a novel food or a byproduct by using these natural strains is quite low. However, an understanding of the functions of many genes and their regulatory systems has helped conduct metabolic engineering in lactic acid bacteria to change the metabolic pathways, and, in some instances, to produce a higher level of byproducts and better products. This approach has a great potential to produce unique bacterial strains, and many studies are being conducted in this field. Several examples are listed here.[5–7]

### *Mixed Acid Fermentation by* Lactococcus lactis

*Lac. lactis* strains metabolize fermentable carbohydrates by the EMP pathway to produce mainly lactic acid. In the terminal step, pyruvate is reduced to lactate by lactate dehydrogenase (LDH), which is encoded by the *ldh* gene. When the *ldh* gene is inactivated by plasmid insertion, the LDH-negative strain reroutes pyruvate to produce acetate, formate, ethanol, and acetoin. Ethanol production is greatly increased by cloning the necessary genes from an ethanol-producing bacterium to the LDH-negative lactic acid bacteria. Similar results are also obtained by inactivating both *ldh* genes in a *Lab. plantarum* strain (which has two LDH enzymes).

### *L(+)-Lactic Acid Production*

Lactic acid bacteria, such as *Lab. acidophilus*, *Lab. plantarum*, *Lab. fermentum*, *Lab rhamnosus*, *Ped. acidilactici*, and *Ped. pentosaceus*, that produce proportionately high quantities of lactic acid in the EMP pathway produce a mixture of both L(+)- and D(–)-lactic acid from pyruvate because they contain both L- and D-lactate dehydrogenases. Because L(+)-lactic acid is produced in the body, it is preferred to D(–)-lactic acid as a food additive, and the strains mentioned are not preferred for commercial production of lactic acid for use in food. Because many of the species mentioned are also used to produce fermented foods, studies are being conducted to produce strains of these species that produce only L(+)-lactic acid. These species have the two genes, *ldh* L and *ldh* D, encoding

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the two LDHs. Strains of some of the species mentioned have been developed in which *ldh* D expressing D-LDH has been inactivated, enabling them to produce only L(+)-lactic acid.

### *Diacetyl Production by* Lactococcus lactis

Diacetyl is associated with a pleasant butter aroma and is used in many nondairy products to give desirable characteristics. It is normally produced by some lactic acid bacteria, including the biovar diacetylactis of *Lac. lactis* species. Other *Lac. lactis* species and strains produce, if at all, very little diacetyl. Metabolic engineering has, however, enabled development of overproducing diacetyl strains of *Lac. lactis*. To develop this strain, first the *nox* gene (NADH oxidase) from a suitable source is cloned under the control of the nisin-inducible *nisA* promoter (NICE) in the *Lac. lactis* strain. In the presence of a small amount of nisin, the *nox* gene in the strain is overexpressed. In the next step, the gene encoding for α-acetolactate decarboxylase (ALDB, which converts α-acetolactate to acetoin, see Figure 12.5) is inactivated. This results in the accumulation of α-acetolactate that is produced from pyruvate. Under the overexpression of the *nox* gene and with inactivated ALDB, α-acetolactate is converted to diacetyl in high levels.

### *Alanine Production from Carbohydrates*

Amino acids from proteins are generally used for growth and synthesis in lactic acid bacteria and do not accumulate in the cells or in the environment. However, by metabolic engineering, a strain of *Lac. lactis* that uses carbohydrate and ammonium sources to produce higher concentrations of alanine has been developed. The alanine dehydrogenase gene from a suitable source was cloned under the control of the NICE system. When the cloned vector was introduced in a LDH-negative strain and the strain was grown in the presence of a small amount of nisin and ammonium supplements, a large amount of carbohydrates was converted to L-alanine. Because L-alanine has a sweet taste, this strain can be used as a starter culture to produce fermented products with different, but agreeable, tastes.

### *Production of Mannitol and Other Polyols*

Mannitol, a sugar alcohol, is produced by some strains of lactic acid bacteria, such as *Lac. lactis* and *Lab. plantarum*, in small amounts. It is produced by the reduction of fructose-6-phosphate (in the EMP pathway) to mannitol-1-phosphate, which then is dephosphorylated by mannitol phosphate dehydrogenase (MPDH) to mannitol. However, 90% of it remains inside the cells. In contrast, *Leuconostoc mesenteroides* secretes most of the mannitol in the environment because it has an efficient mannitol transport system (it also produces high amounts of mannitol). Overproduction of mannitol by an LDH-deficient *Lac. lactis* strain has been achieved by metabolic engineering for overproduction of MPDH and low production of phosphofructokinase. By introducing the mannitol-transfer system from *Leu. mesenteroides*, the engineered *Lac. lactis* strain is able to excrete most of the mannitol in the environment. By similar techniques, *Lac. lactis* strains that are able to produce large amounts of sorbitol and tagatose have been developed. Mannitol, sorbitol, and tagatose (as well as L-alanine, discussed previously) can be used as low-calorie sweeteners in food.

### *Production of Folic Acid and Riboflavin*

Many lactic acid bacteria, such as *Lac. lactis* and *Str. thermophilus*, while growing in milk and other fermented foods, synthesize low levels of folate, but most of it is retained inside the cells.

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The multienzyme biosynthetic pathways of tetrahydrofolate from glutanyltriphosphate (GTP) in *Lac. lactis* have been identified. With this information, a strain of *Lac. lactis* that overexpresses the genes involved in the process has been developed. Initially, the engineered strain produces three times more folate than the wild strain does and excretes most of it into the environment. In the same manner, a *Lac. lactis* strain that produces a higher amount of riboflavin has been engineered. This way, the nutritional value of the fermented foods can be increased.

### *Enhancing Proteolysis by Cell Lysis*

The desirable flavor of cheese is the result of proteolysis of milk proteins by extracellular and intracellular proteolytic enzymes of a starter culture during ripening. As the intracellular enzymes are released slowly (after death and lysis of starter-culture cells during ripening), the process is relatively slow. Methods, including metabolic engineering, are being studied to enhance the ripening of cheese. In a metabolic engineering method, the lytic genes of bacteriophages are used to lyse the starter cells. This has been achieved by cloning the phage genes that encode for lysin and holin (cause cell lysis) under the nisin-induced promoter in *Lac. lactis*. In the presence of nisin, the cells lyse, releasing the proteinases and peptidases, which then help accelerate the ripening of cheese.

## *Protein Targeting*

There is evidence now that many heterologous proteins, both from prokaryotes and eukaryotes, can be expressed and produced in high levels in lactic acid bacteria by cloning the genes in suitable expression vectors and using existing or new secretory signals. The products can be excreted into the environment, attached onto the cell wall and membrane, or even remain inside the cells. The possibilities are many and include many enzymes, antimicrobials, flavor compounds, and important bioactive molecules for use as pharmaceuticals (immunity protein vaccine) and in agriculture (insecticides). Genes of many heterologous proteins have been cloned in *Lactococcus lactis* strains, such as chicken egg lysozyme, bovine prochymosine, α-amylase, pediocin (a bacteriocin), luciferase, interleukin 2 (IL-2), IL-10, cholera toxin B, tetanus toxin C (produced by *Clostridium tetani*), Internalin A protein from *Listeria monocytogenes*, low-calcium V antigen (LcrV) from *Yersinia*, and Cry 1A insect toxin (produced by *Bacillus thuringiensis*). Many of the genes express at low to high levels, but a few do not express to the level of detection.[8–10]

### *Expression of Interleukins*

A murine interleukin 2 (*mIL2*) expression vector, pL2MIL2, was constructed from pLET2N (originally developed from pLET2, the lactococcal T7 polymerase-based system). The plasmid, pL2MIL2, encodes the part of the *mIL2* gene that codes for the mature protein, fused with the lactococcal usp 45 secretory leader signal. *Lac. lactis* MG 1820 was transformed with this plasmid. When grown in an appropriate growth medium, the cells synthesized the USP 45-mIL2 fusion protein and secreted the mature mIL2 into the growth medium. The purified mIL2 was found to be biologically as active as the natural mIL2. By similar procedures, other murine interleukins (such as mIL-6 and mIL-10), murine interferon, and human interleukin have been produced by *Lac. lactis*.[11] Recent studies have shown that daily ingestion of *Lac. lactis*, which can produce biologically active mIL-10 interleukin, has cured and prevented enterocolitis and alleviated allergic diseases in mice.

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### Drug-Delivery System

Many lactic acid bacteria are normally present in the gastrointestinal (GI) tract of humans and food animals and birds, and some of them have beneficial effects on the health and well being of the hosts (see Chapter 16). Currently, studies are in progress to anchor antigens of pathogens associated with enteric diseases in humans on the cell wall of suitable lactic acid bacteria (see Chapter 16). The live cells then can be used as a drug-delivery vehicle to deliver antigens to stimulate antibody production via the digestive tract. One method used to anchor proteins is to use *Lac. lactis* Prt P, which normally remains attached to the cell wall with its carboxy-terminal domain. The effectiveness of the carboxy-terminal cell wall anchor of *Staphylococcus aureus* protein A to the cell wall of *Lac. lactis* is also being investigated. Genes for certain virulence factors from enteric pathogens are also cloned and expressed in lactic acid bacteria to provide protection against enteric diseases in the gastrointestinal tract.[12]

### Production of Pediocin in Heterologous Hosts

The bacteriocin pediocin PA-1/AcH is produced by many strains of *Ped. acidilactici*. The molecule is translated as a 62-amino acid prepediocin from which the 18-amino acid leader peptide at the $NH_2$ terminal is enzymatically removed during membrane translocation through the ABC transporter system (Chapter 17). The 44-amino acid pediocin is then released in the growth medium. The phenotype is plasmid linked, and the structural gene with three other genes is arranged in an operon system, and the protein encoded by the last gene acts as the ABC transporter. The DNA fragment with all four genes has been cloned in several Gram-negative vectors and in a shuttle vector, and the host strains produce active pediocin molecules, generally at lower concentrations.

In a separate study, only the nucleotide segment of the matured pediocin (without the leader segment) was fused to the carboxy terminus of the maltose-binding protein (MBP), a secretory protein, and cloned in a Gram-negative vector. *Escherichia coli* transformed with the cloned vector secreted the chimeric MBP–pediocin by the Sec-dependent transport system in the growth media. The MBP–pediocin molecule, although much larger, retained the bactericidal property as that of natural pediocin. The pediocin operon has also been cloned in suitable vectors and transformed in *Lac. lactis* and *Str. thermophilus* strains, which produced pediocin. Generally, the production is low, and the phenotype is unstable in the host in the absence of selective pressure, such as antibiotics (the genes encoding the resistance to antibiotics are used as selective markers in the cloning vectors).[13]

## Protein Engineering

Many studies are being conducted to change the amino acid sequences of proteins of lactic acid bacteria to determine the influence on the physical, chemical, and biological properties (structures and functions) of the proteins.

### Production of Hybrid Prepediocin

Like the prebacteriocin molecules of lactic acid bacteria, prepediocin molecules have an 18-amino acid leader segment at the $NH_2$ terminus and a 44-amino acid segment at the carboxyl end. Following translation, the leader peptide directs the molecule for translocation through the ABC transporter. In one study, the promoter and nucleotide sequences of the leader segment of the pediocin structural gene were replaced with a similar segment associated with the production of

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the bacteriocin lactococcin A in a *Lac. lactis* strain. The structural gene was cloned in the same *Lac. lactis* strain, which produced propediocin with a different leader peptide but produced matured pediocin by using the lactococcin A-transport system. However, the level of production was very low, suggesting the important roles of leader peptides and specific transporters in high-level production of pediocin (Chapter 17).[13]

### *Bioengineered Bacteriocins*

By random as well as site-directed PCR, mutagenesis of nucleotides is used to improve bacteriocin activity.[14] This strategy was used in the mature pediocin segment; many variant molecules have been isolated that have one or two different amino acids among the 44 amino acids. The variants have different levels of activity: Some do not have detectable activity, many have reduced activity, and one has greater activity. These results indicate the importance of specific amino acid sequences of a bacteriocin for its normal level of activity.[13] Amino acid substitution in the bioengineered bacteriocins also helped improve solubility or made bacteriocins resistant to intestinal proteolytic enzymes, ensuring their activity in the gastrointestinal tract.[14]

## Genome Mapping and Sequencing

Because of the importance of many lactic acid bacteria in the production of bioprocessed foods, food preservatives and different food additives, enzymes and nutraceuticals; maintenance of intestinal health; and as agents for drug-delivery systems, considerable research with many species of lactic acid bacteria has been conducted in the last 20–30 years. To understand the characteristics at the molecular level, for the last several years, there have been efforts to sequence the complete genome (chromosome) of some strains of important lactic acid bacteria. Availability of new techniques and necessary facilities (equipment, computerized programs, and others) has greatly helped generate information in a relatively short time. These techniques have also helped sequence the genomes of many phages and prophages of lactic acid bacteria. The available information is briefly summarized here.

### *Lactic Acid Bacteria*

As of 2011, complete genome sequences of 35 lactic acid bacteria have been completed, and 100 more are in progress.[9,15,16] These results have provided much important information, such as the size of each genome; the number of ORFs a genome carries; and the frequency of prophages, IS-elements, and rRNA operons in a genome (Table 13.1). In addition, other genetic information, such as biosynthetic pathways of amino acids, vitamins, nucleotides, and polysaccharides, synthesis of bacteriocins, and sugar transport and metabolism systems, is now available for these strains. The *Lac. lactis* IL 1403 genome is the first to be completely sequenced.[17] The genome size is approximately 2.4 Mb and has 2310 ORFs with 138 potential regulators, five prophages, six different types of IS elements, and six rRNA operons. The genome encodes nucleotide sequences for de novo biosynthetic pathways of 24 amino acids, folate, riboflavin, purines and pyrimidines, complete PTS-sugar transport systems, and partial components of aerobic respiration. The genome appears to have a fluid structure and is able to undergo changes through point mutation, DNA rearrangements, and horizontal gene transfer. The genome organization has resulted from the evolutionary pressure to enable the species to grow optimally in a nutritionally rich medium.

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**Table 13.1 Some Features of Sequenced Genome of Lactic Acid Bacteria**

| *Genus* | *Species* | *Strains* | *Genome Size (bp)* | *% (GC)* | *ORFs* | *Prophages* | *IS Element Types* | *rRNA Operons* | *Ref Seq.* | *Gene Bank* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Bifidobacterium* | *longum* | NCC 2705 | 2,256,640 | 60 | 1727 | some | 5 | 4 | NC_004307 | AE014295 |
| *Lactococcus* | *lactis* | IL 1403 | 2,365,589 | 35 | 2321 | 5 | 6 | 6 | NC_002662 | AE005176 |
| | *lactis* ssp. *cremoris* | SK11 | 2,438,589 | | 2384 | | | 6 | NC_008527 | CP000425 |
| *Lactobacillus* | *acidophilus* | NCFM | 1,993,564 | 34 | 1864 | | | | NC_006814 | CP000033 |
| | *brevis* | ATCC367 | 2,291,220 | 46 | 2185 | 1 | | 5 | NC_008497 | CP000416 |
| | *casei* | ATCC334 | 2,895,264 | 46 | 2751 | 2 | | 5 | NC_008526 | CP000423 |
| | *delbrueckii* ssp. *bulgaricus* | ATCC 11842 | 1,864,998 | 49 | 1562 | | | | NC_008054 | CR954253 |
| | *delbrueckii* ssp. *bulgaricus* | BAA365 | 1,856,951 | 49 | 1721 | 0 | | 9 | NC_008529 | CP000412 |
| | *gasseri* | ATCC 33323 | 1,894,360 | 35 | 1755 | 1 | | 6 | NC_008530 | CP000413 |
| | *johnsonii* | NCC 533 | 1,992,676 | 34 | 1821 | 0 | 6 | 6 | NC_005362 | AE017198 |
| | *plantarum* | WCFS1 | 3,308,274 | 44 | 3009 | 2 | 2 | 5 | NC_004567 | AL935263 |
| | *sakei* | 23K | 1,884,661 | 41 | 1879 | | | | NC_007576 | CR936503 |
| | *salivarius* ssp. *salivarius* | UCC118 | 1,827,111 | 32 | 1717 | | | | NC_007929 | CP000233 |
| *Leuconostoc* | *mesenteroides* | ATCC 8293 | 2,038,396 | 37 | 1970 | 1 | | 4 | NC_008531 | CP000414 |
| *Oenococcus* | *oeni* | PSU-1 | 1,780,517 | 37 | 1691 | 0 | | 2 | NC_008528 | CP000411 |
| *Pediococcus* | *pentosaceus* | ATCC 25745 | 1,832,387 | 37 | 1755 | | | 5 | NC_008525 | CP000422 |
| *Streptococcus* | *thermophilus* | LMD-9 | 1,856,368 | 39 | 1710 | | | 6 | NC_008532 | CP000419 |
| | *thermophilus* | LMG18311 | 1,796,846 | 39 | 1889 | | | | NC_006448 | CP000023 |
| | *thermophilus* | CNRZ1066 | 1,796,226 | 39 | 1915 | | | | NC_006449 | CP000024 |

*Sources:* Adapted from Klaenhammer, T.R. et al., *FEMS Microbiol. Rev.*, *29*, 393–409, 2005; Makarova, K. et al., *Proc. Natl. Acad. Sci. USA*, *103*, 15611–15616, 2006.



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As the genome sequence of different species and strains of lactic acid bacteria become available, the information can be used to compare and understand various important characteristics of lactic acid bacteria, such as determining the evolutionary relationship among the species and strains in various genera, the loss of genes, the presence of pseudogenes, the function of each gene and its essential and nonessential nature, and the function of the noncoding region on the regulation and expression of a gene or an operon system. This information can then be used to modulate gene expression and efficiently conduct metabolic engineering to develop new strains for use in producing novel fermented products and important byproducts.[18,19]

## *Bacteriophages*

The genomes of several important temperate and virulent bacteriophages of lactic acid bacteria (from genera *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Enterococcus*, and *Streptococcus*) have been completely sequenced. Most have genomes ranging from 20 to 55 kb. The information has helped locate and understand the functions of many ORFs and regulatory regions. Some of them are the genes involved in packaging phage DNA in the heads, genes associated with the lysis of host cells, and genes controlling the lysogenic and lytic cycles of a phage. An understanding of these factors has helped develop phage-resistant lactic acid bacterial strains for use in food fermentation and to improve acceptable characteristics of some fermented foods (e.g., accelerated cheese ripening) and transferring and improving expression of genes in lactic acid bacteria.[20] Several methods of developing phage-resistant starter-culture bacteria are listed in Chapter 14.

Analyses of genomes of lactic acid bacteria reveal the presence of many prophages. Although they pose a metabolic burden to host cells and, if induced, may lyse the host cells, it is surprising that the cells carry them. However, the cells carrying the prophage are resistant to attack by a temperate phage and will be thus dominant in the population. In addition, a mutation in the prophage induction system will enable the cell to maintain resistance without being lysed. This could lead to development of phage-resistant lactic acid bacterial strains.[20]

## *The* Lac *and* Las *Genes*

The genes associated with metabolism of lactose to lactic acid in different lactic acid bacteria are located in at least two operon systems and are grouped as *lac* genes and *las* genes. The *lac* genes are associated with the transport and hydrolysis of lactose to two hexoses and partial metabolism of some hexoses, whereas the *las* (lactic acid synthesis) genes are involved in the production of lactic acid.[21,22]

The *lac* genes, depending on species and strain, can be located either on a chromosome or on a plasmid. In most *Lac. lactis* strains, they are plasmid linked, but in *Lab. delbrueckii* ssp. *bulgaricus* and *Lab. helveticus* they are on the chromosomes. Whereas in *Lac. lactis* they are inducible (also in *Lab. acidophilus*), in the two *Lactobacillus* species, they are constitutive. Limited studies have revealed *lac* genes to be plasmid linked in *Leu. lactis* and chromosomally linked in *Str. thermophilus*.

In the *Lac. lactis* strains, lactose fermentation usually includes its transportation by the PEP-PST system as lactose phosphate, hydrolysis by phospho-β-galactosidase to glucose and galactose-6-phosphate, and conversion of galactose-6-phosphate to tagatose-6-phosphate and then to two triosephosphates before entering the EMP pathway (see Chapter 12). Glucose is also converted to triosephosphate in the EMP pathway. Triosephosphates are finally converted to lactic acid. In *Lab. delbrueckii* ssp. *bulgaricus*, lactose is transported (along with a proton) by lactose permease

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and hydrolyzed by β-galactosidase to glucose and galactose. Glucose is metabolized through the EMP pathway, whereas galactose is first metabolized by the Leloir pathway to glucose-1-phosphate before entering the EMP pathway. In *Str. thermophilus*, following transport of lactose by lactose permease and hydrolysis by β-galactosidase to glucose and galactose, glucose is metabolized by the EMP pathway; in most strains, galactose is excreted into the environment.

The plasmid-linked *lac* genes in *Lac. lactis* strains have been characterized, and the function of each gene has been determined. A total of eight genes are arranged in an operon system and designated as *lacABCDFEGX*. Four genes, *lacABCD*, encode the three enzymes in the tagatose-6-phosphate pathway: The *lacAB* is involved in the conversion of glucose-6-phosphate to tagatose-6-phosphate, *lacC* in the conversion of tagatose-6-phosphate to tagatose-1,6-diphosphate, and *lacD* in the conversion of the latter to glyceroldehyde-3-phosphate and dihydroacetone phosphate. The *lacFE* genes encode the PEP-PTS, and the *lacG* encodes the hydrolysis of lactose-6-phosphate. The function of *lacX* is not known. The promoter of the operon is located upstream of *lacA*, and the regulator gene (*lacR*) is located immediately upstream of the promoter. It encodes the repressor protein that regulates the expression of *lac* operon, which is inducible. The arrangement, location, and regulation of *lac* genes in other lactic acid bacteria have not yet been properly identified.

The *las* genes, or the genes encoding for lactic acid synthesis, have been identified and characterized on the chromosome of *Lac. lactis*. Five genes are involved for encoding the enzymes necessary in the process: *pfk*, for phosphofructokinase; *pyk*, for pyruvate kinase; *ldh*, for L(+)-lactate dehydrogenase; *tpi*, for triosephosphate isomerase; and *gap*, for glyceraldehyde-3-phosphate dehydrogenase. The three genes *las pfk*, *las pyk*, and *las ldh* are organized in an operon (the *las* operon) with the promoter located upstream of *las pfk* and a terminator located downstream of *las ldh*. The genes *las tpi* and *las gap* are located separately on the chromosome, and each is expressed as a monocistronic gene.

The five genes are highly biased in their codon usage, suggesting that they are strongly expressed in *Lac. lactis*. Limited results have indicated that the expression of the three genes in the *las* operon is under a genetic regulatory mechanism, which limits their expression and the level of lactic acid production.

## Conclusion

The importance of many strains of lactic acid bacteria in the fermentation of diverse types of food, production of different food additives, use as probiotics, and application to deliver drugs has necessitated the understanding of the molecular basis of these desirable characteristics. With a modest beginning in early 1970s, research activities on the genetics of lactic acid bacteria have exploded. Currently, studies on the genome sequence of lactic acid bacteria are generating important information on the fluid nature of the DNA that can change with environment. Comparative genome analysis, with more information, will enable researchers to determine the minimum genetic information and the arrangements of genetic codes necessary to design a unique starter-culture strain for a specific use. Production of starter cultures and problems associated with bacteriophages are discussed in Chapter 14.

### QUESTIONS

1. Discuss the important characteristics of plasmids in lactic acid bacteria.
2. Discuss the characteristic differences between large and small plasmids in lactic acid bacteria.
3. List five commercially important phenotypes that are plasmid linked in lactic acid bacteria and discuss their importance in relation to plasmid stability.

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4. Define and list the advantages and disadvantages of the following methods used in gene transfer: transduction, transfection, transformation, and protoplast fusion in lactic acid bacteria.
5. Describe the process involved in conjugal transfer of DNA in lactic acid bacteria. What are the advantages and disadvantages of this method?
6. Describe the technique involved in electrotransformation of DNA in lactic acid bacteria. Discuss the advantages of transferring DNA by this method over the other methods in lactic acid bacteria.
7. Define the following terms and briefly discuss their importance in lactic acid bacteria: cryptic plasmid, plasmid replication, electrotransformation, IS-elements, and transposons.
8. Define the term "metabolic engineering." With a proper example, explain how metabolic engineering in lactic acid bacteria can be used to produce diacetyl, L(+)-lactic acid, and folic acid in strains that have a low ability to produce them.
9. Explain with one example the importance of (a) protein targeting and (b) protein engineering research in lactic acid bacteria.
10. Explain how information on the genome sequence of lactic acid bacteria is helpful in their application in strain development.
11. List three important applications that can be developed from the genome sequences of phages of lactic acid bacteria.
12. Briefly discuss the functions of (a) *lac* genes and (b) *las* genes.

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# Chapter 14

# Starter Cultures and Bacteriophages

## Introduction

"Starter culture" is a generic term and has changed its meaning over the years. Currently, it means a selected strain of food-grade microorganisms of known and stable metabolic activities and other characteristics that is used to produce fermented foods of desirable appearance, body, texture, and flavor. Some starter cultures are also used to produce food additives, such as probiotics, and for drug delivery. Toward the end of the 19th century, the term meant inoculating a small amount of fermented (sour) cream or milk (starter) to fresh cream or milk to start fermentation in the production of butter and cheese, respectively.[1] This process was found to result in better products than those produced through natural fermentation of the raw materials. These starters were mixtures of unknown bacteria. Processing plants started maintaining a good starter by daily transfer (mother starter) and produced product inoculum from these. However, the bacteriological makeup of these starters (types and proportion of the desirable as well as undesirable bacteria) during successive transfers was continually susceptible to changes as a result of strain dominance among those present initially as well as from the contaminants introduced during handling. This created difficulties in producing products of consistent quality and resulted in product failure as a result of bacteriophage attack on the starter bacteria. Some private companies started supplying mixed cultures of unknown bacterial composition for cheese manufacture both in the United States and in Europe. Subsequently, the individual strains were purified and examined for their characteristics, and starter cultures with pure strains were produced by these commercial companies. Initially, such starter cultures were developed to produce cheeses. Currently, starter cultures for many types of fermented dairy products, fermented meat products, some fermented vegetables, fermented baking products, alcohol fermentation, and other purposes (especially with genetically modified organisms, GMOs) are commercially available. In this chapter, a brief discussion of the history, current status, bacteriophage problems, and production of concentrated cultures is presented.



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## History

Initial development of starter cultures resulted from the needs of and changes in the cheese industry. Before the 1950s, small producers were producing limited amounts of cheese to satisfy local consumers. A plant used to maintain a bottle of mother culture by daily transfer, which it received from a culture producer or another neighboring processor. From the mother culture, the plant used to make bulk culture through several transfers to meet the inoculation volume (to meet the need of 1%–2% of the volume of milk to be processed for cheese). These starters were a mixture of undefined strains of bacteria, and it was difficult to produce a product of consistent quality. There were also problems with starter failure from bacteriophage attack. To overcome the problem of quality, the single-strain starter (a desired strain isolated from a mixture) was introduced. Good sanitation and newly designed processing equipment were introduced to overcome phage problems.[1]

Since the 1950s, large cheese operations have replaced the small producers. They needed products of consistent quality and could not afford to have too many starter failures from phage attack. Starter-culture producers developed single-strain cultures and supplied these in dried form to the cheese processors, who, in turn, used them to produce mother cultures and bulk cultures. To overcome phage problems, rotation of strains (such as using different strains each day) to prevent the buildup of a particular phage as well as multiple-strain cultures (if one strain is killed by a specific phage, another will work) were practiced. Later, defined media to produce bulk cultures that reduced phage attack were introduced. Even then, daily production of large amounts of bulk cultures (some cheese processors were handling more than 1,000,000 gal of milk daily and needed more than 10,000 gal of bulk cultures) and maintaining a large number of defined bacterial strains for use in rotation or multiple-strain cultures (some were keeping 30 or more strains) and using them in proper combinations (so that they are compatible) demanded defined and large facilities, expert microbiologists, and a large crew for the operation. This was partially overcome with the introduction in the 1960s of frozen concentrate cultures that could be shipped by air from culture producers to cheese processors in dry ice and could be used directly to produce bulk cultures. This, along with the availability of several types of phage inhibitory media (PIM) to produce bulk cultures, helped cheese processors overcome the cost and labor necessary to maintain a microbiological laboratory and a large number of starter strains. These dairy-based media contain a high concentration of phosphate to chelate calcium in milk and thus make the divalent cation unavailable for the adsorption of phages to the bacterial cells and cause infection. To obtain high cell density by maintaining a high pH, the media had either internal or external pH control systems. Subsequently, frozen concentrated cultures, containing $10^{11-12}$ cells/mL were introduced; they could be directly inoculated into milk in the cheese vat (direct vat set, DVS), eliminating the need to produce bulk starter by a cheese processor.

From the 1970s, the popularity of several types of fermented dairy products (particularly buttermilk and yogurt), as well as fermented sausages, some fermented ethnic products, and fermented health products, stimulated their production by large commercial processors. To meet their need, different types of frozen concentrated cultures for direct inoculation into the raw materials were developed.

Efforts have been made to produce freeze-dried or spray-dried concentrated cultures.[2] Dried cultures can eliminate the bulk problem in transporting frozen concentrated cultures in dry ice as well as their accidental thawing, which would thus prevent their use. Dried cultures can also be used directly for product manufacture or can be used to produce bulk starters. However, many strains do not survive well in the dried state. Thus, their use as dried cultures in large commercial operations has been limited. They are available in small packages for use directly by the small processors of fermented foods or for use to produce bulk cultures before inoculation in the raw material.

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A fairly recent advance has been the availability of custom-designed starter cultures to meet the specific needs of a food processor. An understanding of the genetic basis of some important desirable traits, as well as traits for phage attack inhibition in starter cultures, has helped produce designer cultures.

## Concentrated Cultures

In controlled fermentation of food, a starter is added to a raw material at a level of approximately $10^{6-7}$ live cells/mL or live cells/g for the fermentation to proceed at the desired rate. In the conventional process, a bulk culture with approximately $10^{8-9}$ cells/mL needs to be inoculated at approximately a 1% level in the raw material. A cheese processor who uses 100,000 gal or more milk per day needs 1000 gal or more bulk starter daily. This large volume is produced from mother cultures through several intermediate transfers, involving more handling at the processing facilities and possible introduction of phages as the phages are more abundant in the processing environment (Figure 14.1). In contrast, in the production of concentrated culture, most handling is done by culture producers under controlled environmental conditions, thereby minimizing phage problems. The strains are grown in suitable media to obtain high cell density (some to more than $10^{10}$ cells/mL). The cells are harvested by centrifugation and resuspended in a liquid at a concentration of approximately $10^{12}$ cells/mL. A 360 mL frozen concentrate culture in the DVS system can be added to a 5000-gal milk vat to get the desired initial concentrations of viable cells ($10^{6-7}$ cells/mL). The

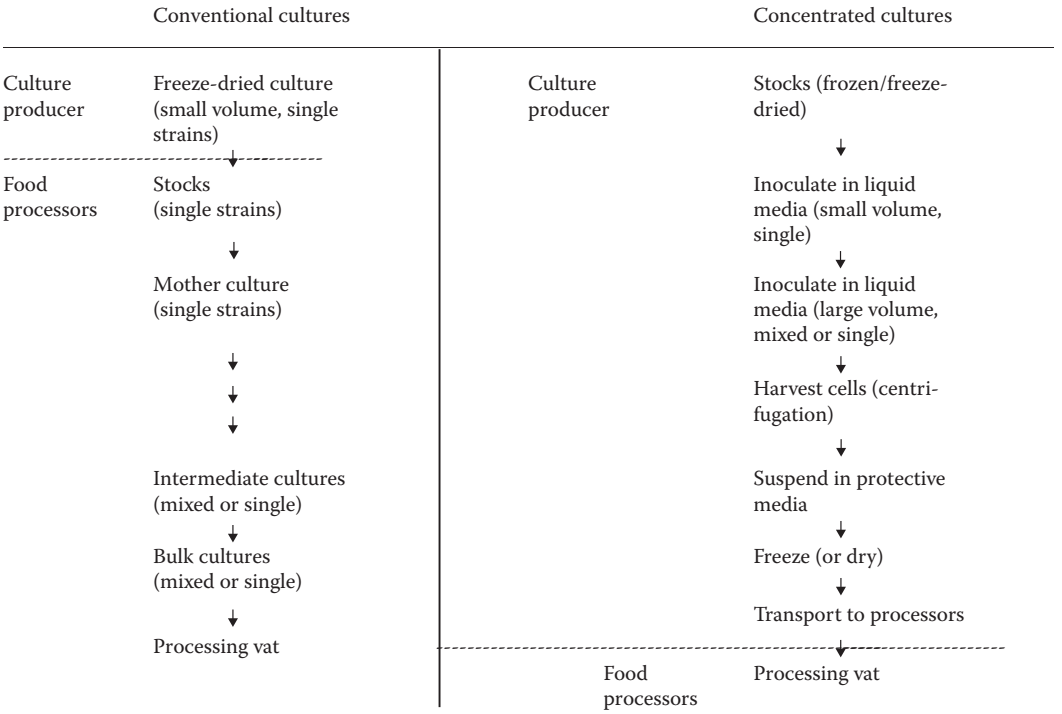



**Figure 14.1 Production steps and use of conventional and concentrated cultures and changes in culture handling by culture producers and food processors.**

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suspending medium contains cryoprotective agents to reduce cell death and injury during freezing and thawing. The cell suspensions in metal containers are frozen, either by using dry ice–acetone (–78°C) or liquid nitrogen (–196°C), and stored at that temperature. They are transported to processors, preferably by air, in Styrofoam boxes with sufficient amounts of dry ice. A processor stores the containers at –20°C or below and uses it within the time specified by the culture producer. Just before use, a container is thawed in warm (45°C), potable water and added to the raw material. The directions for use of these cultures are supplied by the culture producers.[3]

To produce freeze-dried concentrates, the liquid cell concentrates are shell frozen (in thin layers or small droplets) in either dry ice–acetone or in liquid nitrogen and then dried under vacuum. The dried materials are packed in plastic bags under vacuum and stored at –20°C or at refrigerated temperatures. They are transported to processors for quick delivery, either at ambient temperature or in boxes containing ice packs. A processor stores the dried cultures at –20°C or in a refrigerator and uses them within the specified time. Just before use, the dried culture is mixed with warm water (preferably boiled and cooled or sterile) and added to the raw materials.[3]

To reduce costs in starter culture preparation, other drying methods, such as spray drying, vacuum drying, drum drying, fluidized bed drying, and air drying methods, are now considered.[2,4] During spray drying, particles are formed as they are dried. Liquid cultures mixed with a carrier molecule, such as gelatin, skim milk, gum Arabic, or starch, are sprayed using a nozzle or rotary atomizer to form droplets, which when come in contact with hot air in a chamber form dry powder. The major consideration of such approach is the viability of the starter cultures for a desirable period of time.[2]

## Starter-Culture Problems

### *Strain Antagonism*

In mixed-strain cultures, in which a starter culture contains two or more strains, dominance of one over the others under a given condition can change the culture profile quickly. Dominance can result from an optimum growth environment or production of inhibitory metabolites (e.g., bacteriocins, acids, peroxides). This can affect product quality and increase starter failure through phage attack. Culture producers test the compatibility between desirable strains and develop mixed-strain cultures with only the compatible strains to avoid strain antagonism.

### *Loss of a Desired Trait*

A strain carrying a plasmid-linked desired trait can lose the trait during storage, subculturing, and under some growth conditions. Physical and chemical stress and long freezing times can also result in loss of a trait. Genetic studies are being conducted to understand the mechanisms of the stability of these traits. Several strains with better stability of some traits have been developed and are being used. In the future, more such strains will be available.

### *Cell Death and Injury*

The effective use of frozen and freeze-dried concentrated cultures, especially for direct use (such as DVS cultures), depends on two important characteristics: (1) Cultures need to have large numbers of viable cells, and (2) cells should have a short lag phase so that they can start multiplying very

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quickly. The cells can be exposed to adverse physical and chemical environments (or stresses) that can reduce survival, growth, and metabolism.[5] Freezing and thawing and freeze-drying and rehydration are known to cause cell damage, leading to cell death and cell injury (Chapter 10). This can lead to situations wherein a concentrated culture fails to meet the needs of initial viable cell concentrations ($10^{6-7}$ cells/mL or cells/g of raw materials) and the relatively short lag in the fermentation process. Culture producers have been successful in reducing cell damage by using cryoprotectants in the suspending menstrua and freezing the cells rapidly at a very low temperature. Some of the causes of cell viability loss are thawing and refreezing, thawing for a long time before using, mixing thawed cultures with or rehydrating dried cultures in concentrated solutions of other constituents (such as curing salt and spice mixtures used in sausage fermentation), and long storage at –20°C or at higher temperatures. Many of these occur in the product-processing environment as a result of lack of knowledge of the people handling the cultures. For the best performance of concentrated cultures, the directions from culture producers need to be strictly followed.

## Inhibitors in Raw Materials

Milk can contain either antibiotics, given to the animals to treat some infections (mastitis), or sanitizers (from the equipment). Meat can contain ingredients used to make fermented sausages, such as phosphate or nitrite, and sanitizers from equipment. These factors can prevent or reduce the growth of starters.

## Bacteriophages of Lactic Acid Bacteria

The role of bacteriophages (or phages) in starter-culture failure in food fermentation has been recognized for a long time. A short discussion on their life cycle that results in starter failure as well as some current practices used to overcome phage problems in food fermentation are presented here.[6–8] Bacteriophages are also used in controlling foodborne pathogens to improve food safety (see Chapter 38) and to detect foodborne pathogens as a diagnostic tool (see Chapter 42).

### Morphology and Characteristics

Bacteriophages are filterable viruses of bacteria widely distributed in the environment, especially in food fermentation environments. A phage contains several proteins (that make the head, tail, tail fiber, and contractile sheath) and DNA, which can be linear or circular, double stranded of approximately 20 to 55 kb in size. The double-stranded DNA molecule is packed in the head, which can be round or hexagonal (prolate or isometric, respectively).

Several schemes have been proposed at different times to classify the bacteriophages of lactic acid bacteria. Currently, a scheme is used in which the family, group, and morphotype are classified together. The three families Myoviridae, Siphoviridae, and Podoviridae are included in three groups, A (with contractile tails), B (with noncontractile long tails), and C (with noncontractile short tails), respectively (Figure 14.2). They can have three morphotypes, namely 1 (with small isometric heads), 2 (with small prolate heads), and 3 (with large prolate heads), respectively. *Lactococcus lactis* ssp. *lactis* and ssp. *cremoris* appear to have many wide varieties of phages. They have been divided into 12 species, of which three are in the Siphoviridae family, and are important because of their virulent nature. The three in the Siphoviridae family are 936-species (all are virulent with small isometric heads, for example, sk1), c2 species (all are virulent with prolate heads, for example, c2), and P335 species (have both virulent and temperate phages and small

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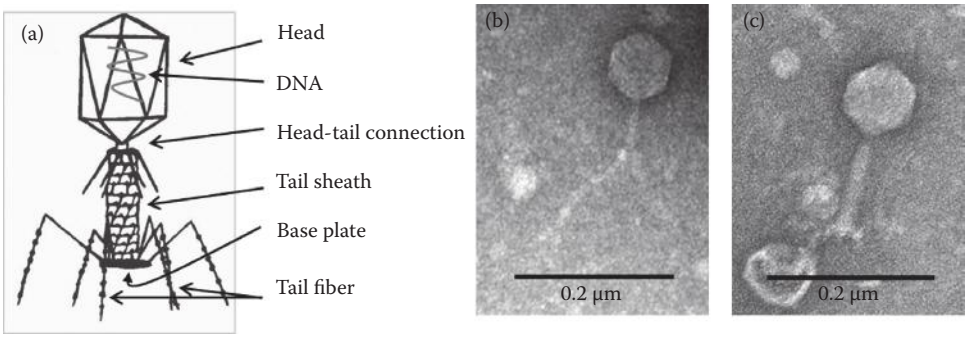



**Figure 14.2 Picture of bacteriophages (a) schematics of T4 phage, (b) transmission electron microscopic (TEM) picture of siphovirus (thin tail), and (c) myovirus (thick tail). (TEM photographs were provided by Dr. Paul Ebner and Yanying Pan, Purdue University.)**

isometric heads, for example, TP901-1). Genome sequence analysis indicates that the species in the same family as well as those in different families vary considerably. In contrast, the genome analysis of phages of *Streptococcus thermophilus* (e.g., sfi 19, sfi 11) indicates a fairly good homology. Phages of several species of *Lactobacillus* have been identified, and genome sequences of some species and strains are now available, namely for *Lactobacillus delbrueckii* (LL-H), *Lab. plantarum* (phi-gle), *Lactobacillus johnsonii* (Lj 965), *Lab. gasseri* (adh), and *Lab. casei* (a2). Very few phages of *Leuconostoc* and *Oenococcus* have been isolated, and none have been isolated from *Pediococcus*.[9]

## Life Cycle

A phage cannot multiply by itself in food. Instead, it attaches (needs $Ca^{2+}$ for adsorption) with its tail on the surface of a bacterial cell (specific host) and injects its DNA inside the cell cytoplasm. If it is a lytic phage, the bacterial cell produces a large number of copies of the phage DNA and phage proteins. Following assembly of the proteins and phage DNA to produce mature phages, the bacterial cell lyses, releasing the phages (as many as 200) into the environment (Figure 14.3). They, in turn, attack other bacterial cells. These are lytic phages. Their growth cycles (lytic cycle) take approximately 20–30 minutes. There are other phages (called temperate phages) that are nonlytic, and their DNA, following injection into the cytoplasm, is integrated with bacterial DNA. The phage DNA (called prophage) is carried by a bacterial cell DNA (lysogeny state), and as a bacterial cell multiplies, the prophage also multiplies without showing the presence of the phage in the bacterial strain (lysogenic strain). As long as the host cell carries the prophage, it is immune to attack by the same phage. Recent genome studies have shown that the DNA of a host strain can have five or more types of prophages.[9] However, a prophage can be induced by a physical (such as UV) or a chemical (such as mitomycin C) agent, causing the phage DNA to separate out of bacterial DNA and resume the lytic cycle (Figure 14.3).

## Host Specificity

Bacteriophages against many species of *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Oenococcus oenos* that are currently used in food fermentation have been discovered. The phages are host-specific, and there can be one specific host (strain) for a specific phage to several related strains for a phage. A bacterial strain can also be the host of many different types of phages. A

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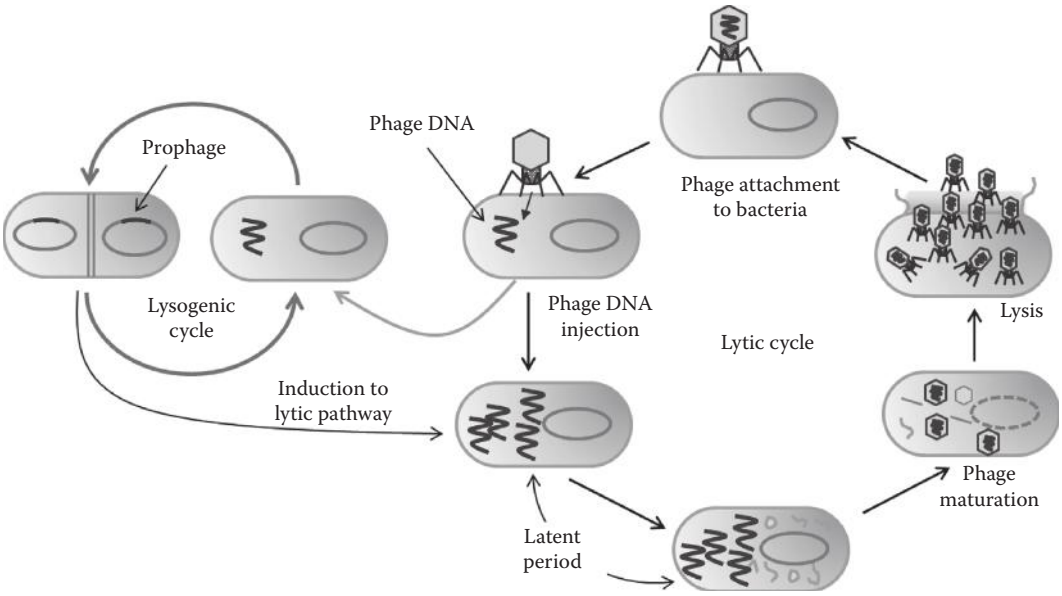



**Figure 14.3 (See color insert.) Schematic presentation of lytic cycle and lysogenic cycle of bacteriophages in bacteria. The steps are attachment (adsorption) of a phage on the bacterial cell wall; injection of phage DNA into the bacterial cell; phage maturation characterized by formation of phage DNA and protein synthesis; maturation of phages and lysis of host cell. The lysogenic cycle includes a temperate phage showing integration of phage DNA in bacterial cell DNA, division of phage DNA during bacterial cell division (prophage), release of phage DNA because of activation, and induction to lytic pathway.**

bacterial strain can have restriction enzymes that can hydrolyze and destroy the DNA of a phage. A phage can be lytic or temperate. All phages require $Ca^{2+}$ for their adsorption on the cell surface of lactic cultures.

## *Control Methods*

Following the discovery of bacteriophage-related starter failure in food fermentation, steps were developed to reduce phage contamination with starter cultures.[6–8] These include proper sanitation to reduce phage buildup in the processing facilities, both during bulk starter preparation and product fermentation, use of phage-insensitive media, rotation of strains, and use of mixed strains to reduce the buildup of a particular phage to a level that causes starter failure. Subsequently, phage-resistant starter strains were developed. Initially, by growing a sensitive bacterial strain in the presence of a specific lytic phage, cells that were not killed by the phage were isolated and supplied to processors. Recent studies have indicated that by genetic techniques, a bacterial strain can be made insensitive to one or more phages. These include modifying the genetic makeup of a starter strain to inhibit phage adsorption, destroying phage DNA by restriction enzyme systems of cells, abortive infection, and the novel restriction-modification system called CRISPR/Cas system before lysis.[8] A CRISPR/Cas (clustered regularly interspaced short palindromic repeats/cas enzyme) system of a host provides a novel mechanism for interference with phage DNA in the host to provide resistance against phage. By combining these traits through genetic manipulation,

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a strain that is resistant to several phages can be developed. Current studies on genome analysis of lactic acid bacteria and bacteriophages will help develop phage-resistant starter strains (see Chapter 13).

## Yeast and Mold Cultures

Specific strains of yeast cultures (e.g., *Saccharomyces cerevisiae*) used to leaven dough in bakery products and to produce alcohol in beer, wine, or distilled liquor have been developed. These yeasts are produced by culture producers as well as by processors. Culture producers grow the yeast in suitable media, concentrate the cells, and supply it in frozen or dried form.

Molds used as starter in some products are also available from culture producers. The strains used should not produce mycotoxins. The molds are grown on the surface of a liquid or solid (bread) media until they sporulate. The spores are collected, dried in powder form, packaged, and supplied to processors.

## Conclusion

Isolation and identification of microorganisms associated with food fermentation have helped the use of specific species and strains in pure culture for controlled fermentation. These starter cultures are currently produced by commercial culture producers for use by food-processing companies directly to start fermentation of raw materials. This has also helped to reduce product loss associated with culture failure, notably from phage attack. Development of phage-resistant starter cultures has also helped overcome the problem. Current studies on the genome sequence of lactic acid bacteria and their bacteriophages will be useful in the future development of bacterial strains for their efficient use in food fermentation. The use of starter culture in food fermentation is covered in Chapter 15.

### QUESTIONS

1. Define starter culture.
2. Briefly list the major advances in starter-culture technology since the 1950s.
3. How are concentrated starter cultures prepared? How does this technology differ from the making of bulk starters?
4. Briefly describe the method used in developing a phage-insensitive medium for the manufacture of bulk starters.
5. List the problems in starter cultures that can affect their performance. What difficulties can occur because of thawing and refreezing a starter culture?
6. Define the terms lytic phage, temperate phage, prophage, lysogeny, and lytic cycle of bacteriophages.
7. Give an example of bacteriophages of each: *Lactococcus*, *Streptococcus*, and *Lactobacillus*.
8. How can starter strains resistant to bacteriophages be produced?

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# Chapter 15

# Microbiology of Fermented Food Production

## Introduction

At the dawn of civilization, humans recognized, probably by accident, that under certain circumstances, when raw foods from plant and animal sources were stored for future consumption, they could change to different but desirable products with longer storage stability. The possibility of such an event occurring might have been after they learned to produce more foods than they could consume immediately and thus needed storage. It was probably the period during which they learned agriculture and animal husbandry as well as making baskets and pottery to store the excess products. On this basis, one can assume that fermented food probably originated in approximately 7000 B.C.–8000 B.C. in the tropical areas of Mesopotamia and the Indus Valley. Subsequently, other civilizations also produced fermented foods from different raw materials, particularly to preserve those that were seasonal and thus available in abundance only for a short harvesting period. Fermented milk products, alcoholic beverages from fruits and cereal grains, and leavened breads became popular among the early civilizations in the Middle East and in the Indus Valley and later among the Egyptians, Greeks, and Romans.[1]

Currently, more than 3500 different fermented foods are consumed by humans worldwide; many are ethnic and produced in small quantities to meet the needs of a group in a particular region. Some are, at present, produced commercially, and only a few are produced by large commercial producers. Production by large producers is now on the rise.

At present, there is interest in the consumption of many types of fermented foods other than cheese, bread, pickles, and alcoholic beverages. One reason for this increase is consumer interest in natural and healthy foods, which fermented foods have been thought to satisfy. Even countries in which many types of fermented foods have been consumed for a long time but mostly produced in small volumes have started commercially producing some products in large volumes. It is anticipated that, in the future, consumption of many fermented foods will increase worldwide.[1]



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## General Method of Production

The production of a fermented product has two related yet separate aspects, one involving the importance of metabolic activities of microorganisms during fermentation and storage of the product and the other involving the parameters used during processing and storage of the product. In this chapter, the microbiological aspects are presented. Processing aspects are generally taught in a food-processing course and are thus covered only in brief here.

Fermentation involves exposing the raw or starting food materials to conditions that favor growth and metabolism of specific and desirable microorganisms. As the desirable microorganisms grow, they utilize some nutrients and produce some end products. These end products, along with the unmetabolized components of the starting materials, constitute the fermented foods having desirable acceptance qualities, many of which are attributed to the metabolic end products.

### *Raw (or Starting) Materials*

A large number of raw materials from plant and animal sources are used to produce fermented foods. These include milk (from cows, buffalo, sheep, goats, and mares), meat (beef, pork, lamb, goat, and fowl), fish (many types), eggs (chicken and duck), vegetables and vegetable juices, many fruits and fruit juices, cereal grains, tubers, lentils, beans, and seeds. Some are used in combination.

### *Microorganisms Used*

Many desirable species and strains of bacteria, yeasts, and molds are associated with fermentation of foods. Depending on a product, fermentation may be achieved by a single predominating species and strain. However, in most fermentations, a mixed population of several bacterial species and strains, or even bacteria and yeasts or bacteria and molds, is involved. When a fermentation process involves a mixed population, the members should not be antagonistic toward one another; rather, they should preferably be synergistic. Maximum growth of a desirable microorganism and optimum fermentation rates are dependent on environmental parameters, such as nutrients, temperature of incubation, oxidation-reduction potential, and pH. In the fermentation process, if the different species in a mixed population need different environmental conditions (e.g., temperature of growth), a compromise is made to facilitate growth of all the species at a moderate rate. Depending on the raw or starting material and a specific need, carbohydrates (dextrose in meat fermentation), salts, citrate, and other nutrients are supplemented. In some natural fermentations, several species may be involved for the final desirable characteristics of the product. However, instead of growing at the same time, they appear in sequence with the consequence that a particular species predominates at a certain stage during fermentation. But analyzing the final product to isolate the species involved in fermentation of such a food does not give the right picture. Instead, samples should be analyzed at intervals to determine predominant types at different times and to know the sequences in their appearance. Finally, some minor flora (secondary flora) can be present in a very low level in the raw material and the final product and might not be detected during regular analysis. However, they may have important contributions for the desirable characteristics, particularly some unique aroma, of the product.

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## Fermentation Process

Foods can be fermented in three different ways, based on the sources of the desirable microorganisms: natural fermentation, back slopping, and controlled fermentation.

### Natural Fermentation

Many raw materials used in fermentation (usually not heat treated) contain both desirable and associated microorganisms. The conditions of incubation are set to favor rapid growth of the desirable types and no or slow growth of the associated (many are undesirable) types. A product produced by natural fermentation can have some desirable aroma resulting from the metabolism of the associated flora. However, because the natural microbial flora in the raw materials may not always be the same, it is difficult to produce a product with consistent characteristics over a long period of time. Also, chances of product failure because of growth of undesirable flora and foodborne diseases by the pathogens are high.

### Back Slopping

In this method, some products from a successful fermentation are added to the starting materials, and conditions are set to facilitate the growth of the microorganisms coming from the previous product. This is still practiced in the production of many ethnic products in small volumes. Retention of product characteristics over a long period may be difficult because of changes in microbial types. Chances of product failure and foodborne diseases are also high.

**Table 15.1 Fermented Food Groups and Examples**

| *Food Groups* | *Examples* |
|---|---|
| Dairy products | Cheeses, yogurt, buttermilk, sour cream, dahi, kumiss, kefir, acidophilus milk |
| Meat products | Salami, pepperoni, chorizo, thüringer, sausage, pickled meat, nahm |
| Cereal products | Breads, pancake, crackers, pizza, nun, idli, dosa, sour rice, miso |
| Fruits and vegetable products | Pickled fruits, pickled vegetables, olives, sauerkraut, kimchi, aachar |
| Legume products | Tofu, fermented soymilk, tempe, soy sauce, koji, mizo, natto, papadam |
| Fish products | Bagoong, fish sauces, pickled fish, tarama, paak, mamoni, izushi |
| Beverages | Beer, wine, distilled spirits, coffee, cocoa, tea |
| Starch crop products | Fermented products from potato, cassava, sweet potato, bananas, plantains |
| Miscellaneous products | Fermented eggs, ghee (from fermented cream), vinegar, red palm oil, bongkrek, dage |

*Source:* Adapted from Campbell-Plat, G., *Fermented Foods of the World*, Butterworths, London, 1987, p. xi.

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### Controlled Fermentation

The starting materials (may be heat treated) are inoculated with a high population ($10^6$ cells/mL or more) of a pure culture of single or mixed strains or species of microorganisms (starter culture). Incubation conditions are set for the optimum growth of the starter cultures. Large volumes of products can be produced with consistent and predictable characteristics each day. Generally, there is less chance of product failure and foodborne diseases. However, there may be no growth of desirable secondary flora. As a result, a product may not have some delicate flavor characteristics.

As indicated before, worldwide there are more than 3500 types of fermented foods. Different methods have been used to arrange them in several major groups. One such method divides fermented foods in nine groups and is presented in Table 15.1.

As it is beyond the scope of this textbook to describe even a few from each group, only the microbiological criteria of several fermented dairy, meat, and vegetable products are briefly discussed here to understand the methods involved in controlled and natural fermentation. For more detailed information, books on fermentation of different food groups can be consulted, some of which have been used as references in this chapter.

## Fermented Dairy Products

Fermented dairy products can be broadly divided into two groups: fermented milk products and cheeses. In fermented milk products, all the constituents of the milk are retained in the final products with the exception of those partially metabolized by the bacteria. In cheeses, a large portion of the milk constituents is removed in whey to obtain the final products.

### *Milk Composition and Quality*

The growth of desirable microorganisms and the quality of a fermented dairy product are influenced by the composition and quality of the milk used in a fermentation process. Cow's milk contains approximately 3.2% protein, 4.8% lactose, 3.9% lipids, 0.9% minerals, traces of vitamins, and approximately 87.2% water. Among the proteins, casein in colloidal suspension as calcium caseinate is present in higher amounts than the other two soluble proteins, albumin and globulin. Lactose is the main carbohydrate and is present in solution, and lipids are dispersed as globules of different sizes in emulsion (fat in water). Minerals are present in solution and as colloid with casein. Water-soluble vitamins are present in the aqueous phase, whereas fat-soluble vitamins are present with the lipids. The solid components (ca. 12.8%) are designated as total solids (TS), and TS without lipids are designated as solid-not-fat (SNF; ca. 8.9%). The whey contains principally the water-soluble components, some fat, and water.

The growth of desirable microorganisms can be adversely affected by several components that are either naturally present or have entered in the milk as contaminants. The natural antimicrobials are agglutinins and the lactoperoxidase-isothiocynate system. The agglutinins can induce clumping of starter-culture cells and slow their growth and metabolism. The lactoperoxidase-isothiocynate system can inhibit starter cultures. Antimicrobials can cause problems only when raw milk is used because both are destroyed by heating milk. Milk can also contain antibiotics, either used in the feed or used to treat animals for some infections, such as mastitis. Their presence can also affect the growth of starter cultures. Some milk can contain heat-stable proteases and lipases produced by some psychrotropic bacteria, such as *Pseudomonas* species, during refrigerated

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storage of raw milk before pasteurization (see Chapter 22). These enzymes remain stable after heating and can cause product defects (low yield of cheese, proteolysis, and rancidity). Before milk is used for fermentation, these aspects need to be considered.

## Fermented Milk Products

Many types of fermented milk products are produced in different parts of the world. A few are produced by controlled fermentation, and the microbial types and their respective contributions are known. In many others, fermented either naturally or by back slopping, the microbial profiles and their contributions are not exactly known. Many types of lactic acid bacteria and some yeasts are found to predominate microbial flora in these products,[3,4] some of which are listed:

1. Buttermilk. Made with *Lactococcus* species without or with *Leuconostoc cremoris*; some can have biovar diacetylactis in place of *Leu. cremoris* (such as ymer in Denmark), whereas some can have a ropy variant of *Lactococcus* species (langfil in Norway) or mold (*Geotrichum candidum* in villi in Finland).
2. Yogurt. Made with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*; some types can also have added *Lab. acidophilus*, *casei*, *rhamnosus*, and *Bifidobacterium* spp.; some may also have *Lactococcus* species and *Lab. plantarum* and lactose-fermenting yeasts (dahi in India).
3. Acidophilus milk. Made with *Lab. acidophilus*.
4. Bifidus milk. Made with *Bifidobacterium* spp.
5. Yakult. Made with *Lab. casei*; may contain *Bifidobacterium* spp.
6. Kefir. Made from *Lab. kefir* (several species of yeasts along with *Leuconostoc*, *Lactobacillus*, and *Lactococcus* spp.).
7. *Kumiss*. Made from *Lab. delbrueckii* subsp. *bulgaricus* and yeasts.

Among these, cultured buttermilk and yogurt are discussed here.

## Microbiology of Cultured Buttermilk Fermentation

It is produced from partially skim milk through controlled fermentation with starter cultures.

### Product Characteristics

Buttermilk should have a pleasant acidic taste (from lactic acid) and a high degree of aroma (from diacetyl) with a slight effervescence (from $CO_2$). It should have a white color with a smooth, thick body and should pour easily.

### Processing

1. Skim milk ≥9% SNF (solid nonfat) + citrate (0.2%)
2. Heated at 185°F (85°C) for 30 min (kills bacterial cells and phages)
3. Cooled to 72°F (22°C), starter added, agitated for 50 min (incorporates air)
4. Incubated at 72°F (22°C) for 12 h, pH 4.7, acidity 0.9%
5. Gel broken, cooled to 40°F (4.5°C), and salted (package)

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### Starter (Controlled Fermentation)

*Lactococcus lactis* ssp. *lactis* or *cremoris* is used for acid and *Leuconostoc mesenteroides* ssp. *cremoris* for diacetyl and $CO_2$. They can be used as direct vat set frozen concentrates. (*Lac. lactis* ssp. *lactis* biovar diacetylactis is generally not used as it may produce too much acetaldehyde, causing a green or yogurt flavor defect.)

### Growth

At 72°F (22°C), there is balanced growth of the two species and balanced production of acid, diacetyl, and $CO_2$. Above 72°F, the growth of *Lactococcus* species is favored with more acid and less flavor; below 72°F, the growth of *Leuconostoc* species is favored with less acid and more flavor.

### Biochemistry

Lactose (transported by the PEP-PTS system) is hydrolyzed by P-β-galactosidase in *Lactococcus* spp. (Figure 15.1). For a desirable flavor, the diacetyl:acetaldehyde ratio should be >3:1 to <4.5:1.

### Genetics

*Lactococcus lactis* strains should transport and hydrolyze lactose ($Lac^+$), metabolize P-galactose by the tagatose pathway and galactose by the Leloir pathway, be phage resistant, not produce slime, and not be very proteolytic.

*Leuconostoc* species should be able to transport and utilize citrate to produce more diacetyl and less acetaldehyde and should ferment lactose, be phage resistant, and not produce slime.

Strains should not produce inhibitory compounds (such as bacteriocins) against each other but can have antimicrobial activity toward undesirable organisms. Through selection and genetic

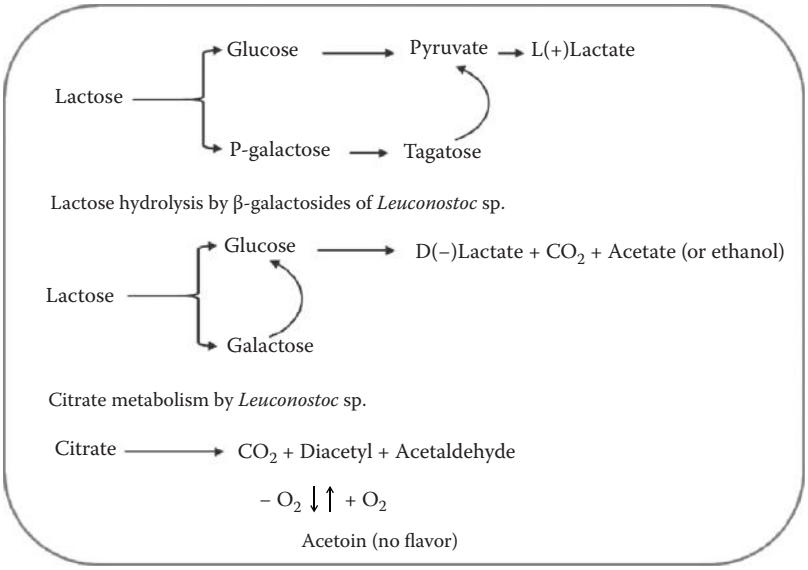



**Figure 15.1 Lactose and citrate metabolism during buttermilk fermentation.**

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manipulation, strains that grow rapidly, produce desirable characteristics, and that are resistant to some phages have been developed. Current information on genome sequences of *Lactococcus* and *Leuconostoc* strains and their phages will help develop better strains in the future.

### Microbial Problems

Because of too much acetaldehyde production (especially if biovar *diacetylactis* is used), green (yogurt flavor) may develop. A slimy texture implies contamination with bacteria that produce slime (*Alcaligenes faecalis*) or that starter cultures (some *Lac. lactic* strains) are slime formers (exopolysaccharides). A yeasty flavor implies contamination with lactose-fermentating yeasts, and a cheesy flavor alludes to contamination with proteolytic psychrotrophs (during storage). Proteolysis by proteases of contaminants in starters can also cause development of a bitter flavor, especially during storage (also see Chapter 20).

## Microbiology of Yogurt Fermentation

### Characteristics

Plain yogurt has a semisolid mass resulting from the coagulation of milk (skim, low, or full fat) by starter-culture bacteria. It has a sharp acidic taste with a flavor similar to walnuts and a smooth mouthfeel. The flavor is a result of the combined effects of acetaldehyde, lactate, diacetyl, and acetate, but 90% of the flavor is a result of the acetaldehyde.

Many types of yogurt are available in the market, for example, plain yogurt, fruit yogurt, flavored and colored yogurt, blended yogurt, sweetened yogurt, heated yogurt, frozen yogurt, dried yogurt, low-lactose yogurt, and carbonated yogurt.[3,5]

### Processing

Yogurt is generally fermented in batches, but a continuous method has also been developed. The batch process for a low-fat (2%) plain yogurt is as follows:

1. Homogenized milk (12% TS) + stabilizer (1%). The stabilizer is added to give desired gel structure.
2. Heated to 185°F (85°C) for 30 min, and cooled to 110°F (43.3°C). Heating helps destroy vegetative microbes and slightly destabilize casein for good gel formation.
3. Starter added, incubated at 110°F (43.3°C) to pH 4.8 for ca. 6 h, acidity ca. 0.9%. Starter used as either direct vat set (frozen) or bulk culture (2%–3%).
4. Quickly cooled to 85°F (29.4°C) in ca. 30 min to slow down further starter growth and acid production, especially by *Lactobacillus* species, agitated, and pumped to filler machine.
5. Packaged in containers and cooled by forced air to 40°F (4.4°C). Final cooling by forced air results in a rapid drop in temperature to stop the growth of starters.
6. Held for 24 h; pH drops to 4.3.

### Starters (Controlled Fermentation)

Frozen concentrates or direct vat set starters can be used. Normally, *Lab. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* are used. Some processors also combine these two with other species, such

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as *Lab. acidophilus* and *Bifidobacterium* spp., *Lab. rhamnosus*, or *Lab. casei*. However, in general, they do not compete well in growth with the two yogurt starters. Therefore, they are added in high numbers after fermentation and before packaging. They may not survive well when present in yogurt with the regular yogurt starter cultures.

For a good product, the two starter species should be added at a *Streptococcus*:*Lactobacillus* cell ratio of 1:1; in the final product, the ratio should not exceed 3:2. However, *Lactobacillus* cells are more susceptible to freezing and freeze-drying. In a frozen concentrate starter for use as DVS, the survivors may not be present in a desired ratio unless they are properly preserved (see Chapter 14).

## Growth

For balanced growth of the two species, the fermentation is conducted at approximately 110°F (43.3°C). At this temperature, both acid and flavor compounds are produced at the desired level. If the temperature is raised above 110°F, the *Lactobacillus* sp. predominates, causing more acid and less flavor production; at temperatures below 110°F, growth of *Streptococcus* sp. is favored, forming a product containing less acid and more flavor.

The two species show symbiotic growth while growing together in milk. Initially, *Streptococcus* sp. grows rapidly in the presence of dissolved oxygen and produces formic acid and $CO_2$. The anaerobic condition, formic acid, and $CO_2$ stimulate growth of *Lactobacillus* sp., which has good exoproteinase and peptidase systems and produces peptides and amino acids from milk proteins (outside the cells) in the milk. Some of the amino acids, such as glycine, valine, histidine, leucine, and methionine, are necessary for good growth of the *Streptococcus* sp., which lacks proteinase enzymes. *Streptococcus* sp. gets these from the milk and grows rapidly until the pH drops to approximately 5.5, at which time the growth of *Streptococcus* sp. slows down. However, growth of *Lactobacillus* sp. continues fairly rapidly until the temperature is reduced to 85°F (29.4°C), following a drop in pH to 4.8. At 85°F, both grow slowly, but *Streptococcus* sp. has the edge. At 40°F (4.4°C) and a pH of approximately 4.3, both species stop growing.

The two species also have a synergistic effect on growth rate, rate of acid production, and amounts of acetaldehyde formation when growing together as compared with when growing individually. The species growing separately in milk produce approximately 8–10 ppm acetaldehyde; when grown together, acetaldehyde production increases to a desirable level of 25 ppm or higher.

## Biochemistry

### Lactose Metabolism

Both species have a constitutive β-galactosidase system, and lactose (transported by permease systems) is hydrolyzed to glucose and galactose (Figure 15.2). Both species are homofermentative and produce lactate from glucose by the EMP pathway. *Lab. delbrueckii* ssp. *bulgaricus* strains have enzymes for the Leloir pathway to metabolize galactose, but while actively metabolizing glucose they do not utilize galactose well. Most *Str. thermophilus* strains do not have the enzymes of the Leloir pathway (or have a very weak system) and thus do not metabolize galactose. As a result, galactose is excreted outside, causing its accumulation in yogurt.

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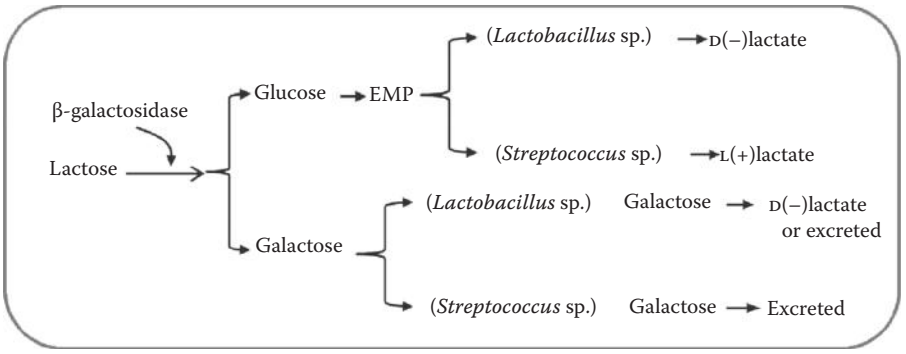



**Figure 15.2 Lactose metabolism during yogurt fermentation.**

## Flavor Production

The major flavor compound in yogurt is acetaldehyde (25 ppm) with some diacetyl (0.5 ppm) and acetate. Acetaldehyde is produced in two ways: from glucose via pyruvate by *Streptococcus* sp. and from threonine (supplied or produced through proteolysis in milk) by *Lactobacillus* sp. (Figure 15.3).

## Formate Production

Formate (necessary for *Lactobacillus* growth) is produced by *Str. thermophilus* from pyruvate by the action of formate lyase (Figure 15.4).

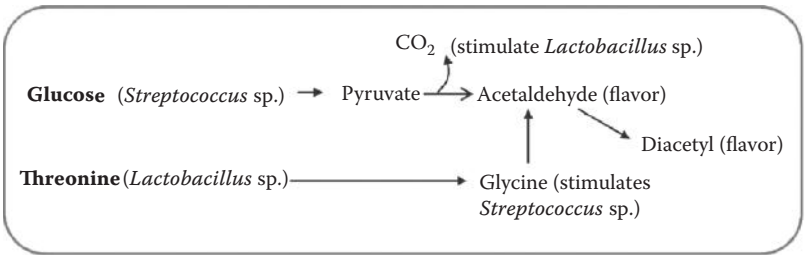



**Figure 15.3 Diacetyl (flavor) production during yogurt fermentation.**

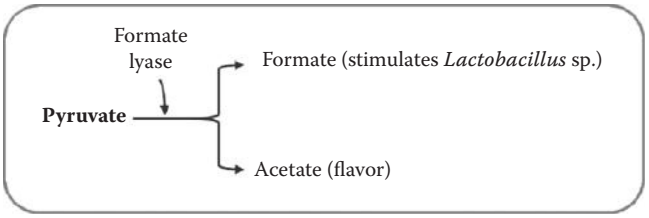



**Figure 15.4 Acetate (flavor) production from pyruvate during yogurt fermentation.**

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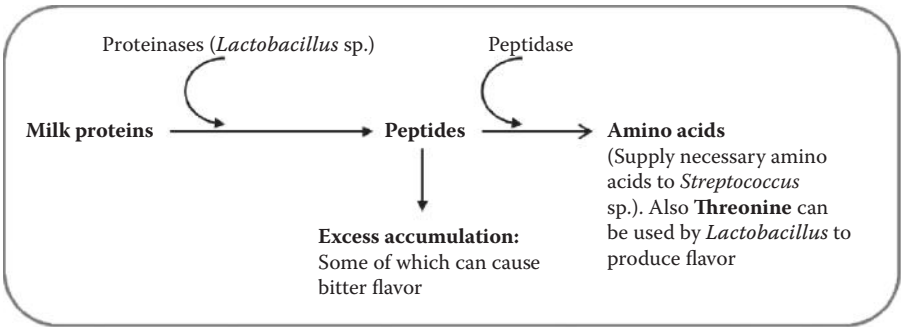



**Figure 15.5 Milk protein hydrolysis and flavor production during yogurt fermentation.**

### Slime Formation (Glycan)

β-Galactosidase in some strains of *Str. thermophilus* polymerizes glucose to produce oligosaccharides and glycan, which may give a viscous texture to yogurt.

### Proteolysis

Milk proteins are hydrolyzed by proteinases or peptidases produced by *Lactobacillus* sp. to produce small peptides (bitter peptide), which contribute to bitterness and also flavoring compounds (Figure 15.5).

## *Genetics*

- *Lac⁺ phenotype*. In both species, the trait (β-galactosidase and permease) is chromosomally linked, constitutive, and quite stable. Some strains can have strong β-galactosidase activity.
- *Gal⁺ phenotype*. *Lab. delbrueckii* ssp. *bulgaricus* is Gal⁺, but *Str. thermophilus* usually is a Gal⁻ phenotype. Strains with a good Gal⁺ phenotype can be developed to reduce galactose accumulation in the product.
- *Pro⁺ phenotype*. *Lab. delbrueckii* ssp. *bulgaricus* strains differ in protein hydrolysis ability. Strains with desirable proteolytic activity should be used. Too much proteolysis can adversely affect the texture and enhance development of bitter flavor.
- *Phage resistance*. Both species have phages; resistant strains need to be developed and used.
- *Symbiotic and synergistic relationship*. Strain selection for the best combinations is necessary.
- *Antagonistic effect*. Strains should not produce inhibitory compounds (such as bacteriocins) against each other.
- *Good survival against freezing and drying*. Possible genetic basis needs to be studied to develop resistant strains to produce concentrated starter cultures.

Complete genome sequences of several strains of each species are available now,[6] which will help in the future to develop more desirable strains for use in yogurt production.

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### Microbial Problems

In plain yogurt, flavor problems can be associated with the concentrations of acetaldehyde. A low concentration gives a chalky and sour flavor, and too much acetaldehyde can give a green flavor. Similarly, too much diacetyl gives a buttery aroma. Too much acid production during storage causes a sour taste. Proteolysis and accumulation of bitter peptides during storage are associated with bitter flavor. Production of exopolysaccharides by the starter can give a viscous and ropy texture (which can be desirable in some situations). Growth of yeasts during storage can also produce a fruity flavor, especially in yogurt containing fruits and nuts. In colored, flavored, and blended yogurt, many of these problems are masked. During long storage, molds can grow on the surface (also see Chapter 20).

## Cheeses

Cheeses are made by coagulating the casein in milk with lactic acid produced by lactic acid bacteria, without or with the enzyme rennin, followed by collecting the casein for further processing, which may include ripening.[6] The process was probably accidentally discovered in the Middle East in approximately 7000 B.C. from the coagulation of milk stored in a calf stomach by lactic acid produced by lactic acid bacteria (and probably rennin in the stomach). At present, many varieties of cheeses are made worldwide, which probably use more than 20% of the total milk produced. In the United States, the total production of different varieties of cheese in 1982 was 4.4 billion pounds (2 billion kg) and, in 1987, this increased to 5.3 billion pounds (2.4 billion kg). Because of the worldwide increase in cheese consumption, cheese production will continue to increase not only in the United States, but also in other countries, especially in dairy-rich countries, such as Europe and New Zealand.

Cheese varieties have been grouped in different ways. Examples of several varieties based on starter cultures used and some important secondary flora are listed here.

### Unripened Cheese

Soft

- Cottage cheese with starters *Lac. lactis* ssp. *lactis* and *cremoris* and *Leuconostoc mesenteroides* ssp. *cremoris*.
- Mozzarella cheese with starters *Str. thermophilus* and *Lab. delbrueckii* ssp. *bulgaricus*.

### Ripened Cheese

Soft

- Brie cheese with starter *Lac. lactis* ssp.; *Penicillium* sp. and yeasts are secondary flora.

Semihard

- Gouda cheese with starters *Lac. lactis* ssp. and *Leuconostoc* spp.; dairy *Propionibacterium* may be secondary flora.
- Blue cheese with starter *Lac. lactis* ssp., *Leuconostoc* spp.; *Penicillium roquefortii*, yeasts, and micrococci are secondary flora.

Hard

- Cheddar cheese with starters *Lac. lactis* ssp.; some lactobacilli and pediococci (and probably enterococci) are secondary (or associative) flora.

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- Swiss cheese with starters *Str. thermophilus*, *Lab. helveticus*, and dairy *Propionibacterium* spp.; enterococci can be secondary (or associative) flora.
- Only cottage, Cheddar, Swiss, and blue cheeses are further discussed to understand the microbiological aspects of unripened and ripened cheeses.

## *Microbiology of Cottage Cheese*[7]

### *Characteristics*

Cottage cheese is made from low-fat or skim milk and has a soft texture with approximately 80% moisture. It is unripened and has a buttery aroma resulting from diacetyl (along with lactic acid and little acetaldehyde).

### *Processing (from Skim Milk)*

1. Pasteurized, cooled to 70°F (22.2°C), starter added, and incubated for 12 h at pH 4.7.
2. Firm curd set, cut in cubes, and cooked at 125°F (51.7°C) for 50 min or more.
3. Whey drained off, stirred to remove more to get dry curd.
4. Salted, creamed, and preservative added.
5. Packaged and refrigerated.

### *Starters (Controlled Fermentation)*

Frozen concentrate for direct vat set can be used. Mixed strains of *Lac. lactis* ssp. *cremoris* and *lactis* are predominantly used for acid. *Leu. mesenteroides* ssp. *cremoris* can be added initially (in which case citrate is added to milk), mainly for diacetyl. *Lac. lactis* ssp. *lactis* biovar diacetylactis can be used for diacetyl but not inoculated in milk because of formation of too much $CO_2$ that causes curd particles to float. Instead, it is grown separately in cream, which is then used to cream the dry curd.

### *Growth, Biochemistry, and Genetics*

Growth, biochemistry, and genetics (including current strain improvements) are similar to those described for the microbiology of buttermilk (see section on microbiology of cultured buttermilk fermentation).

### *Microbial Problems*

- *Slow growth*. Weak or loss of $Lac^+$ phenotype, phage attack or less viable cells in frozen concentrate, antagonistic effect among starter strains for reasons described previously.
- *Floatation of curd*. Caused by too much $CO_2$ production by flavor-producing starters.
- *Harsh flavor*. Caused by more acetaldehyde and less diacetyl production from metabolic imbalance and reduced environment.
- *Low yield*. Caused by partial proteolysis of casein by heat-stable proteinases produced in refrigerated raw milk by psychrotrophs, such as *Pseudomonas* spp.
- *Flavor loss*. Caused by reduction of diacetyl to acetoin in a reduced environment as well as by growth of undesirable bacteria during storage, such as *Pseudomonas* spp.

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- *Spoilage*. Because of high moisture and low acid content, spoilage of psychrotrophic bacteria (such as *Pseudomonas* spp.), yeasts, and molds can grow during storage. Preservatives, such as sorbates, can be used to extend shelf life under refrigerated storage (also see Chapter 19).

## *Microbiology of Cheddar Cheese*[7]

### *Characteristics*

Cheddar cheese is made from whole milk, contains less than 39% moisture, 48% fat, is generally orange-yellow in color (a result of added color, annatto), and ripened. Smoothness of texture and intensity of characteristic flavor vary with the starters used and the period of ripening. The typical flavor is the result of a delicate balance among flavor components produced during ripening through enzymatic breakdown of carbohydrates, proteins, and lipids in unripened cheddar cheese.

### *Processing*

1. Pasteurized; color (annatto) and starter added.
2. Incubated at 86°F (30°C) for acidity to increase by 0.2%; rennet added.
3. Incubated for coagulation (ca. 30 min) and cut in cubes.
4. Cooked at 100°F (37.8°C), whey drained, cheddaring to lose whey and curd to mat.
5. Milled, salted, put in form, pressed for 16 h to drain whey, and removed.
6. Dried for 5 days at 50°F (10°C), waxed, vacuum packaged, and cured at 40°F (4.4°C) for 2–12 months.

### *Starters (Controlled Fermentation)*

Starters are selected mixed strains of *Lac. lactis* ssp. *cremoris* or *lactis*. *Leuconostoc*, which may be added for flavor. Starters can be used as frozen concentrates (direct vat set).

### *Growth*

Growth is accomplished by incubation at 86°F (30°C) for mesophilic starters to start early growth and produce lactic acid to a 0.2% level by 60 minutes. This is important for the coagulation of casein to occur rapidly following the addition of rennin. During curing, cells of the starter (also of secondary flora) slowly die and release intracellular enzymes into the cheese.

### *Biochemistry*

A large number of biochemical reactions occur during the different stages of processing, from the initial incubation to the end of ripening, many not yet properly understood. Some are described here.

Initially, lactose metabolism produces lactic acid as well as some diacetyl, acetate, ethanol, and acetaldehyde, especially under aerobic conditions. Rennin destabilizes casein to produce paracasein that coagulates at low acidity and low temperatures. In addition, extracellular proteinases and peptidases of the starter cultures metabolize milk proteins to peptides and amino acids and transport them into the cells. Normally very little, if any, lipolysis occurs as a result of microbial growth.

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During curing, breakdown of remaining lactose in the curd continues. However, a large change occurs in proteins and other nitrogenous compounds. By the actions of rennin (retained in curd) and cellular exoproteinases and endoproteinases and peptidases, peptides of different sizes and amino acids are released. Further breakdown of amino acids produces hydrogen sulfide, methanethiol and related sulfur compounds, amines, and other products. Lipids also undergo lipolysis, releasing fatty acids, including the C4–C8 fatty acids (which are present in milk fat). Other reactions produce lactones, ketones, and thioesters. Some of the reactions are nonenzymatic. The typical Cheddar cheese flavor is the result of a delicate balance among the products produced from carbohydrate, protein, and lipid breakdown during processing and curing. The concentrations of these components change with curing time.

Some secondary microflora that survive heating or gain entrance later in the milk and curd during processing have definite roles in the flavor of Cheddar cheese. These include some enterococci, lactobacilli, pediococci, micrococci, and some Gram-negative rods. They probably contribute to the typical intense flavor that could be missing in cheese made with defined starter strains only. Some of these flora are known to produce several flavor compounds rather rapidly and at higher concentrations (e.g., volatile fatty acids and $H_2S$).

## *Genetics*

Phenotypic characteristics, as described before for these species, should be considered. $Lac^+$ strains capable of producing lactic acid rapidly at the initial stage are preferred. Also, strains with weak proteinase ($Pro^+$) activity are desirable because they do not cause rapid proteolysis with the accumulation of some peptides and the appearance of bitter flavor in the products. In mixed starters, they should not have an antagonistic effect. Also, the strains should preferably be resistant to multiple phages.

## *Microbial Problems*

Bitter flavor in Cheddar cheese, especially in the aged product, results from the accumulation of bitter peptides that are approximately 1000–12,000 Da and rich in hydrophobic amino acids. Starters capable of hydrolyzing proteins rapidly (fast starters) tend to produce bitter peptides more than slow starters do. Their enzymes hydrolyze proteins quickly, releasing large amounts of peptides that are subsequently hydrolyzed slowly to smaller peptides and amino acid by peptidases, resulting in the accumulation of peptides. Because they are hydrophobic, bitter peptides are generally hydrolyzed slowly, causing them to accumulate. Use of slow starters for protein breakdown at a slow rate or treatment of cheese with peptidase, or both, are effective in reducing bitterness (also see Chapter 20).

Mold growth on the surface or in air pockets inside the cheese can occur after removing the packing material from cheese. The spores are generally present in the raw material or get in the product during processing and before sealing. It is not possible to determine from the colonial morphology whether they are mycotoxin producers. It is better not to consume cheese with heavy growth.

*Staphylococcus aureus*, following contamination of milk after heating, can grow during processing Cheddar cheese and produce enterotoxins. The toxins remain in the cheese even after the death of cells during curing. Food poisoning can occur from consuming such cheese (even if they are heated in some preparations).

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Biological amines (histamine from histidine and tyramine from tyrosine) can form by decarboxylation (by starter decarboxylases) of some amino acids, especially in cheese ripened for a long time. Allergic reactions can occur from consuming such cheese. Secondary flora may have an important role in such amine formation.

## Microbiology of Swiss Cheese[7]

### Characteristics

Swiss cheese is made from partially skimmed milk (cow's milk) and coagulated with acid and rennin; it is hard and contains approximately 41% moisture and 43% fat. The cheese should have uniformly distributed medium-sized eyes (openings). It has a sweet taste, resulting from proline, and a nutty flavor.

### Processing

1. Pasteurized, starter added, and incubated at 90°F (32.2°C) for acidity to increase by 0.2%.
2. Rennin added, incubated for firm coagulation, cut in 1/8-in. cubes, and cooked for 1 h at 125°F (51.7°C).
3. Whey removed, curd pressed for 16 h, cut in blocks, and exposed in brine at 55°F (12.8°C) for 1–3 days.
4. Surface dried, vacuum packaged, stored for 7 days at 55°F (12.8°C), and transferred to 75°F (23.9°C) for 1–4 weeks.
5. Cured at 37°F (2.8°C) for 3–9 months.

### Starters (Controlled Fermentation)

Starters are *Str. thermophilus* and *Lab. helveticus* as primary for acid and *Propionibacterium* sp. as secondary for eye formation, taste, and flavor.

### Growth

Primary starters grow during fermentation, cooking, and processing; growth of *Str. thermophilus* is favored. Propionibacteria grow well during storage at 75°F (23.9°C). During curing, none of the starters grow. The cells slowly die and release intracellular enzymes.

### Biochemistry

Lactose is hydrolyzed by β-galactosidase of both lactic acid bacteria and metabolized by the EMP pathway. *Str. thermophilus* produces L(+)-lactic acid, and *Lab. helveticus* produces D(–)-lactic acid. Some acetate also forms. Production of large amounts of lactate is very important to facilitate growth of propionibacteria. During storage at 75°F (23.9°C), propionibacteria convert lactate (by lactate dehydrogenase) to pyruvate, which is then converted to propionic acid, acetate, and $CO_2$. Eye formation occurs from the production of $CO_2$, and the size and distribution of eyes depends on the rate of $CO_2$ production. Proteins are hydrolyzed by rennin, intracellular proteinases, and peptidases of starters, especially propionibacteria, resulting in the production of small peptides and amino acids, which impart the nutty flavor and sweet taste (sweet taste is attributed to a

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relatively high concentration of proline produced by propionibacteria). Very little lipolysis occurs during curing.

### Genetics

Rapid production of lactate in large amounts from lactose by lactic acid bacteria and their resistance to phages (some *Lab. helveticus* strains have temperate phages that are activated at high processing temperatures, causing starter failure) are important considerations. *Propionibacterium* strains should produce $CO_2$ at proper rates for desirable numbers and sizes of eye formation (uniform distribution of medium-size eyes preferred).

### Microbial Problems

Spores of *Clostridium tyrobutyricum*, present in raw milk or entering as contaminants, can germinate, grow, and cause rancidity and gas blowing in this low-acid cheese. Nisin has been used as a biopreservative to control this problem (also see Chapter 20).

## Microbiology of Blue Cheese[7]

### Characteristics

Blue cheese is a semihard (46% moisture, 50% fat), mold-ripened cheese made from whole milk (cow's milk). It has a crumbly body, mottled blue color, and sharp lipolytic flavor.

### Processing

1. Homogenized, pasteurized, starter added, and incubated at 90°F (32.2°C) for acidity to increase to 0.2%.
2. Rennet added, incubated for firm set, cut, cooked at 100°F (37.8°C), and whey drained.
3. Curd collected in hoop, drained 16 h, and salted in brine for 7 days.
4. Spiked to let air get inside. Mold spores added, stored at 50°F (10°C) in high humidity for 4 weeks.
5. Stored at 40°F (4.4°C) for curing for 3 months.

### Starters and Growth (Controlled Fermentation)

*Lac. lactis* ssp. *cremoris* or *lactis* and *Leu. cremoris* or *lactis* serve as primary starters. *Pen. roquefortii* spores serve as secondary starters. The lactic starters grow until curing, and from lactose, they produce lactate, diacetyl, acetate, $CO_2$, and acetaldehyde. Mold spores, during storage at 50°F (10°C) in high humidity, germinate quickly, produce mycelia, and spread inside to give the mottled green appearance. Their growth continues during curing. Puncturing the inside of the cheese helps remove $CO_2$ and let air in to help the growth of molds.

### Biochemistry, Genetics, and Problems

*Lactococcus* species produce mainly lactic acid from lactose, whereas *Leuconostoc* species produce lactic acid, diacetyl, $CO_2$, and acetate. Proteolysis is quite limited by the lactic starters. Molds

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produce extracellular lipases and proteinases and cause lipolysis and proteolysis during curing. Fatty acids are both oxidized and reduced to produce methyl ketone and D-lactone, respectively. These, along with volatile fatty acids, contribute to the sharp flavor of blue cheese.

The desired genotypes of the lactic acid bacteria starters used have been previously described. A white variant of the mold has been isolated and used to produce this cheese without the blue mottled color. *Pen. roquefortii* strains that produce mycotoxins have been identified. Strains that do not produce mycotoxins need to be selected. Ripening for a long time can lead to formation of biologically active amines from some amino acids (e.g., histamine from histidine).

## *Accelerated Cheese Ripening*[8]

During curing of hard and semihard cheeses, milk components are degraded through enzymatic (from starters and secondary flora) and nonenzymatic reactions. This is necessary to develop a desirable flavor of these cheeses. However, because of a long storage time, it is not quite economical. Thus, methods are being studied that will speed up the ripening process. Some of these methods are briefly described.

### *Curing at High Temperature*

Because enzymatic (also nonenzymatic) reactions increase as the temperature is increased, studies were performed to cure cheese above the usual 5°C–6°C (40°F). Ripening some cheeses at 13°C–16°C (55°F–61°F) reduced the curing time by 50% or more. However, at higher temperatures, growth of spoilage bacteria has been a problem in some cheeses, and there is some concern about the growth of foodborne pathogens.

### *Addition of Enzymes*

As intracellular enzymes of starters have an important role in curing, enzymes obtained from cell lysates of starter-culture bacteria have been added to increase the rate of curing. In Cheddar cheese, the curing time has been substantially reduced but has resulted in bitter flavor.

### *Slurry Method*

Cheddar cheese slurry has been prepared by mixing water with cheese to 40% solids (in place of the usual 60%). The slurry is incubated at 30°C for 4–5 days with agitation. This method increases the flavor greatly and the product can be used to make processed cheeses. The major disadvantages are the inability to properly control the enzymatic reactions to produce uniform products and possible growth of spoilage and pathogenic bacteria.

### *Novel Methods*

In recent years, application of a suitable bacteriocin (of lactic acid bacteria) to cheese and exposing cheese to high hydrostatic pressure to lyse the cells of starter cultures are being studied to enhance ripening by the released intracellular enzymes.

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## Fermented Meat Products

### Types

Fermented meat products are produced by first mixing meat, fat, salt, sugar, curing agents, and spices; filling the mixture in a casing; and fermenting it either naturally or by adding (during mixing) selected starter-culture bacteria.[3] The acids produced by the starters during fermentation and the curing agents used help control the growth of pathogenic and spoilage bacteria that might be present in the meat. Depending on the type, the fermented products may be dried to reduce $A_W$ or smoked or heated to ensure the safety and shelf life of the products.

Meat fermentation probably originated in the Mediterranean countries and later spread to European countries and North America. In the United States, semidry sausages are the most popular although some dry sausages are also produced. Following fermentation, semidry sausages are heated (also sometimes smoked) before consumption. For dry sausages, following cooking, the products are dried to reduce the $A_W$. Even now, fairly large amounts of fermented sausages in the United States are produced by natural fermentation, especially those produced by small processors. However, more processors now use selected starter cultures and controlled fermentation. Commercial starter cultures are available as both frozen and freeze-dried concentrates for direct inoculation in the meat mixture.

Semidry and dry sausages include many types, such as pepperoni, Genoa salami, hard salami, summer sausage, beef sticks, beef logs, thuringer, cervelat, and Italian salami. Most are made with beef and pork, but in recent years, some have been made with meat from chicken and turkey. The microbiology of semidry sausages is described here.

### Microbiology of Semidry Sausages

#### Characteristics

Semidry sausages include summer sausage, thuringer, and semidry salami. The average composition is approximately 30% fat, 20% protein, 3% minerals (salts), and 47% water. They have a tangy taste with a desirable flavor imparted by the combined effect of lactate, acetate, and diacetyl and some breakdown components from proteolysis and lipolysis. The use of spices also contributes to the flavor. Those containing nitrite have a pinkish color in contrast to the grayish color in products without it.

#### Processing

1. Meat, salts, glucose, cure, spices, and starter mixed uniformly.
2. Stuffed in casings, fermented at 85°F–110°F (29.4°C–43.3°C) with 80%–90% relative humidity.
3. Incubated until the pH drops to ca. 5.2–4.6, cooked to 140°F (60°C) internal temperature, and cooled to 50°F.
4. Stored at 40°F–50°F (4.4°C–10°C) for 3–4 days, vacuum-packaged, and consumed directly.
5. Cures contain nitrite to give a final concentration of ca. 100 ppm. Fermentation can be carried out in a smokehouse. Fermentation time is usually 8–12 h, during which the pH is dropped to the desired level.

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### Starters (Controlled or Natural Fermentation)

In controlled fermentation, frozen or dried concentrates are used directly in a $10^{6-7}$ cells/g mix. Starters should not be mixed with salt, cure, or spices as they can kill injured cells. Instead, they should be thawed and immediately put into the meat. Starters vary, depending on the fermentation temperature and final pH of the product desired. For high temperature and low pH, *Pediococcus acidilactici* strains are preferred; for low temperature and high pH, *Lab. plantarum* strains are preferred. *Ped. pentosaceus* strains can be used under both conditions. Some starters can have both *Pediococcus* and *Lactobacillus* species. In addition, selected *Micrococcus* spp. or *Sta. carnosus* strains are added as secondary flora for their beneficial effects on desired product color.

In naturally fermented sausages, *Lab. sake*, *Lab. curvatus*, and *Leuconostoc* spp. present in raw materials are important starter bacteria, especially when fermentation is set at lower temperatures (60°F–70°F [15.6°C–21.1°C]) for several days and the final pH reached is not below 5.0.

### Growth

Because the raw meat used may contain pathogens and spoilage bacteria, it is extremely important that starter culture grows rapidly and produces acid in large amounts to reduce pH from the initial 5.7 to approximately 5.3 very quickly to retard their growth. This can be achieved by adding large numbers of active starter cells, adding dextrose to the mix, and setting the temperature of fermentation optimum for the starters used. The optimum growth temperatures for *Ped. acidilactici*, *Ped. pentosaceus*, and *Lab. plantarum* are approximately 40°C, 35°C, and 30°C (104°F, 95°F, and 86°F), respectively. *Micrococcus* spp. and *Sta. carnosus* grow well at approximately 32.2°C (90°F). Cooking to an internal temperature of 60°C (140°F) kills *Lab. plantarum* and probably *Ped. pentosaceus* but probably not *Ped. acidilactici*, *Micrococcus*, or *Sta. carnosus*. However, low pH and low $A_W$ prevent their growth in the finished products.

### Biochemistry

Both pediococci are homolactic fermentors and metabolize glucose to mainly lactic acid (DL forms) with small amounts of acetate and diacetyl. *Lab. plantarum*, being facultatively heterofermentative, metabolizes glucose to principally lactic acid (DL); however, it can also produce substantial amounts of acetate, ethanol, and diacetyl. Strains of all three species can produce $H_2O_2$, which can discolor the product by oxidizing myoglobin during fermentation. *Micrococcus* spp. or *Sta. carnosus* have catalase that can destroy $H_2O_2$. *Micrococcus* spp. or *Sta. carnosus* and some strains of *Lab. plantarum* can also reduce nitrate to nitrite. If nitrate is used in place of nitrite in cure, these bacteria can produce nitrite and help develop the agreeable pinkish color of the product. If the products are cured or stored for long periods of time, some of the intracellular enzymes of the lysed cells of starters are able to cause proteolysis and lipolysis and produce biologically active amines (such as histamine).

### Genetics

Rapid acid-producing lactic acid bacterial strains at temperatures of fermentation and non-$H_2O_2$ producers are desired. Strain selection can also be done for nonproducers of biogenic amines. Strains producing bacteriocins can be used to control pathogens and spoilage bacteria. *Ped. acidilactici* strains that cannot hydrolyze sucrose ($Suc^-$) can be used to produce sweet and sour products

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by supplementing the meat mixture with both glucose and sucrose. Exopolysaccharide-producing strains can improve the texture of products.

### Microbial Problems

Slow acid production can be a serious problem if the starters used are not metabolically active or have lower numbers of viable cells or as a result of other factors, such as low glucose and high salts in the mix. Sour or no flavor can occur if the starter, especially *Ped. acidilactici*, grows very rapidly and reduces the pH to below 4.5. Gas formation can occur because of growth of *Leuconostoc* spp. during fermentation and during storage in vacuum packages. *Leuconostoc* spp. are usually present in raw meat. Pathogens, when present in meat, can grow if acid production is slow during fermentation. Acid-resistant pathogens can also survive in the products and cause health hazards. During long storage or curing, biogenic amines can form. Also, mycotoxin-producing molds can grow on the product surface during curing (also see Chapter 20).

## Fermented Vegetable Products

Almost all vegetables can be fermented through natural processes because they harbor many types of lactic acid bacteria. Worldwide, many types of vegetables are fermented, mostly in small volumes. However, some are produced commercially. Vegetable fermentation originated in the early years of human civilization and, even now, is widely used by many cultures. Examples of some fermented products and vegetables used currently for fermentation are sauerkraut and kimchi[9,10] (from cabbage), olives, cucumbers, carrots, celery, beans, peas, corn, okra, tomatoes, cauliflower, peppers, onions, citron, beets, turnips, radishes, chard, Brussels sprouts, and their blends. Figure 15.6 shows assorted

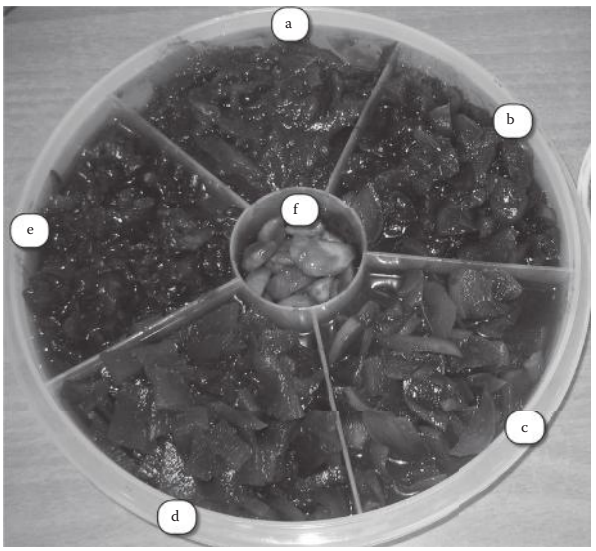



**Figure 15.6 (See color insert.) Traditional fermented vegetable products of South Korea. (a) Codonopsis lacneolata roots, (b) Japanese apricot, (c) Persimmon, (d) Daikon radish, (e) Cucumber, and (f) Garlic.**

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traditional fermented vegetable products from South Korea. Most are produced by natural fermentation; however, some, such as cucumbers, are currently produced in limited amounts by controlled fermentation. Production of sauerkraut by natural fermentation is described here as an example.

## Microbiology of Sauerkraut[3]

### Characteristics

Sauerkraut is produced by fermenting shredded cabbage. The product has a sour taste with a clean acid flavor.

### Processing

1. Cabbage cleaned, trimmed, and shredded finely and uniformly.
2. Packaged tight to exclude air in vat and layered with salt (2.25%).
3. Top covered to exclude air, fermented at 18°C (65°F) for 2 months.

Fine shredding helps the sugars (3%–6%) come out of the cabbage cells. Tight packaging helps create an anaerobic condition, thus preventing the growth of aerobes. Salt stimulates growth of some lactic acid bacteria and discourages the growth of some undesirable bacteria and pectinase (in cabbage) action. The top is covered to exclude air and prevent growth of some aerobes. Fermentation at 18°C (65°F) discourages the rapid growth of some undesirable bacteria (facultative anaerobic or anaerobic), but it encourages the growth of desirable lactic acid bacteria. Natural inhibitors in cabbage also discourage the growth of undesirable Gram-negative and Gram-positive bacteria.

### Starters (Natural) and Growth

The raw material has a large number of undesirable organisms and a small population of lactic acid bacteria (<1%). Among the lactic acid bacteria, most are *Lactococcus* spp. and *Leuconostoc* spp., and a small fraction is *Lactobacillus* spp. and *Pediococcus* spp. During fermentation, sequential growth of these lactic acid bacteria occurs. The presence of 2.25% salt, large amounts of fermentable sugars (sucrose, hexoses, pentoses), an absence of oxygen, and a low fermentation temperature facilitate *Leuconostoc* spp., primarily *Leu. mesenteroides*, to grow rapidly. When the acidity has reached approximately 1% (as lactic acid), growth of *Leu. mesenteroides* slows down. Then *Lab. brevis* starts growing rapidly until acid production reaches approximately 1.5%. Then *Ped. pentosaceus* takes over and increases the acidity to approximately 1.8%. Finally, *Lab. plantarum* starts growing and brings the acid level to approximately 2%.

### Biochemistry

*Leuconostoc* spp. metabolize sucrose, hexoses, and some pentoses in the raw material to lactate, acetate, ethanol, $CO_2$, and diacetyl. *Lab. brevis* (obligatory heterofermentative, such as *Leuconostoc* spp.) ferments sucrose, hexoses, and pentoses to products similar to those by *Leuconostoc* spp. *Ped. pentosaceus* metabolizes hexoses to form mainly lactic acid and some pentoses to lactic acid, acetate, and ethanol. *Lab. plantarum* also produces products from sucrose, hexoses, and pentoses similar to those by *Ped. pentosaceus*. *Leuconostoc* spp. produces D(–)-lactate, whereas the other three species produce DL-lactate.

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The characteristic flavor of sauerkraut is the result of the combined effects of lactate, acetate, ethanol, $CO_2$, and diacetyl in proper amounts.

### *Genetics*

If starters are developed in the future for controlled fermentation, some of the characteristics that will be important are rapid acid production, good flavor production, low $CO_2$ production (to reduce any gassy defect), and the ability to produce antimicrobial compounds, especially against pathogens.

### *Microbial Problems*

Off-flavor, a soft texture, and discoloration of sauerkraut can occur because of the growth of molds and yeasts when air is not completely excluded. A slimy texture of sauerkraut can occur as a result of the overgrowth of *Leuconostoc* spp. in the presence of sucrose; they metabolize fructose but synthesize dextrans from glucose (also see Chapter 20).

## Conclusion

Food fermentation involves the use of specific starter cultures in different types of foods as starting materials. The starting materials can be milk, meat, fish, fruits, vegetables, grains, seeds, and others. They are used separately or in combination. The current trend is to use controlled fermentation in place of natural fermentation. Improvement of strains has helped overcome some of the drawbacks of controlled fermentation. Information on genomes will help improve strains in the future.

**QUESTIONS**

1. Define and discuss the advantages of different methods used to produce fermented foods.
2. Briefly discuss the factors to be considered while selecting milk to produce fermented dairy products.
3. List the characteristics of buttermilk and describe how they help select specific starter cultures.
4. Describe the symbiotic growth of starter cultures used in the production of yogurt. What genetic improvement will be important in these strains?
5. Most commercial yogurt now contains *Lab. acidophilus* and *Bifidobacterium* with regular yogurt bacteria. However, they are added to yogurt after fermentation. Briefly discuss the reasons.
6. In creamed cottage cheese, cream ripened with diacetyl-producing lactic starter is often used instead of using this starter during fermentation. Discuss the reasons.
7. Discuss the biochemical basis of the typical flavor of Cheddar cheese. What is the basis of bitter flavor formation? How can it be reduced by genetic improvement of the starters?
8. Discuss the role of starter cultures in the development of desired characteristics in Swiss cheese.
9. Describe the role of molds in blue cheese production. What precautions should be taken in selecting the mold strains?
10. Discuss the methods used to accelerate cheese ripening.

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11. List the primary and secondary starter cultures used in controlled fermentation of semidry sausages and discuss their specific roles.
12. Describe the sequential growth of lactic acid bacteria during the natural fermentation of sauerkraut.

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# Chapter 16

# Intestinal Bacteria and Probiotics

## Introduction

Since the discovery of food fermentation, our ancestors recognized that the process yielded products that had not only better shelf life and desirable qualities but also some health benefits, especially to combat some intestinal ailments. The belief in the health benefits of fermented foods continued throughout civilization and, even today, remains of interest among many consumers and researchers.[1] There are differences between early beliefs and the current interest: Whereas the early beliefs probably emerged from associated effects (getting a benefit from consuming) without knowing the scientific basis, current interest is based on understanding the microbiology and biochemistry of fermented foods, the microbial ecology of the human gastrointestinal (GI) tract, and the roles of some bacteria in the GI tract and in fermented foods in human health. Researchers, however, are divided in their opinions. Some advocate the different health benefits that consumers can have from consuming fermented foods, especially fermented dairy products, but others doubt those attributes, especially when "cure-all" claims are made by overzealous individuals with no or very little scientific background in the area.

In Western countries, the beneficial role of fermented milk (yogurt) in the prolongation of life was first advocated by Metchnikoff in 1907. He suggested that the bacteria and their metabolites in yogurt neutralized the harmful products yielded from foods in the GI tract and provided protection to human health. However, later studies produced controversial results.

In recent years, especially since the 1970s, consumers in many developed countries have been particularly interested in the health benefits of foods. There is an increased interest in foods that are not harshly processed and preserved, but are natural, and fermented foods are considered natural and healthy. Consumers' interest in and demand for fermented foods have resulted in a large production increase of many products that had very small markets before. Many ethnic products have earned commercial status. In addition, consumers' interest in the health benefits of some bacterial species has stimulated production of new products containing live cells of these bacteria. These products are generally designated as probiotics. The term has been used loosely for a while,



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but is currently defined as a product containing living microorganisms, which on ingestion in certain numbers exert health benefits beyond inherent general nutrition.[1]

This trend will continue not only in the developed countries but also in many developing countries, particularly where fermented foods have been consumed for a long period of time. However, it will be extremely important to resolve the present controversies on the health benefits of probiotics, which can only be possible by understanding the current problems, conducting well-designed experiments, and analyzing the results scientifically. In this chapter, the current status of our knowledge on intestinal microbial ecology, the beneficial microorganisms of the GI tract, the beneficial effects of fermented milk products, and the probable causes of some controversies are briefly presented.

## Microbiology of the Human GI Tract[2]

The GI tract of humans contains more than $10^{14}$ microorganisms, many more than the total number of our body cells, and they coexist as mutualists (Figure 16.1). They are metabolically diverse and active; thus it is quite likely that they have a great influence on our well being. The human GI tract harbors three dominant phyla, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, and approximately 1000 microbial species, but only 30–40 species constitute 95% of the population.[1,2] Normally microbial cells in the stomach are $<10^2$ cfu/g, in the small intestine, duodenum ($10^{1-3}$ cfu/g), jejunum ($10^{3-4}$ cfu/g) and ilium ($10^{7-9}$ cfu/g), and in the large intestine (cecum, colon, rectum) approximately $10^{10-12}$/g of the content. Aerobic microbes are present in the upper part of the GI tract while anaerobic bacteria are present in the lower part of the GI tract. The most predominant types in the small intestine are several species of *Lactobacillus* and *Enterococcus*, and in the large intestine, they are several genera of Enterobacteriaceae, different species of *Bacteroides*, *Fusobacterium*, *Clostridium*, *Eubacterium*, *Enterococcus*, *Peptostreptococcus, Ruminococcus, Propionibacterium, Bifidobacterium*, and *Lactobacillus*.

The intestine of a fetus in the uterus is sterile. At birth, it is inoculated with vaginal and fecal flora from the mother. Subsequently, a large variety of microorganisms enter in the digestive tract

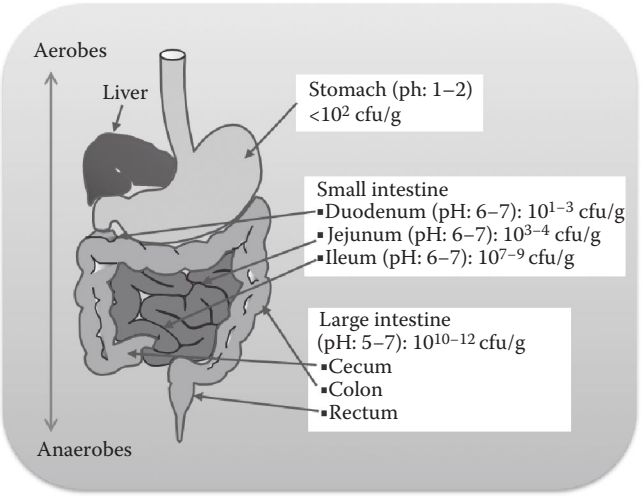



**Figure 16.1 Microbial counts in different parts of the gastrointestinal tract.**

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of infants from the environment. From these, the normal flora of the GI tract are established. In both breast-fed and formula-fed babies during the first couple of days, *Escherichia coli* and *Enterococcus* appear in large numbers in the feces. Then, in the breast-fed babies, large numbers of *Bifidobacterium* species and a lower level of both *Esc. coli* and *Enterococcus* species appear. In formula-fed babies, in contrast, *Esc. coli* and *Enterococcus*, together with *Clostridium* and *Bacteroides*, predominate, with *Bifidobacterium* being almost absent; this situation may lead to diarrhea. As breast-fed babies are introduced to other foods, the levels of *Esc. coli*, *Enterococcus*, *Bacteroides*, *Clostridium*, and others increase, but *Bifidobacterium* still remains high. When breast-feeding is completely stopped, *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* species predominate, along with some *Esc. coli*, *Enterococcus*, *Clostridium*, and others. By the second year of life, the different microflora establish themselves at their specific ecological niche in the GI tract, and the population resembles that of adult GI tracts.

The intestinal microflora are divided into indigenous (autochthonous) and transient (allochthonous) types. Many indigenous species can adhere to intestinal cells, which helps maintain them in their specific niche. Whereas the indigenous types are permanent inhabitants, the transient types are either passing through or temporarily colonizing a site from where the specific indigenous type has been removed because of some inherent or environmental factors (such as antibiotic intake).

Among the indigenous microbial flora, several species of *Lactobacillus* in the jejunum and ilium, and *Bifidobacterium* in the large intestine are thought to have beneficial effects on the health of the GI tracts of the hosts. From the intestines and intestinal content of humans, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus rhamonosus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Lactobacillus plantarum*, *Bifidobacterium bifidus*, *Bif. longum*, *Bif. adolescentis*, *Bif. infantis*, and others have been isolated. However, age, food habits, and health conditions greatly influence the species and their levels. There is some belief that a portion of the intestinal *Lactobacillus* species is transient. Initially, it was considered that *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and some *Bifidobacterium* are the main indigenous species. At present, several other *Lactobacillus* species, such as *Lactobacillus casei* and *Lactobacillus rhamnosus*, have been tested to be beneficial. The presence of high numbers of the indigenous *Lactobacillus* species in the feces (and content of the large intestine) probably results from their constant removal from the small intestine.

## Gut Microbiota, Health, and Chronic Diseases

In recent years, enormous interest has been generated in understanding the role of the intestinal microbial community in brain development, psychological well being, infectious diseases, inflammatory bowel diseases, and chronic metabolic disorders, such as obesity and diabetes.[2] The human microbiome project conducted by the National Institute of Health[3] revealed that microbial communities from different parts of the body (skin, gut, and vagina) differ greatly among healthy individuals. Furthermore, the variation may also be attributed to diet, host genetics, race and ethnicity, and environment. Metagenomic data, however, pointed to the stability in microbial metabolic pathways despite variation in microbial community structure.[3] A diverse colonization of the microbial community in the gut early in life promotes a balanced immune response and prevents allergic diseases, obesity, and diabetes.[4] In obese hosts, the microbial community consists of a high ratio of Firmicutes to Bacteriodetes,[5] and this dominant phylum is responsible for enhanced hydrolysis of complex carbohydrates to produce energy and increased nutrient adsorption. Gut microbiota are also responsible for insulin resistance, increased adsorption of triglycerides, and

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chronic low-grade inflammation and show a propensity for development of diabetes.[6] Long-term feeding with a specific diet can also alter the microbial community structure. Diets consisting of high fat and low fiber tend to increase *Bacteriodes* while high fiber and low fat diets enhance the *Prevetella* population.[7] Scientists believe maintaining a healthy community of microbes in the gut is essential to prevent obesity and chronic metabolic disorders, such as diabetes and microbial diseases.

## Important Characteristics of Beneficial Bacteria

Some relevant characteristics of *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *Bifidobacterium* species are briefly discussed here. All three are found in the GI tract of humans as well as in animals and birds. They are Gram-positive rods and grow under anaerobic conditions. *Lab. acidophilus* is an obligatory homolactic fermentator; *Lab. reuteri* is a heterolactic fermentator and produces lactic acid, ethanol, and $CO_2$; and *Bifidobacterium* species produce lactic and acetic acids (in a 2:3 ratio). They are less sensitive to stomach acid than many other bacteria under a given condition and highly resistant to bile, lysozyme, and pancreatic enzymes present in the GI tract (small intestine). The two *Lactobacillus* species are present in low numbers in the jejunum but in relatively high numbers in the ilium, especially toward the distal part, whereas *Bifidobacterium* species are present in the proximal part of the colon (near the ilium). All three are able to colonize in their respective niches, but studies with *Lab. acidophilus* reveal that all strains do not adhere to the GI mucosa of the host. Other studies have shown that the ability to adhere to the intestinal epithelial cells by *Lab. acidophilus* strains can be species specific; that is, a specific strain can adhere to a particular species only, and the trait may be lost during prolonged culturing under laboratory conditions.

Under normal conditions, these three species are thought to help in maintaining the ecological balance of GI tract microflora by controlling the growth rate of undesirable microflora. This effect is produced through their ability to metabolize relatively large amounts of lactic and acetic acids. In addition, they can produce specific inhibitory substances, several of which are recognized. Many strains of *Lab. acidophilus* are known to produce bacteriocins although they are most effective against closely related Gram-positive bacteria. Also, because of the sensitivity of bacteriocins to proteolytic enzymes of the GI tract, their actual role in controlling undesirable Gram-positive bacteria in the GI tract is disputable. Some strains also produce compounds that are not well characterized but have been reported to have an antibacterial effect on both Gram-positive and Gram-negative bacteria. Some *Lactobacillus reuteri* strains, while growing in glycerol, produce reuterine, which is inhibitory to both Gram-positive and Gram-negative bacteria. Antibacterial metabolite production by a few *Bifidobacterium* species is also reported. Some strains of *Lactobacillus acidophilus* are able to deconjugate bile acids to produce compounds that are more inhibitory than the normal bile acids. Some *Lactobacillus* strains can also produce $H_2O_2$ but probably not under anaerobic conditions in the GI tract. Identification of specific antibacterial compounds other than acids (such as reuterine) in these beneficial bacteria will help in understanding their role in maintaining intestinal health.

Several studies have indicated that beneficial effects of these bacteria are produced when they are present in relatively high numbers in the intestinal tract ($\geq 10^{6-7}$/g intestinal content). Diets rich in foods from plant sources, as opposed to those rich in foods from animal sources, seem to favor their presence in higher numbers. Many other conditions in a host also can reduce bacterial numbers in the GI tract, such as antibiotic intake, mental stress, starvation, improper dietary habits, alcohol abuse, and sickness and surgery of the GI tract. These, in turn, can allow the undesirable

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indigenous or transient bacteria to grow to high levels and produce enteric disturbances, including diarrhea, flatulence, and infection by enteric pathogens.

## Beneficial Effects of Probiotics

The word "probiotic" is derived from the Greek word meaning "for life." Probiotics are defined as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host. In the last 40 years, studies have been conducted to determine specific health benefits from the consumption of live cells of beneficial bacteria. Live cells have been consumed from three principal sources: (1) as fermented milk products, such as yogurt, which contains live cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* and is supplemented with *Lab. acidophilus* and others, and pasteurized milk, which contains *Lactobacillus acidophilus*; (2) as supplementation of foods and drinks with live cells of one, two, or more types of probiotics, such as *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus casei*, and *Bifidobacterium* species; and (3) as pharmaceutical products of live cells of a monoculture or a mixture in the form of tablets, capsules, granules, and freeze-dried sachets. For example VSL #3 is a commercial product, and it consists of four species of *Lactobacillus* (*casei*, *plantarum*, *acidophilus*, and *delbrueckii* subsp. *bulgaricus*), three species of *Bifidobacterium* (*longum*, *breve*, and *infantis*), and one strain of *Streptococcus salivarius* subsp. *thermophilus*. Probiotic preparations consisting of multiple species or strains are functionally superior because of their synergistic effects, and if one culture fails, others can compensate, which is not possible for probiotics of a monoculture. The beneficial effects from consuming these live cells were attributed to their ability to provide protection against enteric pathogens, supply enzymes to help metabolize some food nutrients (such as lactase to hydrolyze lactose), detoxify some harmful food components and metabolites in the intestine, stimulate intestinal immune systems, improve intestinal peristaltic activity, reduce tumerogenesis (colorectal cancer), and ameliorate chronic disorders, such as ulcerative colitis (UC) and inflammatory bowel disease (IBD). Some of the benefits are briefly discussed here (Figure 16.2). In addition, many other benefits have been claimed by entrepreneurs without any scientific basis, and they are not discussed.

### *Lactose Hydrolysis*

Lactose-intolerant individuals, because of a genetic disorder, are unable to produce lactase (β-galactosidase) in the small intestine. When these people consume milk, lactose molecules are not hydrolyzed in or absorbed from the small intestine but are passed to the colon. They are then hydrolyzed in the colon by lactase of different bacteria to glucose and galactose and then further metabolized to produce acids and gas, resulting in fluid accumulation, diarrhea, and flatulence. Consumption of yogurt, acidophilus milk, and live cells of *Lactobacillus*, especially *Lactobacillus acidophilus* in fresh milk and pharmaceutical products, reduces the symptoms in lactose-intolerant individuals. This benefit is attributed to the ability of beneficial bacteria to supply the needed lactase in the small intestine. However, as *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* do not survive stomach acidity well and are not normal intestinal bacteria, the benefit of consuming normal yogurt is considered to be a result of the reduced amounts of lactose in yogurt as compared to milk and to the supply of lactase from the dead cells. In contrast, the intestinal bacteria, especially some *Lactobacillus* species, could, under proper conditions, colonize the small intestine and subsequently supply lactase. Different studies, however, did not unequivocally prove

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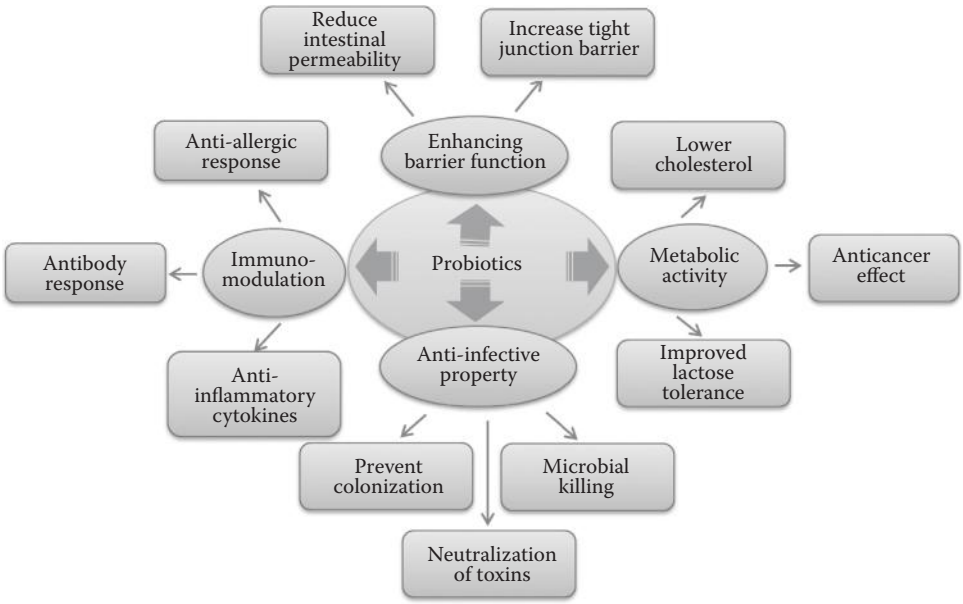



**Figure 16.2 Diagram summarizing probiotic function.**

the desired benefits, probably because of differences in study methods, including the use of strains that lack β-galactosidase, use of strains that are not host-specific, use of species that are not of the intestinal type or unable to adhere to the intestine, use of preparations with low viable cells, and lack of expertise in microbiology and gastroenterology research.

## *Reducing Serum Cholesterol Levels*

Consumption of fermented dairy products (some containing unknown microorganisms) and high numbers of live cells of beneficial intestinal bacteria has been associated with low levels of serum cholesterol in humans. This is attributed to two possible factors. One is the ability of some intestinal lactobacilli to metabolize dietary cholesterol, thereby reducing the amounts absorbed in blood. The other possibility is that some lactobacilli can deconjugate bile salts and prevent their reabsorption in the liver. The liver, in turn, uses more serum cholesterol to synthesize bile salts and indirectly helps reduce cholesterol levels in serum. However, results of several studies by different researchers do not always favor this hypothesis. Again, this can be a result of differences in experimental design, such as use of strains that do not metabolize cholesterol or deconjugate bile acid, and other reasons described previously.

## *Reducing Colon Cancer*

Many of the undesirable bacteria in the colon have enzymes that can activate procarcinogens, either present in food or produced through the metabolism of undesirable bacteria, to active carcinogens that, in turn, can cause colon cancer. Beneficial intestinal bacteria, both *Lactobacillus* and *Bifidobacterium* species, by their ability to control the growth of undesirable bacteria in the colon, can reduce the production of these enzymes. Also, beneficial bacteria, by increasing

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intestinal peristaltic activity, aid in regular removal of fecal materials. This, in turn, lowers the concentrations of the enzymes and carcinogens in the colon and reduces the incidence of colon cancer. Several studies have shown that oral consumption of large numbers of live cells of the beneficial bacteria reduces fecal concentrations of enzymes such as β-glucuronidase, azoreductase, and nitroreductase of undesirable colon bacteria. However, the relationship between reduction of these enzymes and reduction in colon cancer from the consumption of beneficial intestinal bacteria has not been studied, and thus contributions of these bacteria in controlling colon cancer are not clearly known. Animal studies have shown that formation of aberrant cysts, considered to be putative precancerous lesions, is reduced by consuming live cells of beneficial GI tract bacteria, especially bifodobacteria. Again, the results are not consistent, which could be a result of the factors listed previously.

## *Prevention and Control of Enteric Infections*

Under certain conditions, as indicated previously, the intestinal population of beneficial bacteria can be reduced. The undesirable bacteria in the intestine and some transient pathogens (such as enteric pathogenic bacteria and viruses) from the environment can then cause enteric disorders, including infection, IBD, and UC. Infection results from the invasion and growth of enteric pathogens in the intestine. Ingestion of large numbers of live cells of beneficial intestinal bacteria over a period of time was reported to reduce these problems. Both infants and adults on oral antibiotic therapy can develop diarrhea because of a loss of desirable bacteria in the intestine and an increase in undesirable pathogenic bacteria and viruses. It was suggested that beneficial bacteria, when consumed in large numbers, establish in the intestine and produce antimicrobial compounds (acids, bacteriocins, reuterine, etc.), which, in turn, control the pathogens. Deconjugation of biles by beneficial species also produces compounds that are more antibacterial than the bile salts; this has also been suggested as a mechanism to control the growth of undesirable enteric bacteria. Probiotic bacteria also increase the specific immunoglobulins, reduce intestinal permeability, and normalize intestinal microflora. In many studies, the results were not always positive. This, again, could be a result of the variation in study methods, as described previously, including the use of strains without the specific trait. Probiotics are reported to control enteric viral and bacterial infections, including rotavirus, Norovirus, *Campylobacter*, *Salmonella*, *Vibrio*, *Escherichia coli* O157:H7 and antibiotic associated *Clostridium difficile*.[8]

## *Modulating Immune Response*

Limited studies have shown that intestinal microorganisms act on intestinal defense barriers and help regulate systemic and local immune response. This is more effective at an early age, during the development of lymphoid tissues in the gut. Normal establishment of GI tract flora at an early age helps develop immunity to oral administration of antigens associated with an inflammatory reaction in the gut. Oral administration of probiotic gut flora also helps overcome some immune responses caused by the undesirable gut microflora. This beneficial effect is possibly produced by changing intestinal permeability, altering gut microbiology, improving the intestinal immunological barrier functions, and alleviating intestinal inflammatory responses. Probiotic bacterial cell wall components, especially the peptidoglycan molecules, are thought to be responsible for immunomodulatory function. Specific immune modulation function of probiotics includes the activation of cellular and humoral immune responses. Cellular immune response includes activation of T helper cells to produce cytokines that, in turn, activate phagocytic cells for clearance of

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pathogenic bacteria from circulation, and activation of macrophages to produce cytokines TNF-α, IL-6, IL-12, and IL-18 to induce immune responses. Humoral response includes increased production of mucosal secretory IgA to prevent attachment of pathogens and antiviral IgG to eliminate viral infection, such as during rotavirus-induced diarrhea.

### *Reducing Allergic Diseases*

Establishment of normal gut flora, which starts after birth and continues up to two years of age, may be important in the development in later life of a counter-regulatory ability against several specific immune responses. The normal flora of the GI tract enters the body through food, water, air, and other environmental sources. Raising infants in an oversanitary environment and feeding semisterile processed foods may interfere with the establishment of normal microflora in the GI tract. This may cause the immune system of infants to develop an inflammatory response to many food antigens. Probiotics containing beneficial gut bacteria can have a suppressive effect to such reaction by stimulating the production of anti-inflammatory cytokines and reducing allergic reactions in sensitive individuals. Many studies suggest that infants with atopic diseases (allergies) have lower counts of lactobacilli, bifidobacteria, and *Bacteriodes*; however, further experimental evidences are needed to establish a strong relationship between gut microbiota and atopic diseases in children.[4]

## Bioengineered Probiotics

The antimicrobial effect of probiotics is generic and redundant, and sometimes the effect is unsatisfactory. Thus probiotics are engineered to express foreign proteins usually from the pathogen of interest to create a competitive environment to exclude the target pathogen from interacting with the host cells.[9,10] The concept started in the last decade when scientists from Stanford University expressed a HIV-1 surface protein receptor (Gp120) on *Lactobacillus jensenii*.[11] This recombinant *Lactobacillus* expressing Gp120 interacted with the host T cell marker CD4 and thus was able to prevent direct viral interaction with the cells in the genital tract of the host. A similar approach was used to develop a toxin receptor mimicry system in a probiotic to combat enteric infections. A toxin receptor, such as an oligosaccharide from a host cell, was expressed in probiotic bacteria and during infection; bacterial toxins bound strongly to the receptor expressed on the probiotic surface thus prevented toxic effects and protected host. This approach was able to control infection caused by enteric pathogens *Escherichia coli*, *Vibrio cholera*, *Clostridium difficile*, and *Clo. perfringens* in animal models. *Helicobacter pylori* infection was also reduced by expressing urease, an adhesion factor in host gastric cells, in probiotic bacteria, creating a competitive environment to exclude *Hel. pylori* colonization. Similarly, expression of *Listeria* adhesion protein on probiotics was able to reduce *Listeria monocytogenes* infection. A probiotic expressing *Esc. coli* fimbrial antigen also provided protection against this pathogen.

## Probiotics as Vaccine Carriers for Infectious Agents

Many probiotics' natural habitat is the intestine; thus they are an ideal delivery vehicle for a vaccine agent to the mucosal surface.[12,13] Probiotics were engineered to deliver varieties of antigens. The first recombinant probiotic oral vaccine was developed by expressing the tetanus toxin antigen in *Lactococcus lactis* to protect the host against tetanus. A *Lactococcus lactis* strain was engineered

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with an invasin protein Internalin A from *Lis. monocytogenes* to deliver immunostimulatory molecules inside the epithelial cells in the GI tract. A recombinant *Lactobacillus lactis* expressing rotavirus spike-protein VP8 induced mucosal IgA and anti-VP8 antibodies and provided 100% protection against a rotavirus challenge. Expression of *Salmonella* flagella in probiotics developed a protective immune response against salmonellosis. Additionally, lactic acid bacteria were used as a vaccine carrier to prevent *Streptococcus pneumoniae*, *Helicobacter pylori*, enterotoxigenic *Esc. coli*, *Bacillus anthracis*, *Bordetella pertussis*, *Borrelia burgdorferi*, HIV, Dengue virus, *Giardia lamblia*, etc.[12] Also, probiotic strains were engineered to express cytokines and other anti-inflammatory molecules to help suppress intestinal inflammation and provide cytoprotection.

## Some Aspects to Consider

The health benefit theory of fermented foods and beneficial intestinal bacteria is controversial. Although an association effect, that is, some benefits from their consumption, cannot be denied, many studies have not been able to prove the benefits without doubts. As suggested previously, the differences in study methods could be one reason for the differences in results. In designing these studies, several aspects have to be recognized (Table 16.1). There are definitely differences in human responses, but there are also differences in bacterial responses. In selecting bacterial strains, the following considerations are important.

### *Strain Variation*

Beneficial strains differ in adherence ability and specificity (Figure 16.3). An adherent strain should probably be favored over a nonadherent strain. Also, strains adherent to humans should be preferred over strains adherent to other species. The selected strains should have a strong adherence

**Table 16.1 Criteria of an Ideal Probiotic**

| |
|---|
| • Accurate taxonomic identification |
| • Normal inhabitant of the targeted species |
| • Nonpathogenic |
| • Generally recognized as safe (GRAS) |
| • Resistant to bile, hydrochloric acid, and pancreatic juice |
| • Ability to survive in both acidic conditions of the stomach and the alkaline conditions of the intestine |
| • Ability to persist in the gut even if it doesn't colonize |
| • Adhesion to epithelium to prevent attachment of pathogens |
| • Immunostimulatory action |
| • Maintain high cell viability and metabolic activity at the target site |
| • Stability of desired characteristics during processing, storage, and delivery |
| • Genetic stability |

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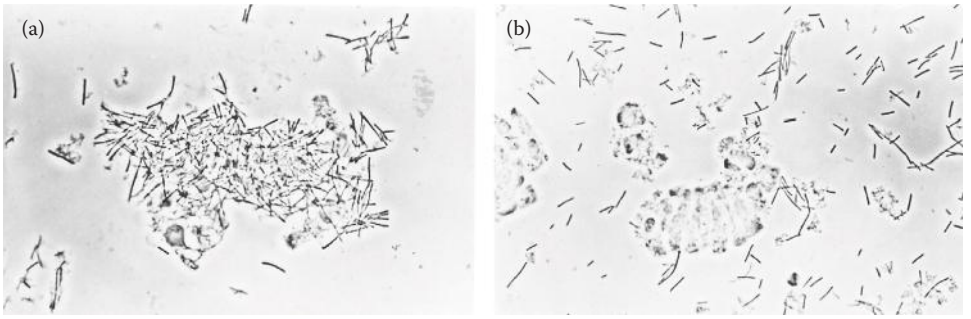



**Figure 16.3 (See color insert.) (a) Cells of adherent strain of *Lab. acidophilus* are associated with calf intestinal epithelial cells. (b) Cells of nonadherent strain of *Lab. acidophilus* remain uniformly distributed and do not show adherence to epithelial cells from the same source.**

property. The adherent property can be lost during long maintenance under laboratory conditions. Many studies have been conducted without even knowing the source and identity of the strains. In selecting a strain for a study, the following factors need to be considered.

## Sensitivity to Stomach Acids, Biles, and Pancreatin

Survivability of strains to acid (low stomach pH), biles, and pancreatin present in the GI tract varies greatly. These effects can be reduced by either reducing stomach acidity with food, by using strains that are proven resistant to an acid environment, or delivered in the protective encapsulated form.

## Viability and Injury of Cells

Cells of beneficial bacteria when frozen, dried, and exposed to low pH, high salts, and many chemicals can die. Among the survivors, many can be injured and killed by stomach acid and bile salts and lysozyme in the intestine. In studies, it may be better to use cells grown for 16–18 hours and maintained before feeding under conditions that retain their maximum viability.

## Dose Level and Duration

Consumption of large numbers of live cells ($10^9$ per day), which are not stressed, over a period of time (ca. 14 days) is advocated to obtain a benefit. Using preparations that have low levels of viable cells, many of which could be stressed, cannot provide expected results. Many products that are currently marketed do not have the needed level of viable cells to produce beneficial effects (Table 16.2). Some products also have bacteria that are associated with improper sanitary practices.

## Induced Lactase Trait

In *Lactobacillus acidophilus*, lactase is an induced enzyme. To study the lactase effect, strains should be grown in lactose-containing media. In commercial preparations, a strain may be grown in glucose and thus not have lactase when consumed.

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**Table 16.2 Viable Cell Counts (CFUs) of *Lab. acidophilus* in Commercial Probiotic Products**

| *Product Type* | *Claimed* | *CFUs/g[a] Actual* | *Beneficial[b]* |
|---|---|---|---|
| **Health Products (Dry)** | | | |
| H1 | Not specified | $1.5 \times 10^3$ c | No |
| H2 | $1 \times 10^8$ | $1.6 \times 10^3$ c | No |
| H3 | $4 \times 10^8$ | $7.3 \times 10^2$ c | No |
| H4 | Not specified | $2.5 \times 10^6$ c | No |
| **Pharmaceutical Products (Dry)** | | | |
| P1 | Not specified | $4.8 \times 10^6$ | Maybe |
| P2 | Not specified | $1.3 \times 10^8$ | Yes |
| P3 | $5 \times 10^9$ | $3.2 \times 10^9$ | Yes |
| P4 | $2 \times 10^7$ | $2.2 \times 10^3$ | No |

[a] Three samples from separate batches for each product type were enumerated for CFUs/mL in MRS-agar supplemented with 0.15% oxgall. Average results are presented.

[b] A strain with a proven record of benefit, needs to be consumed at $10^{8–9}$ viable cells/day for the expected results. On that basis only, consumption of P2 and P3 (provided the strains are beneficial types) can be expected to produce benefits.

[c] The samples had (differed with products) coliform, lactose-negative Gram-negative rods, *Bacillus* spp., and *cocci*. This suggests unsanitary practices in the production of these products.

## *Antibacterial Substances*

Many studies have reported that strains of beneficial intestinal bacteria produce metabolites that are active against many Gram-positive and Gram-negative bacteria. Some of these were identified, such as several bacteriocins, organic acid, and reuterine. Other substances need to be identified and examined in purified form for their antibacterial effectiveness.

## *True Species and Strains*

Many species and strains used by many probiotic food producers do not have either proper identity or information on their original sources. Also, many species that were previously regarded as *Lactobacillus acidophilus* have been found to be different species, and many are not of intestinal origin. Before selecting a strain for a study, one needs to be sure, through testing by recommended methods, that the strain being used is what it is supposed to be.

## *Expertise in Research Areas*

Lack of an understanding in research in the areas of microbiology, gastroenterology, immunology, oncology, and related fields may result in faulty experimental design and interpretation of data.

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In addition, differences in response by humans and animals in feeding trials can produce data of little value. Such studies are not expected to produce reproducible results.

Research conducted by considering these factors will help reduce bacterial variability. This, in turn, will help compare results of different studies and determine whether the health benefits of these bacteria are real or imaginary.

## Current Developments

Diverse types of probiotic products are now available on the market, and their consumption has gained popularity, especially in some European and Southeast Asian countries. Although a long list of health benefits are claimed by manufacturers, most have not been authenticated by well-designed scientific studies. In addition, as mentioned before, many products do not use species and strains that have been studied for the possible beneficial effects, and many products do not have the high viable cell numbers required to produce a benefit (Table 16.2). As a result, doubts about the beneficial effect of these products remain.

### *Standard of Identity*

There has been a concerted effort to develop a scientific basis for probiotic bacteria. Through a joint effort by scientists in this area, a standard of identity has been established for the beneficial probiotic bacteria, especially those from the genus *Lactobacillus*. This includes the ability to (1) adhere to GI epithelial cells, (2) interfere with adherence of enteric pathogens to GI epithelial cells, (3) persist and multiply in the GI tract without disturbing the normal microbial balance, (4) produce antibacterial metabolites, (5) coaggregate, (6) form a normal flora of the GI tract, and (7) be safe to the hosts. Following a critical evaluation of the data of scientific studies, five strains of lactobacilli are considered effective for use as probiotics: (1) *Lactobacillus acidophilus* NCFM, (2) *Lactobacillus casei* Shirota, (3) *Lactobacillus casei* CRL431, (4) *Lactobacillus rhamonosus* GG, and (5) *Lactobacillus reuteri* MM53. In the last few years, several other species or strains of *Lactobacillus* and *Bifidobacterium* have been included in the list (Table 16.3).

### *Microorganisms Used as Probiotics*[14]

Several microorganisms have been studied for their beneficial attributes, and the list continues to grow. However, claims are not supported by valid scientific studies and have created disbelief among consumers. It was suggested that, to overcome the current controversy, future research should be directed toward (1) identifying the phylogeny of a strain, (2) conducting health benefit studies with humans, (3) explaining the exact mechanisms by which a strain produces a benefit, (4) finding the dose level of viable cells and duration required for a benefit, (5) determining the ability of a strain to adhere to GI epithelial cells, (6) studying the influence of a probiotic strain on normal GI tract microflora, and (7) determining the proper and effective delivery systems of a probiotic strain. A list of commonly used probiotics is provided (Table 16.3).

There is no doubt that some bacterial species and strains do have beneficial influence on the health of the GI tract and probably the overall health of humans. But they have to be identified through valid scientific studies. The indiscriminate use, imaginative claims, and unscrupulous methods of a few have created the disbelief. A move for current collaborative scientific studies to define the standards of identity of probiotic strains as well as the suggestions outlined in the status

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**Table 16.3 Probiotics for Human and Animal Use**

| *Lactobacillus* | *Bifidobacteriam* | *Others* |
|---|---|---|
| *Lactobacillus* (LA-5) | *Bif. adolescentis* | *Escherichia coli* Nissle 1917 |
| *Lab. acidophilus* | *Bif. animalis* ssp. *lactis* | *Enterococcus faecalis* |
| *Lab. casei* | *Bif. animalis* | *Enterococcus faecium* |
| *Lab. gasseri* | *Bif. bifidum* | *Lactococcus lactis* |
| *Lab. johnsonii* | *Bif. breve* | *Streptococcus thermophilus* |
| *Lab. helveticus* | *Bif. infantis* | *Propionibacteria* |
| *Lab. paracasei* | *Bif. longum* | *Bacillus cereus* Toyoi spores |
| *Lab. reuteri* | | *Saccharomyces cerevisiae* (Boulardii) |
| *Lab. plantarum* | | |
| *Lab. lactis* | | |
| *Lab. fermentum* | | |

*Source:* Adapted from de Vrese, M. and Schrezenmeir, J., *Adv. Biochem. Eng. Biotechnol.*, *111*, 1–66, 2008.

report regarding the research requirements before claiming a strain to have benefits, are the correct steps to remove the myths surrounding beneficial properties of probiotic bacteria.

## *Safety of Probiotics*

There have been a few reports indicating the involvement of certain strains of probiotics, such as *Bacillus subtilis*, *Lactobacillus rhamonosus*, and *Saccharomyces cerevisiae* (Boulardii) in causing infections (bacteremia, endocarditis, and septic shock) in humans with underlying conditions.[15,16] Lactic acid bacteria, especially those used in food fermentation and as probiotics, are considered food grade and have been given the GRAS (generally regarded as safe) status internationally. In this regard, involvement of these bacteria in health hazards raised questions about their safety. The best answer to this doubt is an aphorism of the 16th-century German scientist Paracelsus: "All substances are poison—the right dose differentiates a poison and a remedy." From ancient civilization on, people have been consuming live cells of many of these bacteria through foods without having major incidence of a health hazard, and they, without being eliminated, remained in our food chain. There are three possible reasons for the infections that result:

1. The true identity of a strain is not known. Many strains currently used, especially as probiotics, have not been correctly identified to the species level (especially by genetic techniques), have not been tested for their beneficial properties to humans, or their sources have not been identified. The products may be contaminated with pathogens because they have been produced under unsanitary conditions.
2. Being overly anxious for the benefit, an individual may consume a product in large volume. If the individual is immunocompromised and has underlying conditions, the large dose can cause opportunistic infection.

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3. Many isolates from infections have not been identified correctly to the genus and species levels by modern genetic techniques. By biochemical fermentation pattern only, it is difficult to identify the genus and species of an isolate in many situations.

The incidence of health hazard from beneficial bacteria, even with all the abuses, is very low. If we consider other beneficial attributes, such as the use of antibiotics in the treatment of diseases, the incidence of health risk is much higher. Considering this, it is justifiable to assume that true food-grade lactic acid bacteria are safe.

Bioengineered probiotics carrying foreign genes also raise several concerns about their safety, most importantly, the consequences of prolonged consumption of probiotics that are carrying adhesion and invasion genes of pathogens. It is also not known whether individuals with suppressed immunity will respond differently than healthy persons. The most important consideration in the use of probiotics should be "do the benefits outweigh the ill effects?" Nevertheless, it is essential to screen probiotics for their safety or long-term health effects prior to their use.[16] Guidelines developed by the FAO (Food and Agriculture Organization) of the World Health Organization (WHO)[17] should be followed: (i) identification of the probiotics at the genus, species, and strain level; (ii) functional characterization using in vitro and/or animal testing; (iii) safety assessment using in vitro cell culture and animal models; (iv) efficacy assessment in a human trial (double blind study); and (v) effectiveness assessment in humans in comparison with a specific treatment under a specific condition. In addition, probiotics should be labeled properly, specifying its genus, species, and strain; bacterial counts at the end of shelf life; storage condition; manufacturer contact information, etc.[8]

## *Probiotics, Prebiotics, and Synbiotics[14]*

### *Probiotics*

The term "probiotics" was initially used with no specific definition. In 1989, Fuller[1] defined the term as products containing living microorganisms that on ingestion (by humans, animals, and birds) in certain numbers exert health benefits beyond inherent general nutrition. In this statement, several requirements were included, such as the microbial cells should be alive and consumed in high numbers (usually $10^9$ cells/day). But it did not include which microorganisms (only intestinal or others) should be used and consumed for how long (daily as a preventative or for two or more weeks as a curative). It indicates that the consumption should produce health benefits as judged by scientific clinical studies by reputed and independent research groups and results published in peer-reviewed journals (not just a claim by somebody). It also does not specify that the mechanisms by which the health benefits are produced should be explained. In the status summary, as discussed before, these aspects have been specifically included.

In recent years, several studies have shown that some specific health benefits can be achieved by also consuming products containing dead cells or cell components of beneficial bacteria (e.g., immune modulation). It has to be decided in the future whether they also constitute probiotics. In order to consider a new microorganism as probiotic or as a new probiotic functional food, the following properties must be known: (1) source or origin, (2) resistance to pH and bile salts, (3) adhesion and colonization, (4) competitive exclusion of pathogens, (5) immune regulation, (6) safety, (7) stability to food processing conditions, (8) sensory assessment, (9) consumer acceptance, and (10) efficacy assessment through human clinical trials.[18]

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### Prebiotics

The beneficial effect of probiotic bacteria depends on their presence in high numbers in the GI tract. This can be achieved either by consuming a large number of viable cells of probiotic bacteria or by stimulating rapid growth of desirable gut bacteria by supplying appropriate nutrients termed as prebiotics.[19] Among the beneficial gut bacteria, *Lactobacillus* species are predominant in the small intestine, whereas *Bifidobacterium* species are predominant in the large intestine (colon). An approach has been taken to stimulate growth of *Bifidobacterium* in the colon by supplying one or more selective carbon and energy sources that are not metabolized by the bacteria in the small intestine as well as by many bacteria found in the colon. This gives *Bifidobacterium* a selective growth advantage and allows it to reach high numbers. Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth or activity, or both, of one or a limited number of bacteria in the colon, which can improve the host's health. Some of the nutrients that have gained importance as prebiotics are lactulose, lactitol, fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, lactosucrose, pyrodextrins, and inulin. Their actual effectiveness is currently being studied.

### Synbiotics

The term "synbiotics" was coined to include both the terms "probiotics" and "prebiotics." It is assumed that instead of using probiotics and prebiotics separately, a product containing both, that is, the beneficial gut bacteria in high numbers as well as nutrient supplements for them will enable them to multiply rapidly in the gut and produce health benefits more effectively. This has to be studied to determine their actual effectiveness.

## Biogenics[20]

The health benefit from the consumption of fermented dairy products is attributed to the lactic acid bacteria used in fermentation as well as the byproducts of the metabolism of milk nutrients by them. The latter aspect becomes important to explain health benefits attributed to fermented products in which non-gut bacteria, such as *Lactococcus lactis*, *Str. thermophilus*, and *Lactobacillus delbrueckii* ssp. *bulgaricus*, are used in fermentation. These products have recently been termed "biogenics" and include components in food that are derived through the microbial metabolic activity of food nutrients and have health benefits. One such group is the peptide produced by the exoproteinases of some lactic acid bacteria, such as *Lac. lactis* in buttermilk and *Lactobacillus delbrueckii* ssp. *bulgaricus* in yogurt. Some of the peptides thus produced and present in the fermented milk can reduce blood pressure in individuals with hypertension. This aspect is currently being studied.

## Genome Sequence of Probiotic Bacteria[21,22]

The bacterial species and strains that are being studied for genome sequence include several *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Pediococcus* species that are currently used as probiotic bacteria. These include *Lactobcillus-acidophilus*, *johnsonii*, *casei*, *gasseri*, and *rhamnosus*; *bulgaricus*, *plantarum*, *helveticus*, *lactis*, *Bif. longum*, *breve*, and *linens*; and *Streptococcus thermophilus*. The complete genome sequences are used to compare the genetic and functional diversities of the GI tract beneficial bacteria, including adhesion traits, resistance to biles, bacteriocin production,

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carbohydrate metabolism, and stress response. This information can then be used to better understand signaling processes among gut microbes and between intestinal cells, food components, microbial metabolic products, and mechanisms of beneficial actions of probiotic bacteria. Finally, they will help design better desirable gut bacteria or specific strains for specific beneficial effects.

## Conclusion

Although fermented foods and some lactic acid bacteria have been considered to help to normalize intestinal microbial ecosystems, there are doubts resulting from differences in results. These are a result of improper uses of bacteria that have not been tested by well-designed studies by well-experienced research groups. Currently, standards of identity of beneficial intestinal bacteria are being advocated to change the myths to reality. In the future, genome sequence information will help better understand the interactions of food, intestinal systems, and microbial species and strains and use them effectively.

### QUESTIONS

1. Discuss the sequential establishment of GI flora in humans from birth to two years of age. How does the predominant flora differ between breast-fed and formula-fed babies?
2. Discuss the following terms with examples: indigenous bacteria, transient bacteria, beneficial bacteria, and undesirable bacteria in the GI tract of humans.
3. List the important characteristics of beneficial bacteria present in the GI tract. Where are they present? What are some of the antibacterial substances they produce?
4. List the factors that could adversely affect the presence of beneficial bacteria in the human GI tract.
5. What is lactose intolerance? Explain the possible mechanisms by which consumption of some fermented dairy products or live cells of beneficial intestinal bacteria can help overcome this problem.
6. How can beneficial intestinal bacteria or some fermented foods possibly reduce serum cholesterol levels?
7. "Beneficial GI tract bacteria may be effective in reducing colon cancer and enteric diseases." What is the basis of this suggestion?
8. Experimental data do not always support the health benefits from the consumption of fermented foods and live cells of beneficial intestinal bacteria. This can be a result of wide differences in experimental methods used. If you are planning to conduct such a study, what are some of the experimental factors you should consider?
9. Discuss advantages and disadvantages associated with bioengineered probiotics.
10. Describe how lactic acid bacteria are used as a vaccine carrier and what are the potential benefits of such an approach?

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# Chapter 17

# Food Biopreservatives of Microbial Origin, Bacteriocin, and Nanotechnology

## Introduction

Even in the early days of food fermentation, our ancestors recognized that fermented foods not only have delicate and refreshing tastes, but also have a longer shelf life and reduced chances of causing sickness from food-borne diseases. Fermentation helped them preserve some foods in their fermented form longer than the raw materials, which may be a major reason for food fermentation being so popular among early civilizations located in high-temperature zones. The knowledge of safety and stability of fermented foods has been transferred through the centuries and helped us understand their scientific basis.

It is now known that the food-grade bacteria associated with food fermentation can produce several types of metabolites that have antimicrobial properties, some of which are listed in Table 17.1. There could be many more that are currently not identified. At present, there is an increased interest in the use of these antimicrobials in nonfermented foods to increase their stability and safety. Some of them, such as lactate and acetate (vinegar), have been used in many foods for a long time, whereas the others have generated much interest as potential food biopreservatives in place of some currently used nonfood preservatives, such as nitrite, sulfite, parabens, diacetate, and ethylformate. Their effectiveness as food biopreservatives is discussed in this chapter. In addition, currently, some yeasts have been found to inhibit the growth of molds in fruits and vegetables. This aspect is also briefly discussed.

## Viable Cells of Lactic Acid Bacteria (LAB) as Preservatives

The process involves the addition of viable cells of mesophilic *Lactococcus lactis*, some *Lactobacillus* species, and *Pediococcus* species in high numbers to control spoilage and pathogenic bacteria



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**Table 17.1 Antimicrobial Compounds of Food-Grade Bacteria**

| *Metabolites* | *Effectiveness* |
|---|---|
| Organic acids: lactic, acetic, propionic | Against bacteria and fungi |
| Aldehydes, ketones, and alcohols: acetaldehyde, diacetyl, ethanol | Against bacteria |
| Hydrogen peroxide | Against bacteria, fungi, and phages |
| Reuterine | Against bacteria and fungi |
| Bacteriocins | Against Gram-positive bacteria, normally |

during the refrigerated storage of a food at or below 5°C.[1] In the presence of mesophilic lactic acid bacteria, the growth of psychrotrophic spoilage and pathogenic bacteria is reported to be controlled. Growth of some of these spoilage and pathogenic bacteria at slightly higher temperatures (~10°C–12°C) is also reduced. Studies were conducted by adding lactic acid bacteria to fresh meat, seafood, liquid egg, and some processed meat products, such as bacon, against *Clostridium botulinum*, *Salmonella enterica* serovars, and *Staphylococcus aureus*. In refrigerated raw milk, meat, egg, and seafood, cells of *Lactobacillus*, *Lactococcus*, and *Leuconostoc* species were added to control the growth of psychrotrophic spoilage bacteria, such as *Pseudomonas* spp. In some studies, growth of the psychrotrophs was inhibited by 90% or more during 4–10 days of refrigerated storage. Addition of cells of lactic acid bacteria in refrigerated raw milk also increased the yield of cheese and extended the shelf life of cottage cheese.

Lactic acid bacteria are also suitable for inhibiting fungi (molds) that are responsible for food spoilage and mycotoxin production. Mycotoxins, such as aflatoxins, fumonosins, ochratoxin, patulin, tricothecenes, and zearalenone, are produced as secondary metabolites on varieties of foods and are carcinogenic, teratogenic, immunotoxic, neurotoxic, hepatotoxic, and nephrotoxic. Hence control of these microbes using lactic acid bacteria is considered a natural "green preservative".[2] Enzymes produced during the growth of molds destroy food architecture, leading to food spoilage, and render food unacceptable for consumption. Several species of *Lactobacillus* (*amylovorus*, *casei*, *delbrueckii*, *paracasei*, *plantarum*, *rhamnosus*, and *sanfranciscencis*) and *Pediococcus acidilactici* have been recommended for use on bread, milk, corn, cottage cheese, pepperoni, salami, yogurt, and silage.[2]

The inhibitory property of LAB is attributed to the release of intracellular antimicrobial compounds, such as organic acids, carboxylic acids, fatty acids, ethanol, carbon dioxide, bacteriocins, and hydrogen peroxide, from the cells by the nonmetabolizing lactic acid bacteria. The antibacterial role of hydrogen peroxide with the lactoperoxidase-thiocyanate system in raw milk is discussed later.

## Organic Acids, Diacetyl, Hydrogen Peroxide, and Reuterine as Food Preservatives

### *Organic Acids*

In Chapter 12, the ability of starter-culture bacteria to produce lactic, acetic, and propionic acids was discussed. Commercially, lactic acid is produced by some *Lactobacillus* spp. capable of producing L(+)-lactate (or DL-lactate), acetic acid by *Acetobacter aceti*, and propionic acid by dairy

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*Propionibacterium* spp. They are on the generally regarded as safe (GRAS) list and are used in many foods as additives to enhance flavor and shelf life and as safety precautions against undesirable microorganisms. These acids and their salts are used in foods at approximately a 1%–2% level[3,4] (also see Chapter 36).

Acetic acid, its salts, and vinegar (which contains 5%–40% acetic acid and many other compounds that give it the characteristic aroma) are used in different foods for inhibiting growth and reducing the viability of Gram-positive and Gram-negative bacteria, yeasts, and molds. Acetic acid is generally bacteriostatic at 0.2% but bactericidal above 0.3% and more effective against Gram-negative bacteria. However, this effect is pH dependent, and the bactericidal effect is more pronounced at low pH (below pH 4.5). It is added to salad dressings and mayonnaise as an antimicrobial agent. It is permitted to be used as a carcass wash.

Propionic acid and its salts are used in food as a fungistatic agent, but they are also effective in controlling growth and reducing viability of both Gram-positive and Gram-negative bacteria. Gram-negative bacteria seem to be more sensitive at pH 5.0 and below, even at acid levels of 0.1%–0.2%. Propionic acid is used to control molds in cheeses, butter, and bakery products and to prevent growth of bacteria and yeasts in syrup, applesauce, and some fresh fruits.

Lactic acid and its salts are used in food more for flavor enhancement than for their antibacterial effect, especially when used above pH 5.0. However, recent studies have shown that they have a definite antibacterial effect when used in foods at 1%–2% levels even at or above pH 5.0. Growth of both Gram-positive and Gram-negative bacteria is reduced, indicating increased bacteriostatic action. Below pH 5.0, lactic acid can have a bactericidal effect, especially against Gram-negative bacteria. It may not have any fungistatic effect in the food environment. It is used in many processed meat products and has also been recommended as a carcass wash.

The antimicrobial effect of these three acids is considered to be a result of their undissociated molecules. The dissociation constants (pKa) are 4.8 for acetic, 4.9 for propionic, and 3.8 for lactic acid. Thus, at most food pH (5.0 and above), the undissociated fractions of the three acids can be quite low, the lowest being for lactic acid. The lower antimicrobial effectiveness of lactic acid is probably a result of its low pKa. The antimicrobial action of the undissociated molecules is produced by dissociation of the molecules in the cytoplasm following their entry through the membrane. $H^+$ released following dissociation initially reduces the transmembrane proton gradient and neutralizes the proton motive force and then reduces the internal pH, causing denaturation of proteins and viability loss. However, other studies have suggested that these weak acids produce antimicrobial action through the combined effects of undissociated molecules and dissociated ions. The acids can also induce sublethal injury of the cells and increase their chance of viability loss. Undissociated molecules as well as dissociated ions can induce cellular injury.[3,4]

Several pathogenic bacterial strains, such as some strains of *Salmonella* Typhimurium, and *Escherichia coli* O157:H7, have been found to be relatively resistant to low pH because of their ability to overproduce some proteins induced by the acid environment. These proteins, also called stress proteins, enable the cells to withstand lower internal pH (see Chapter 10). The presence of such a strain in food, which is freshly acidified at a 1%–2% level to control microorganisms, may pose a problem.

## *Diacetyl*

Diacetyl is produced by several species of lactic acid bacteria in large amounts, particularly through the metabolism of citrate (see Chapter 12).[5] Several studies have shown that it is antibacterial against many Gram-positive and Gram-negative bacteria. Gram-negative bacteria are particularly sensitive at pH 5.0 or below. Diacetyl is effective at approximately 0.1%–0.25%. Recent studies

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have shown that in combination with heat, diacetyl is more bactericidal than when used alone. Diacetyl has an intense aroma, and thus its use is probably limited to some dairy-based products in which its flavor is not unexpected. Also, it is quite volatile and thus may lose its effectiveness in foods that are expected to have a long storage life. Under reduced conditions, it is converted to acetoin, which may have reduced antibacterial effects. This will pose difficulties in its use in vacuum-packaged products. The antibacterial action is probably produced by deactivating some important enzymes. The dicarbonyl group (–CO–CO–) of diacetyl reacts with arginine in the enzymes and modifies their catalytic sites.[5]

## Hydrogen Peroxide

Some lactic acid bacteria produce $H_2O_2$ under aerobic conditions of growth, and, because of the lack of intracellular catalase, pseudocatalase, or peroxidase, they release it into the environment to protect themselves from its antimicrobial action. Some strains can produce, under proper growth conditions, enough $H_2O_2$ to induce bacteriostatic (6–8 μg/mL) but rarely bactericidal action (30–40 μg/mL).[6] It is a strong oxidizing agent and can be antimicrobial against bacteria, fungi, and viruses (also bacteriophages). Under anaerobic conditions, very little $H_2O_2$ is expected to be produced by these strains. In refrigerated raw milk, the antibacterial action produced by adding nongrowing cells of mesophilic lactic acid bacteria is thought to be a result of the ability of $H_2O_2$ of bacteria to activate the lactoperoxidase-thiocyanate system in raw milk.[7] Raw milk contains lactoperoxidase enzymes and thiocyanate ($SCN^-$). In the presence of $H_2O_2$, lactoperoxidase generates a hypothiocyanate anion ($OSCN^-$), which, at milk pH, can be in equilibrium with hypothiocyanous acid (HOSCN). Both $OSCN^-$ and HOSCN are strong oxidizing agents and can oxidize the –SH group of proteins, such as membrane proteins of Gram-negative bacteria that are especially susceptible. In addition, this system has inhibitory activity against other bacteria, fungi, and viruses. It is inactivated by pasteurization.

Hydrogen peroxide is permitted in refrigerated raw milk and raw liquid eggs (ca. 25 ppm) to control spoilage and pathogenic bacteria. Before pasteurization of these foods, catalase (0.1–0.5 g/1000 lb [455 kg]) is added to remove the residual $H_2O_2$. Its antibacterial action is attributed to its strong oxidizing property and its ability to damage cellular components, especially the membrane. Because of its oxidizing property, it can produce undesirable effects in food quality, such as discoloration in processed meat, and thus has limited use in food preservation. However, its application in some food processing and equipment sanitation is being studied.

## Reuterine

Some strains of *Lactobacillus reuteri*, found in the gastrointestinal tract of humans and animals, produce a small molecule, reuterine (β-hydroxypropionaldehyde; $CHO–CH_2–CH_2OH$), which is inhibitory to many Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*) and Gram-negative (*Salmonella enterica* serovar Choleraesuis, *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Campylobacter jejuni*) bacteria.[6,8] It produces an antibacterial action by inactivating some important enzymes, such as ribonucleotide reductase. Reuterin is active over a wide range of pH and resistant to a variety of proteolytic and lipolytic enzymes present in foods. However, reuterine is produced by the strains only when glycerol is supplied in the environment, which limits its use in food preservation. In limited studies, food supplemented with glycerol and inoculated with reuterine-producing *Lab. reuteri* effectively controlled growth of undesirable bacteria. Addition of reuterine to certain foods also effectively controlled growth of undesirable bacteria.[6]

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## Bacteriocins of Lactic Acid Bacteria as Food Preservatives

The term *bacteriocin* is currently used to refer to a group of bioactive peptides produced by many bacterial strains from Gram-positive and Gram-negative groups. The bacteriocins produced by many strains of lactic acid bacteria and some propionic acid bacteria are of special interest in food microbiology because of their bactericidal effect, normally, to different Gram-positive spoilage and pathogenic bacteria and, under stressed conditions, to different Gram-negative bacteria important in food. Chemically, bacteriocin peptides are ribosomally synthesized, cationic, amphipathic, have α-helical or β-sheet structures, or both, and can have thioethers, disulfide bridges, or free thiol groups. The presence of an amphipathic α-helical structure with opposing polar and nonpolar faces along the long axis enables bacteriocins to interact with both the aqueous and lipid phases when bound to the surface of the membrane of a sensitive bacterial cell, leading to its functional destabilization and death of the cell. This aspect is discussed later.[9]

### *Bacteriocin-Producing Strains*

Many bacteriocin-producing strains from the different genera and species of lactic acid bacteria have been isolated. Some of the species include *Lac. lactis*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lab. plantarum*, *Lab. sake*, *Lab. curvatus*, *Leuconostoc mesenteroides*, *Leu. carnosum*, *Leu. gelidium*, *Pediococcus acidilactici*, *Ped. pentosaceus*, *Ped. parvulus*, *Tetragenococcus halophilus*, *Carnobacterium piscicola*, *Enterococcus faecalis*, *Ent. faecium*, and *Bifidobacterium bifidum*. It appears now that bacteriocin-producing strains of lactic acid bacteria are quite common in the food environment. A proper procedure and experience in differentiating between a bacteriocin producer and a nonproducer (including one that produces other types of antibacterial compounds) are important to successfully isolate a bacteriocin-producing strain from the natural environment as well as to identify a strain already present in culture collections. Some important factors to be considered in the isolation from the natural environment are boosting the number of producer cells over the associative microorganisms (such as incubating in low-pH broth), selecting sensitive strains of indicators (Gram-positive, preferably from the lactic acid bacterial group and more than one strain), using a known producer strain as a control, and recognizing the zone of growth inhibition around a colony on an agar-medium plate produced by a bacteriocin producer and a producer of another antibacterial compound (a circular clear zone for bacteriocins as opposed to a fuzzy zone for organic acids).[9]

Bacteriocins of lactic acid bacteria are bactericidal to sensitive cells, and death occurs very rapidly at a low concentration. Normally, Gram-positive bacterial strains are sensitive, the spectrum or range of which can vary greatly. Although Gram-negative bacterial cells are normally resistant to bacteriocins of lactic acid bacteria, they become sensitive following impairment of the cell surface lipopolysaccharide structure by physical and chemical stresses. Many bacteriocins are bactericidal against a few related species and strains, but several are effective against many strains from different species and genera. Some other general features of bacteriocins of lactic acid bacteria can be grouped as follows:[9–12]

- A producer strain is immune to its own bacteriocin but can be sensitive or resistant to other bacteriocins (e.g., pediocin AcH producing *Ped. acidilactici* is immune to pediocin AcH, sensitive to nisin A, and resistant to sakacin A).
- A strain can produce more than one bacteriocin (e.g., a *Lac. lactis* strain produces *lactococcin* A, B, and M); many strains of the same species can produce the same or different

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bacteriocins (e.g., pediocin AcH is produced by many *Ped. acidilactici* strains, but sakacin A and sakacin P are produced by different strains of *Lab. sake*).

- Strains from different species and genera can produce the same bacteriocins (e.g., pediocin AcH is produced by strains of *Ped. acidilactici*, *Ped. pentosaceus*, *Ped. parvulus*, *Lab. plantarum*, *Bacillus coagulans*).
- Strains from different subspecies can produce different bacteriocins (e.g., different *Lac. lactis* ssp. *lactis* produce nisin A and lacticin 481).
- Different species from a genus can produce different bacteriocins (e.g., enterococcin EFS2 and enterocin 900 are produced by strains of *Ent. faecalis* and *Ent. faecium*, respectively).
- Natural variants of the same bacteriocin can be produced by different strains and species (e.g., nisin A and Z by *Lac. lactis* strains; pediocin AcH and coagulin by *Ped. acidilactici* and *Bac. coagulans*, respectively).
- Many bacteriocins named differently before their amino acid sequences are determined to be the same (e.g., pediocin AcH and pediocin PA of *Ped. acidilactici* strains).[9,10]

## *Characteristics of Bacteriocins*[10–16]

Although isolation of a large number of bacteriocins of lactic acid bacteria has been reported in the literature, for most the amino acid sequences have not been determined. Amino acid sequencing studies have shown that some of the bacteriocins that were initially given different names have the same amino acid sequences. Some examples are pediocin AcH and pediocin PA-1, curvacin A and sakacin A, and sakacin P and bavaricin A. In general, they contain less than 60 amino acids, but their bactericidal efficiency is not related to the number of amino acids in the molecule. They are cationic, and the net positive charge is higher at low pH. Because of their hydrophobic nature, the molecules have a tendency to aggregate, especially when stored in the liquid state and at high concentrations. The bactericidal property is higher at lower pH, relatively stable at high temperature, and not affected by organic solvents. Anions in high concentrations can reduce bactericidal efficiency of some cationic bacteriocins by competitive exclusion. Different proteolytic enzymes can hydrolyze these peptides, leading to a loss of activity. They are fairly stable at frozen and refrigerated storage, but some with methionine can be oxidized to methionine sulfoxide, which reduces the potency (e.g., pediocin AcH). The monomers, especially with disulfide bonds, can form dimers and trimers by bond exchange but retain their bactericidal efficiency.

Bacteriocins of lactic acid bacteria are characterized as ribosomally synthesized peptides as they undergo very little structural change following translation. In general, a molecule, as translated, is designated as prebacteriocin, which contains an N-terminus leader peptide and a C-terminus probacteriocin. The leader peptide is removed during transportation of the molecules from the cytoplasm side to outside through the membrane-bound ABC transporter; the ABC transporter, acting as an endopeptidase, excises the leader peptide. Although many bacteriocins have one peptide chain, some have two peptide chains. The function of the leader peptide is to direct the transport of a molecule through the ABC transporter. Once the bacteriocin molecules are released into the environment, depending on the pH, they either remain bound with the anionic molecules on the cell surface or are released into the environment. Recent studies have shown that some bacteriocins can be transported by the sec-dependent secretory system, whereas a few do not have the leader sequence.

Bacteriocin molecules, based on the molecular structures, are subdivided into several groups. Broadly, they are grouped as Class I, which contains lanthionine rings, and Class II, which lacks

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lanthionine (Table 17.2). Lanthionine rings are formed after translation of prebacteriocin in the cytoplasm and before transportation through the ABC transporter by enzymatic dehydration of L-serine to dehydroalanine and L-threonine to dehydrobutyrine and formation of thioether rings (Table 17.2 and Figure 17.1). These bacteriocins, also designated as lantibiotics, may contain one or more thioether rings. In general, the bactericidal efficiency is higher in lantibiotics containing more thioether rings. Bacteriocins without lanthionine are grouped under Class II, currently containing five subgroups (Table 17.2). The subgroup of cystibiotics has, at present, 14 bacteriocins and has two or four cysteines that form one or two disulfide rings in an oxidized environment (outside cytoplasm; Table 17.2). Cystibiotics with two disulfide rings have greater

**Table 17.2 Classification of Bacteriocins of Lactic Acid Bacteria**

| *Groups* | *Structural Basis* | *Bacteriocin Example* | *Producer Strain* |
|---|---|---|---|
| Class I: (Lanthionine containing) | Five thioether rings | Nisin A | *Lac. lactis* ATCC11454 |
| Lantibiotics | Three thioether rings | Lacticin 481 | *Lac. lactis* CNRZ481 |
| | Two thioether rings | Lactocin S | *Lab. sake* L45 |
| Class II: (no lanthionine) | | | |
| <10 kDa | | | |
| IIa: Cystibiotics (also pediocin-like) | Four cysteines with two disulfide bonds | Pediocin AcH | *Ped. acidilactici* H |
| | Two cysteines with one disulfide bond | Leucocin A | *Leu. gelidum* UAL 187 |
| IIb: Two peptides[a] | Two separate peptides | Plantaricin S | *Lab. plantarum* LCP 010 |
| II: Non-subgroup | Some contain thiol group (one or more) | Carnobacteriocin A | *Car. piscicola* LV17A |
| II: No leader peptide[b] | Translated as probacteriocin | Enterocin L50 | *Ent. faecium* L51 |
| II: Sec-dependent[c] | Leader peptide recognizes sec-secretory system | Enterocin P31 | *Ent. faecium* P13 |
| Class III: Large molecules[d] (>30 kDa) | Heat labile | Helveticin J | *Lab. helveticus* J |

[a] All other bacteriocins have one peptide.
[b] All other bacteriocins have leader peptides.
[c] All other bacteriocins are transported by specific ABC transporters.
[d] All other bacteriocins are heat stable.

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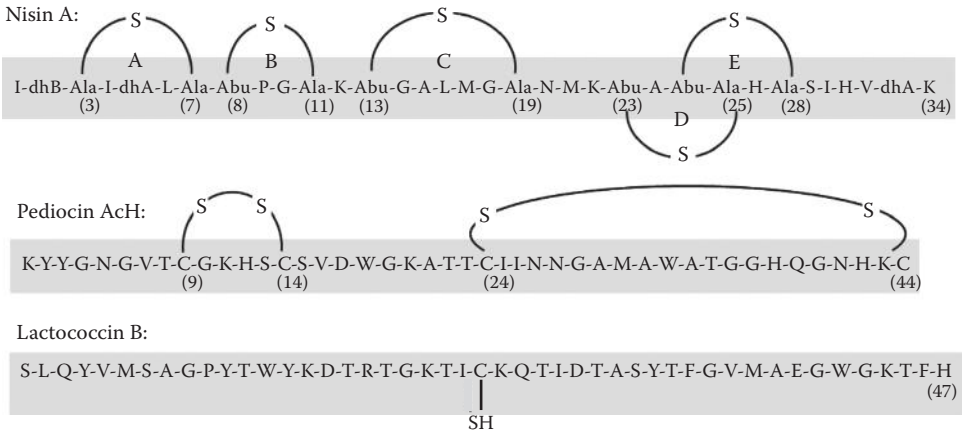



**Figure 17.1 Sequence of three bacteriocins is presented. Sequence of nisin showing thioether rings labeled as A, B, C, D, E, and also disulfide bonds, and thiol groups in other bacteriocins of lactic acid bacteria are shown. Abu, Aminobutyrate, dhA, dehydroalanine; dhB, didehydrobutyrine.**

bactericidal effectiveness than those with one disulfide ring (Figure 17.1). A bacteriocin with two peptides (that have some similarities in amino acid sequence with each other) needs both peptides for full bactericidal activity. However, each peptide has a lower bactericidal effect. The bacteriocins under "nonsubgroup" in Class II do not have any common characteristics; however, a few can have a free thiol group (Figure 17.1). A few bacteriocins, quite different from those described previously, are translated as probacteriocin (only the main part) without any leader peptide at the N-terminus region. Finally, several bacteriocins of lactic acid bacteria are found to be secreted by the Sec-dependent secretory mechanism as opposed to ABC transporter systems of the other bacteriocins.

A comparison of the amino acid sequences of leader peptides revealed very few similarities among both Group I and Group II bacteriocins (Table 17.3). This is more evidenced with Group I bacteriocins. But some Group II bacteriocins have –G–G– at the C-terminus end of the leader peptide (at –1 and –2 positions). The double glycine is the recognition site for the endopeptidase activity of the ABC transporter to remove the leader peptide from the prebacteriocin molecule. Because a leader peptide propels the prebacteriocin molecule toward an ABC transporter in the membrane, it is possible that a specific amino acid sequence of a leader peptide recognizes a specific ABC transporter system for the most efficient transport of the probacteriocin (prebacteriocin without the leader peptide) in the environment; an interchange of leader peptides with other bacteriocins may not function very effectively. The amino acid sequences of probacteriocins (or bacteriocins) of lantibiotics have very few similarities (Table 17.4). In contrast, bacteriocins in the cystibiotic subgroup have several sequence homologies. The most important is the –YGNGV– sequence in the N-terminal halves of the molecules, which is known to have an important role in the bactericidal properties of the molecules. In addition, there are at least two cysteins, usually at positions +9 and +14, that form a disulfide bond and are important for the bactericidal property. In several cystibiotics (e.g., pediocin AcH or PA-1, enterocin A) there are two more cysteine molecules in the C-terminal half of the molecule (at +24 and +44 positions

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**Table 17.3 Amino Acid Sequences of Leader Peptides of Bacteriocins of Lactic Acid Bacteria**

| *Bacteriocin* | *Amino Acid Sequence* |
|---|---|
| Nisin A (23) | MSTKDFNLDLVSVSKKDSGASPR |
| Lacticin 481 (24) | MKEQNSFLLQEVTESELDLILGA |
| Pediocin AcH (18) | MKKIEKLTEKEMANIIGG |
| Leucocin A (24) | MMNMKPTESYEQLDNSALEQVVGG |
| Sakacin P (18) | MEKFIELSLKEVTAITGG |
| Lactococcin A (21) | MKNQLNFNIVSDEELSEANGG |

*Note:* Numbers in parentheses are the number of amino acids. The last amino acid at the carboxyl end (right) is designated as 1. Class II bacteriocins have GG at positions –1 and –2.

**Table 17.4 Amino Acid Sequences of Selected Probacteriocins of Lactic Acid Bacteria**

| *Probacteriocin* | *Amino Acid Sequence* |
|---|---|
| Nisin A (34) | ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK |
| Lacticin 481 (27) | KGGSGVIHTISHECNMNSWQFVFTCCS |
| Pediocin AcH/PA-1 (44) | KY**YGNGV**TCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC |
| Leucocin A (37) | KY**YGNGV**HCTKSGCSVNWGEAFSAGVHRLANGGNGFW |
| Sakacin P (43) | KY**YGNGV**HCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK |
| Lactococcin A (55) | KLTFIQSTAAGDLYYNNTNTHKYVYQQTQNAFGAAANTIVNGWMGGAA GGFGLHH |
| Bifidocin B (37) | KY**YGNGV**TCGLHDDCRVDRGKATCGIINNGGMWGDIG |
| Carnobacteriocin B2 (48) | VN**YGNGV**SCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP |
| Bavaricin MN (42) | TKY**YGNGV**YCNSKKCWVDWGQAAGGIGQTVVXGWLGGAIPGK |
| Plantaricin 423 (37) | KY**YGNGV**TCGKHSCSVNWGQAFSCSVSHLANFGHGKC |

*Note:* Numbers in parentheses are number of amino acids. The first N-terminal amino acid at the left is designated as +1. Y G N G V are indicated in bold letters, and underlined cysteine residues are involved in disulfide bond formation.

in pediocin AcH or PA-1); the presence of an extra disulfide bond at the C-terminus makes the molecules more potent bactericidal agents than those with one disulfide bond at the N-terminus region (e.g., leucocin A). In general, the amino acid sequence of a bacteriocin determines the formation of α-helix and β-sheet domains in the molecule, which are important for its bactericidal efficiency.

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## Genetics and Gene Organization

The limited available information has revealed that the structural genes encoding prebacteriocin molecules of lactic acid bacteria can be encoded in a plasmid (e.g., pediocin AcH or PA-1), in the genome (e.g., sakacin P), or in a genome-integrated transposon (e.g., nisin A) of a producer strain. With plasmid-encoded bacteriocins, several variations are observed: A single plasmid can encode only one bacteriocin (e.g., pediocin AcH or PA-1) or more than one bacteriocin (e.g., lactococcin A, B, and M); the same bacteriocin can be encoded in different-size plasmids in the same species (e.g., lactococcin A in three separate strains of *Lac. lactis*) or different species (e.g., pediocin AcH in *Ped. acidilactici*, *Ped. pentosaceus*, *Ped. purvulus*, and *Lab. plantarum*); or a strain producing more than one bacteriocin that can be encoded in different plasmids (e.g., carnobacteriocin A and carnobacteriocin B1 and B2 are encoded in two plasmids in a *Car. piscicola* strain).

In addition to the structural gene encoding for a bacteriocin, the DNA (plasmid or genome) also encodes several other genes related to its production (Figure 17.2). Some of them are quite simple (e.g., pediocin AcH), whereas others can be very complex (e.g., nisin A). There are many other differences in the organization of the genes associated with bacteriocin production in lactic acid bacteria. The genes could present in a single operon (e.g., in pediocin AcH and nisin A) or in more than one operon (sakacin P), and all genes are arranged in the same direction (e.g., sakacin P) or in opposite directions (e.g., leucocin A). In some, one or more genes can overlap (e.g., nisin A and leucocin A). The gene organization in the simplest form, as observed for pediocin AcH or PA-1 production, consists of an operon, with the promoter located upstream, and four genes, encoding for four proteins: *pap* or *pedA* for prepediocin, *pap* or *pedB* for immunity protein (PapB or PedB confer immunity against pediocin), *pap* or *pedC* for helper protein (PapC or PedC necessary for the efficient transport of pediocin), and *pap* or *pedD* (PapD or PedD for the ABC-transporter protein). Almost similar gene expressions, but with two promoters, are present in the production of leucocin A. In nisin A production, the genetic expression is quite different. Eleven genes, *nisA-BTCIPRKFEG*, are present in the operon with the promoter located upstream and *nisA* encoding for prenisin (Figure 17.2). The functions of the other proteins from the other genes are as follows:

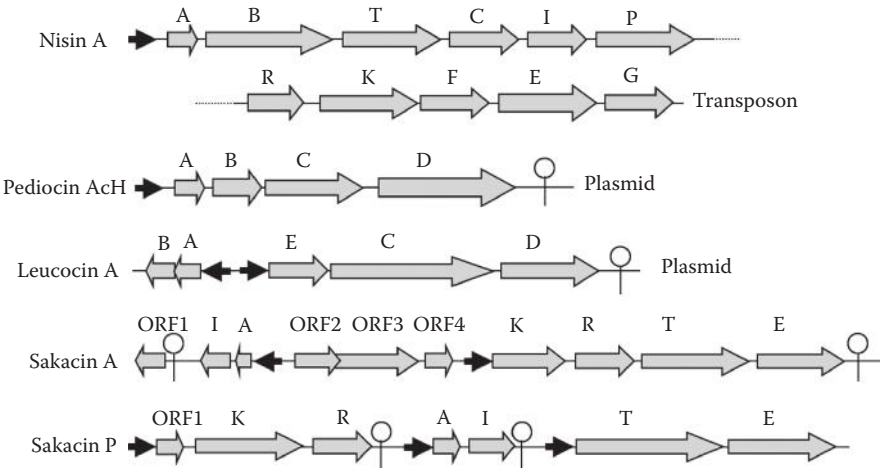



**Figure 17.2 Organization of several well-characterized bacteriocin gene clusters. Structural genes are shown as shaded arrows, promoters as black arrowheads, and rho-independent terminators as lollipop symbols. Genes of unknown functions are designated ORFs.**

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NisB and NisC are involved in the dehydration of serine and threonine to dehydroalanine and dehydrobutyrine and thioether ring formation; NisT is the ABC transporter involved in the transportation of nisin; NisI is responsible for producer cell immunity to nisin; NisP removes the leader peptide from prenisin following its transport by NisT; NisR and NisK are regulatory proteins and modulate transcription of nis-operon; and NisE, NisF, and NisG provide accessory protection to the producer cells against nisin. Similar genes for regulatory proteins are found in other bacteriocin operons, such as sakacin P.

Biosynthesis of a bacteriocin, involving transcription, translation, structural modification, and secretion, occur in a concerted way. In pediocin, AcH or PA-1 and similar bacteriocins, probably a single mRNA, is produced for all four genes, which are then translated into four separate proteins (Figure 17.2). As soon as the 62-amino-acid prepedicin is translated in the cytoplasm, the leader peptide directs transport of the molecule outside the cell by the help of Pap or PedD (ABC transporter) and C (helper). During transportation, the catalytic site of protein D recognizes –G–G– at the –1 and –2 positions of prepediocin and cleaves the leader peptide (18 amino acids); the transporter then secretes the 44-amino-acid pediocin into the environment. In an oxidized environment, the four cysteine molecules form two disulfide bonds, one at positions +9 and +14 and another at +24 and +44. The location and mechanism of the immunity protein Pap or PedB in the producing cells are not known. In nisin A production, initially, proteins NisR and NisK induce transcription of the cluster, probably to produce a single mRNA, which is then translated into 11 separate proteins. NisB dehydrates serine and threonine to their respective dehydroamino acids, and NisC with NisB then enables dehydroamino acids to form thioether rings with cysteine residues in the molecule. The modified molecule is then translocated through the membrane by NisT, and NisP removes the 14-amino-acid leader peptide, releasing the 34-amino-acid nisin outside the cells. NisE, NisF, and NisG provide extra protection to producer cells against nisin.

## *Mode of Action*[10–17]

Bacteriocins of lactic acid bacteria kill sensitive bacterial cells very rapidly and the highly potent one at a very low concentration [minimum inhibitory concentration (MIC) ~0.01 μg/mL for pediocin AcH to *Listeria monocytogenes*]. Their antibacterial actions can be summarized as follows:

1. They differ greatly in the spectra of antibacterial activity against sensitive Gram-positive bacteria (e.g., nisin and pediocin have wider spectra than leucocin or sakacin).
2. Their relative potency (MIC) against a sensitive strain differs greatly (e.g., pediocin is more potent than leucocin against *Lis. monocytogenes*).
3. Bactericidal efficiency of a bacteriocin increases at acidic pH, at higher temperatures, in the presence of a detergent, and against exponentially growing cells.
4. Even in the population of a most sensitive strain, there are variant cells that are resistant to a bacteriocin, but this property is not stable because in the absence of the bacteriocin they become sensitive again.
5. A sensitive strain resistant to one bacteriocin can be sensitive to a second bacteriocin.
6. Gram-negative and resistant Gram-positive bacteria injured by a physical or chemical stress become sensitive to a bacteriocin.
7. Bacterial spores of a sensitive bacterium are resistant to a bacteriocin but become sensitive following germination and outgrowth.

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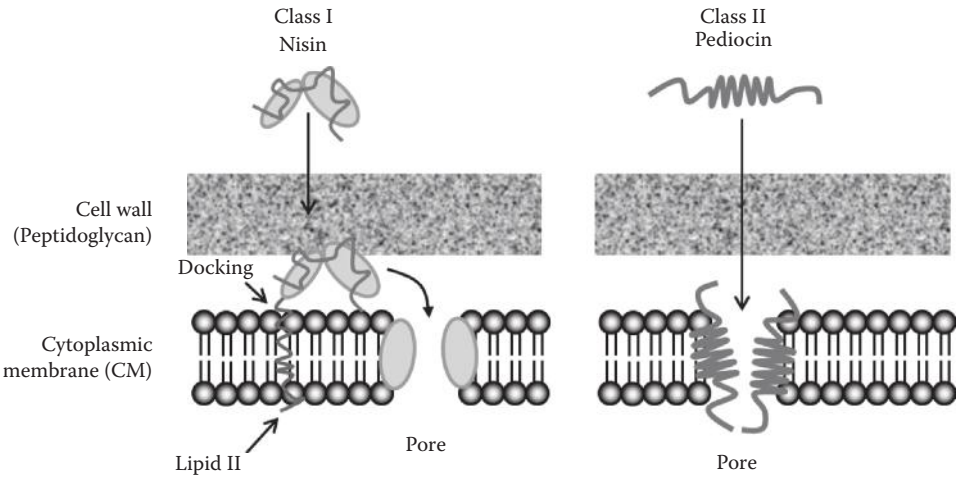



**Figure 17.3 Mode of action of Class I (nisin) and Class II (pediocin) on bacterial cells. (Schematic theme was redrawn based on Cotter, P.D. et al., *Nat. Rev. Microbiol., 3*, 777–788, 2005.)**

The bactericidal effect of a bacteriocin toward a sensitive bacterial cell is produced primarily by destabilization of the function of the cytoplasmic membrane (Figure 17.3). It is now accepted that, in general, the bacteriocin molecules are initially adsorbed on the membrane surface and form transient pores, leading to loss of proton motive force as well as the pH gradient across the membrane. This alters the permeability of the membrane, causing leakage of small nutrient molecules as well as affecting the transport of nutrients and synthesis of ATP. These changes finally cause the cell to lose viability. In addition, some bacteriocins can cause lysis of sensitive cells. The exact mechanisms by which these changes are brought about differ with bacteriocins and are explained here by using nisin A and pediocin AcH or PA-1 as two examples. In the case of nisin, several molecules initially bind (dock) to the lipid II of the cell wall (Figure 17.3). This subsequently helps the molecules to come in contact with the membrane, leading to pore formation. Pore formation by nisin requires a voltage difference between the inside and outside of the membrane. Nisin is thus more potent against growing cells as opposed to resting cells of a target population. In contrast, the action of pediocin is not dependent on the voltage difference of the membrane and is thus effective against both growing and resting cells. Pediocin also forms pores on the cytoplasmic membrane of target cells. As the molecules come in contact with the membrane, their random conformation changes to a defined structure. Several molecules then assemble in a cluster, leading to formation of a pore in the membrane.

## *Bioengineered Bacteriocin*[14,18]

Bacteriocins' use as food preservative is sometimes limited because of their narrow antimicrobial activity spectra, low solubility and stability, and resistance to intestinal enzymes. Modification of the bacteriocins through bioengineering, such as mutation or substitution of amino acid residues, has been attempted to improve bacteriocin functionality. For example, mutation in amino acids in ring A of nisin (Figure 17.1) improved its antimicrobial spectrum. Likewise, site-directed mutagenesis in nisin-producing strains yielded increased antimicrobial activity against *Listeria monocytogenes* and *Staphylococcus aureus*. Solubility of nisin Z was improved by substituting asparagine at

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position 27 and histidine at position 31 with lysine residues. Sensitivity of microcin J25, produced by *Escherichia coli*, to chymotrypsin was achieved by substitution of glycine at position 12 with tyrosine without affecting its antimicrobial activity. Introduction of a disulphide bridge in the C-terminal end of sakacin P broadened its antimicrobial spectrum. A chimeric bacteriocin consisting of the C-terminal half of pediocin and the N-terminal half of enterocin A yielded improved antimicrobial activity against *Leuconostoc lactis*, a dairy product spoilage bacterium. Continued efforts are needed in developing desirable bacteriocins through bioengineering to enhance antimicrobial spectrum and stability during food preservation.

## Production and Purification

Bacteriocin production in lactic acid bacteria is directly related to the cell mass. Generally, the parameters that help generate more cell mass produce more bacteriocin molecules. The parameters include nutritional composition, initial and terminal pH and O-R potential of a broth, and incubation temperature and time. The species and the strains of a species growing under similar conditions differ greatly in the amount of bacteriocin production. In general, production of nisin A or pediocin AcH is much higher than leucocin or sakacin A. A nutritionally rich medium is always better, and, for many bacteriocins, growing the strains in a fermentor under a controlled terminal pH produces more bacteriocin. For example, nisin A production is much higher at pH 6.0, but pediocin AcH production is much higher at a lower terminal pH of 3.6. It is important to determine the optimum parameters for growth and bacteriocin production for an unknown strain to obtain a high yield of bacteriocin.

Bacteriocin molecules following secretion by a producer strain remain either adsorbed on the cell surface or free in the medium, depending on the pH of the environment. More molecules remain free in the environment at pH 1.5–2.0, whereas at pH 6.0–7.0, they remain bound to the cell surface. Based on this pH-dependent adsorption-desorption of bacteriocin on the cell surface of a producer strain, an effective and easy method has been developed to purify bacteriocin molecules in large amounts. A second method of purification involves precipitation of the molecules by ammonium sulfate followed by stepwise gel filtration. This gives a highly purified preparation but in small amounts.

## Applications[8–11,15,18]

Bacteriocins of food-grade lactic acid bacteria are considered safe food biopreservatives and have the potential to be used to kill sensitive Gram-positive food spoilage and food-borne pathogenic bacteria. Among the bacteriocins, only nisin has received regulatory approval for use in select foods. In 1969, the Joint FAO/WHO Expert Committee on Food Additives approved its use and gave it the food additive number 234. The US FDA gave nisin GRAS status and reported nisin as safe for human consumption at 2.94 mg/day and is approved for use in cooked meat and poultry products at 5.5 mg nisin/kg (FDA GRAS Notice No. GRN 000065). In foods that can contain injured Gram-negative bacteria, bacteriocins can also be effectively used to kill them. They are more effective when used in minimally heat-processed foods in suitable combinations of two or more (e.g., nisin and pediocin together). Table 17.5 presents the effectiveness of a preparation containing nisin and pediocin against pathogenic and spoilage bacteria in processed meat products during refrigerated storage. The results show that the bacteriocin preparation effectively reduced the Gram-positive spoilage (*Leu. mesenteroides*) and pathogenic (*Lis. monocytogenes*) bacteria as well as Gram-negative pathogens (*Salmonella* and *Esc. coli* O15:H7) during six weeks of storage at 4°C. Bacteriocins are examined in combination with other antimicrobials, such as acids, salts, spice

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**Table 17.5 Reduction in Viability and Growth of Food Spoilage and Foodborne Pathogenic Bacteria in Refrigerated Vacuum-Packaged Process Meats**

| *Products*[a] | *Test Bacteria* | *Treatment* | *$Log_{10}$ CFU/g Following Storage at 4°C* | | |
|---|---|---|---|---|---|
| | | | *1 day* | *2–4 weeks* | *6 weeks* |
| Hot dogs | *Leuconosoc mesenteroides* | Control | 3.5 | 4.8 | 6.6 |
| | | Treated | 1.0 | <1.0[b] | <1.0 |
| | *Lactobacillus viridescens* | Control | 2.7 | 6.8 | 7.8 |
| | | Treated | <1.0 | <1.0 | <1.0 |
| | *Listeria monocytogenes* | Control | 3.4 | NT | 5.4 |
| | | Treated | <1.0 | <1.0 | <1.0 |
| Roast beef | *Listeria monocytogenes* | Control | 3.5 | 5.4 | 6.9 |
| | | Treated | <1.0 | <1.0 | <1.0 |
| | *Salmonella* Typhimurium | Control | 2.7 | 2.5 | 1.7 |
| | | Treated | 2.3 | 1.8 | <1.0 |
| | *Escherichia coli* O157:H7 | Control | 3.2 | 3.0 | 2.5 |
| | | Treated | 1.4 | <1.0 | <1.0 |
| Turkey roll | *Leuconostoc mesenteroides* | Control | 3.8 | 4.1 | 4.3 |
| | | Treated | 3.4 | 1.8 | 1.5 |
| Ham | *Listeria monocytogenes* | Control | 2.9 | 3.2 | 5.4 |
| | | Treated | <1.0 | <1.0 | 1.8 |

[a] Processed products were vacuum packaged and inoculated with cells of test strains with and without the bacteriocin mixture (pediocin+nisin in a 1:1 ratio to 5000 activity units/g and 1% lactate). The products were stored at 4°C and enumerated for survivors in suitable agar media.

[b] Two samples were used for each test and average results are presented. <1.0 means no CFU was obtained from 0.4 g equivalent of a product.

extracts, high pressure, or other bacteriocins, for synergistic effects to control both Gram-positive and Gram-negative foodborne pathogens in food (Table 17.6). There are many other food products in which bacteriocins of lactic acid bacteria can be used effectively as preservatives (Table 17.5).

Bacteriocins are now evaluated for therapeutic use as a substitute for antibiotics and have shown some promising results.[14,15] Use of bacteriocin-producing probiotics is desirable as a prophylactic for their ability to colonize the GIT and secrete bacteriocin to inactivate pathogens. However, for therapeutic use, purified bacteriocin produced mixed results because some bacteriocins are unstable in *in vivo* environments. For example, feeding of laboratory animals with purified pediocin did not affect the composition of the gut microbiome, which is responsible for maintaining intestinal homeostasis, while nisin did affect microbiome composition. Intragastric administration of purified pediocin was effective in reducing *Listeria monocytogenes* infection in a mouse model.

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**Table 17.6 Bacteriocins Used with Other Hurdles for Pathogen Inactivation**

| *Bacteriocins* | *Antimicrobials* | *Target Pathogens* |
|---|---|---|
| Divergicin | Chitosan | *Listeria monocytogenes* |
| Enterocin | NaCl (6 or 7%) | *Staphylococcus aureus* |
| Enterocin | Nitrite 150 ppm | *Bacillus cereus* |
| Enterocin | Sodium lactate | *Bacillus cereus* |
| Enterocin | Sodium tripolyphosphate | *Escherichia coli* O157:H7 |
| Enterocin | Spice extracts | *Listeria monocytogenes* |
| Lacticin | Sodium citrate or sodium lactate | *Clostridium perfringens* |
| Lacticin | Pressure (250 MPa) | *Listeria innocua* |
| Nisin | Sodium lactate | *Listeria monocytogenes, Salmonella* |
| Nisin | EDTA | *Escherichia coli*<br>*Salmonella*<br>*Listeria monocytogenes* |
| Nisin | Spice extracts | *Listeria monocytogenes*<br>*Escherichia coli* O157:H7<br>*Salmonella enterica* |
| Nisin | Pediocin | *Bacillus spores* |
| Nisin | Lactoperoxidase | *Listeria monocytogenes* |
| Nisin | Curvatacin | *Listeria monocytogenes* |
| Nisin | Lysozyme | *Staphylococcus aureus* |
| Nisin | Pressure (500 MPa) | *Escherichia coli*<br>*Listeria innocua*<br>*Salmonella* |
| Nisin | Lactoperoxidase | *Listeria monocytogenes* |
| Nisin | Curvatacin | *Listeria monocytogenes* |
| Nisin | Lysozyme | *Staphylococcus aureus* |
| Nisin | High pressure (500 MPa) | *Escherichia coli*<br>*Listeria innocua*<br>*Salmonella* |
| Nisin | High-intensity pulsed electric field (800–1200 μs) | *Staphylococcus aureus* |

*(continued)*

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**Table 17.6 Bacteriocins Used with Other Hurdles for Pathogen Inactivation (Continued)**

| *Bacteriocins* | *Antimicrobials* | *Target Pathogens* |
|---|---|---|
| Nisin | Diacetyl (2.5 mM/L) | *Cronobacter sakazakii* |
| Pediocin | High pressure (345–400 MPa) | *Listeria monocytogenes*<br>*Salmonella enterica* |
| Pediocin | Sodium lactate or potassium sorbate or phytic acid or citric acid | *Listeria monocytogenes* |

*Source:* Adapted and revised from Mills, S. et al., *Ann. Rev. Food Sci. Technol.*, *2*, 299–329, 2011.

Likewise, intravenous injection of piscicolin also reduced *Lis. monocytogenes* counts but prolonged protection was not observed. Therefore, before bacteriocins can be used, additional research showing their effectiveness and safety has to be conducted thoroughly. Finally, approval by regulatory agencies will be necessary before they can be used as food biopreservatives and therapeutic agents.

## Encapsulation and Delivery of Bacteriocin[19–23]

Bacteriocins, in general, are highly susceptible to food environments, including microbial enzymes, pH shift (to neutral or alkaline pH), salts, and other food components. Therefore, it is essential to develop technologies to preserve the activity of bacteriocins in food for at least a certain duration before the expiration of the shelf life of a product. Scientists have employed numerous strategies to effectively deliver antimicrobial peptides, including bacteriocins on varieties of foods. For example, bacteriocins have been encapsulated in macrostructures or nanostructures for maintaining activity and controlled release over a period of time. The major advantages of such strategies include loading of large amounts of antimicrobials, homogenous dispersion into the food system, accessibility to microorganisms, increased interaction and attachment to microbes because of smaller sizes, and protection from food environments and controlled release of active molecules. Some select encapsulation methods are briefly discussed below.

## Food Packaging[20]

Synthetic polymers are widely used as packaging materials, and more recently, biopolymers have been used because of their high water vapor permeability, environmental concerns, and their potential for incorporating active components. Biopolymers are made of natural materials, including carbohydrates, proteins, lipids, and composites. Advances in nanotechnology also helped develop packaging materials with improved functionality, such as active packaging. Active packaging became an industry trend because of its ability to modify the internal environment, resulting from direct interaction of packaging materials with the food. This enabled production of high-quality products with fresh-like appearance and extended shelf life. Incorporation of antimicrobial compounds, such as nisin, into the packaging material (bioactive packaging) to control microbes on the surface of food products can improve safety and prolong shelf life. Another important attribute of active packaging is that it can be fabricated to allow controlled or slow release of the active compounds for long duration. This will slow down the bacterial growth kinetics, rendering it safe for human consumption.

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## Liposomes[19]

Liposomes are vesicles composed of a phospholipid bilayer containing aqueous media. They are prepared as multilamellar or unilamellar vesicles with sizes varying from 20 nm to 1 μm. The polar headgroups in phospholipids (phosphatidyl choline or phosphatidyl glycerol) are exposed to an aqueous phase, and the hydrophobic hydrocarbon tails are forced to face each other, forming a vesicle (Figure 17.4). Liposomes are able to encapsulate both lipophilic and hydrophilic functional components, and they are widely used in the food industry as delivery vehicles for vitamins, enzymes, antioxidants, nutraceuticals, and flavors. Liposomes have been used to encapsulate bacteriocins, such as nisin and pediocin. Most studies have used nisin for encapsulation because nisin is the only bacteriocin approved as a food preservative. Nisin is encapsulated into liposomes by the thin-film hydration method in which nisin interacts with phospholipids via electrostatic interaction.

## Nanoencapsulation[21–23]

Nanoencapsulation is becoming an attractive strategy for delivery of antimicrobial peptides, including bacteriocins for food preservation and in pharmaceutical applications. Nanostructures usually fall within the 1 nm–100 nm range; however, size may increase (<500 nm) after the functional molecule incorporation. The physicochemical properties, such as optical property, catalytic property, interaction with host tissues and food, of nanostructures differ greatly from the bulk materials. For encapsulation and delivery of antimicrobials for food application, several nanoencapsulation strategies have been developed (Figure 17.4): nanoliposome, nanoemulsion, lipid nanovesicles, nanofibers, carbohydrate dendrimers, and self-assembled peptides. In addition, nanostructures using silver, zinc, and gold have been used to carry antimicrobial peptides for other applications. Nanoemulsions have been used for delivery of nisin, lysozyme, etc. on their surface. It consists of two immiscible liquids, such as oil and water, which are prepared in the form of droplets. Similarly, solid lipid nanoparticles (SLN ~ 140 nm) are produced using a lipid, and it was able to maintain nisin activity for 20 days. Carbohydrate nanoparticles (60–90 nm) were prepared using phytoglycogen from corn, which was chemically modified to create a dendrimer structure to carry nisin. The nisin-loaded dendrimer maintained nisin activity over 40 days and

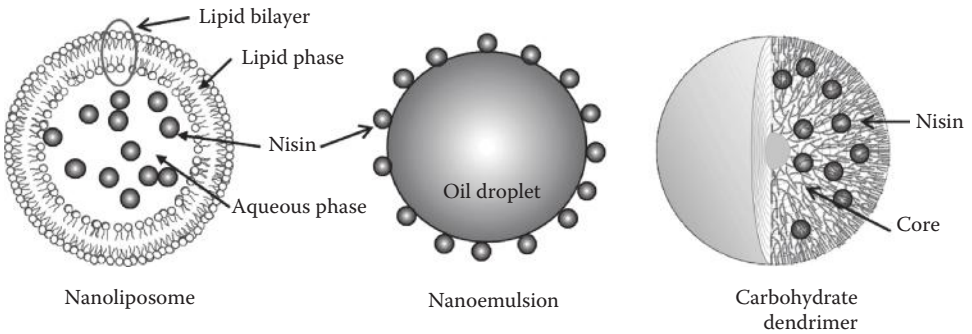



**Figure 17.4 (See color insert.) Schematics of nanostructures used for delivery of bacteriocin (nisin). Carbohydrate dendrimer structure was provided by Dr. Yuan Yao (Purdue University). (From Bi, L. et al., *J. Control. Release*, *150*, 150–156, 2011.)**

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effectively inactivated *Listeria monocytogenes* in a model food system.[23] Nanofibers are made of poly (D,L-lactide) and polyethylene oxide dissolved in *N,N*-dimethylformamide and are used for delivery of several bacteriocins.

## Safety Concerns with Nanotechnology[20,24]

The physicochemical properties of engineered nanomaterials (ENMs) that may be altered as compared to bulk materials include optical property, catalytic activity, surface property, mechanical strength, increased stability, altered reactivity, improved functionality, and adsorption potential. Hence, there is a potential for increased systemic bioavailability to organs and tissues. Furthermore, solubility and biopersistence are other concerns. The application of nanotechnology in food has focused on the uncertainties and unknowns and lack of toxicological data. Even though many of the biomaterials used in food application (encapsulation) are GRAS listed, yet chemical or physical modification of such molecules may alter their physicochemical properties; thus their safety aspects should be thoroughly examined before food use. Recently a detailed guideline has been developed by European scientists to assess the safety of ENMs in food employing a systemic tiered approach. This is divided into five key steps:[24] (1) characterization of bulk materials from which the ENM is derived; (2) characterization of the physicochemical properties of ENMs; (3) identification of ENMs requiring focused toxicological assessments; (4) toxicological assessment (adsorption, distribution to tissues and organs, metabolism and excretion, and cellular interaction, that is, cytotoxicity, inflammation, oxidative stress and genotoxicity); and (5) safety evaluation of the ENM as used in its intended food matrix.

## Yeast Metabolites as Preservatives

Certain yeasts, including strains of *Saccharomyces cerevisiae*, produce several proteins that have limited antimicrobial properties.[25] These proteins (designated as killer toxins or zymocins) can, through genetic manipulation, be altered to have a wider antimicrobial spectrum, especially against fungi. However, very few studies are currently being conducted in this area.

Several yeast isolates normally present on the surface of fruits and vegetables are reported to prevent spoilage of the produce by molds. Some of the inhibitory compounds are small proteins, whereas others are enzymes. Cells of one such yeast isolate was found to adhere tightly with the mold mycelia and produce β-gluconase, which degrades the cell wall of the molds and kills them. Because many of these yeasts are normally present in fruits and vegetables that are eaten raw, they are not considered pathogenic, and thus can be used in place of fungicides to enhance the preservation of fruits and vegetables.[26]

## Conclusion

Food-grade bacteria and yeasts produce different antimicrobial compounds, such as organic acids, diacetyl, $H_2O_2$, reuterine, bacteriocins, and enzymes, that have bacteriostatic, bactericidal, fungistatic, and fungicidal action against microorganisms involved in food spoilage and food-borne diseases. They differ in the mode of antimicrobial action. Because they are from safe microorganisms,

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they can be used as food biopreservatives. Although some are currently used in a limited way, approval by regulatory agencies will be necessary for the use of others in food.

**QUESTIONS**

1. List the antimicrobial compounds produced by different starter-culture bacteria.
2. Discuss the mode of antibacterial action of organic acids produced by starter-culture bacteria. At pH 6.0, why are propionic and acetic acids more antibacterial than lactic acid? List the advantages of using these acids in food preservation.
3. Describe the mode of antibacterial action of diacetyl and discuss why it has limited use as a food biopreservative.
4. Under what conditions can some lactic acid bacteria generate sufficient quantities of $H_2O_2$ to inhibit other microorganisms in a food system? What are some disadvantages of using $H_2O_2$ in food?
5. Discuss the advantages of using small amounts of $H_2O_2$ in raw milk to inhibit psychrotrophic bacteria, such as *Pseudomonas* species. What needs to be done before pasteurizing this milk?
6. Define bacteriocins of lactic acid bacteria and list their four general characteristics.
7. How are bacteriocins grouped? Give one example of each group.
8. Define the terms prepeptide, propeptide, leader peptide, thioether rings, disulfide bonds, and thiol group in relation to the structure of bacteriocins.
9. List two important similarities in the amino acid sequence of cystibiotics that are related to their bactericidal property.
10. List three features of plasmid-encoded bacteriocins of lactic acid bacteria.
11. Briefly list differences in gene organizations associated with bacteriocin production in lactic acid bacteria.
12. Using either pediocin AcH or nisin A as an example, briefly explain the function of genes in the production of active molecules.
13. Briefly discuss how bacteriocin production of a producer strain of lactic acid bacteria can be enhanced.
14. List five potential applications of bacteriocins of lactic acid bacteria as food preservatives.
15. Briefly explain (a) potential therapeutic uses of bacteriocins and (b) use of antimicrobial properties of yeasts to preserve foods.
16. What strategies are used to improve functionality of bacteriocins? Use examples.
17. Define nanoencapsulation technology. What are the advantages of using nanoencapsulation for bacteriocin delivery?

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# Chapter 18

# Food Ingredients and Enzymes of Microbial Origin

## Introduction

Many microbial metabolites can be used as food additives to improve nutritional value, flavor, color, and texture. Some of these include proteins, essential amino acids, vitamins, aroma compounds, flavor enhancers, salty peptides, peptide sweeteners, colors, stabilizers, and organic acids. Because they are used as ingredients, they need not come only from microorganisms used to produce fermented foods but can be produced by many other types of microorganisms (also algae) with regulatory approval for safety before use in foods. Many enzymes from bacteria, yeasts, molds, as well as from plant and mammalian sources, are currently used for the processing of foods and food ingredients. Some examples are production of high-fructose corn syrups, extraction of juice from fruits and vegetables, and enhancement of flavor in cheese.

Recombinant DNA technology (or biotechnology) has opened up the possibilities of identifying and isolating genes or synthesizing genes encoding a desirable trait from plant and animal sources or, from microorganisms that are difficult to grow normally, clone it in a suitable vector (DNA carrier) and incorporate the recombinant DNA in a suitable microbial host that will express the trait and produce the specific additive or enzyme economically.[1] In addition, metabolic engineering, by which a desirable metabolite can be produced in large amounts by a bacterial strain, is being used to produce food additives from new sources. The metabolites can then be purified and used as food additives and in food processing, provided they are a safe-to-use, generally regarded as safe (GRAS)–listed compound.[2] Some of these aspects involving genetic manipulations are discussed in Chapter 13. The microbiology of the production and uses of some additives and enzymes are discussed here.



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## Microbial Proteins and Food Additives

### *Single-Cell Proteins (SCPs)*[3]

Molds, yeasts, bacteria, and algae are rich in proteins, and the digestibility of these proteins ranges from 65% to 96%. Proteins from yeasts, in general, have high digestibility as well as biological value. In commercial production, yeasts are preferred. Some of the species used are from genera *Candida*, *Saccharomyces*, and *Torulopsis*. Some bacterial species have been used, especially from genus *Methylophilus*.

The use of microbial proteins as food has several advantages over animal proteins. There may not be enough animal proteins to feed the growing human population in the future, especially in many developing countries. Also, microbial proteins can be produced under laboratory settings. Thus, land shortage and environmental calamities (such as drought or flood) can be overcome. They can be produced on many agricultural and industrial wastes. This will help alleviate waste disposal problems and also reduce the cost of production. Microbial proteins can be a good source of B vitamins, carotene, and carbohydrates.

There are some disadvantages of using microbial proteins as human food. They are poor in some essential amino acids, such as methionine. However, this can be corrected by supplementing microbial proteins with the needed essential amino acids. The other problem is that proteins from microbial sources can have high-nucleic acid content (RNA and DNA; 6%–8%), which, in the human body, is metabolized to uric acid. A high serum-acid level can lead to kidney stone formation and gout. However, through genetic manipulations, the nucleic acid content in microbial proteins has been reduced.

Even though, at present, the use of microbial proteins as a protein source in human food is limited, they are being used as a protein source in animal feed. An increase of microbial proteins will automatically reduce the use of grains (such as corn and wheat) as animal feed, which then can be used as human food.[3]

### *Amino Acids*

Proteins of most cereal grains are deficient in one or more of the essential amino acids, particularly methionine, lysine, and tryptophan. To improve the biological values, cereals are supplemented with essential amino acids. Supplementing vegetable proteins with essential amino acids has been suggested to improve the protein quality for people who either do not consume animal proteins (people on vegetarian diets) or do not have enough animal proteins (such as in some developing countries, especially important for children). To meet this demand, as well as for use as nutrient supplements, large amounts of several essential acids are being produced. At present, because of economic reasons, they are mostly produced from the hydrolysis of animal proteins followed by purification.

In recent years, bacterial strains have been isolated, some of which are lactic acid bacteria that produce and excrete large amounts of lysine in the environment. Isolating high-producing strains of other amino acids and developing strains by genetic and metabolic engineering that will produce these amino acids in large amounts can be important for economical production of essential amino acids.

### *Nutraceuticals and Vitamins*

Many vitamins are added to foods and also used regularly by many as supplements. Thus, there is a large market for vitamins, especially some B vitamins and vitamins C, D, and E. Some of these

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are obtained from plant sources, several are synthesized, and a few are produced by microorganisms.[4] Vitamin C is now produced by yeast by using cheese whey. Microorganisms have also been a source of vitamin D. Many are capable of producing B vitamins.

The possibility of using gene-cloning techniques to improve production of vitamins by microorganisms may not now be very practical or economical. Vitamins are produced through multienzyme systems, and it may not be possible to clone the necessary genes. In recent years, through metabolic engineering, strains of lactic acid bacteria have been developed that when used in dairy fermentation produce high amounts of folate and some cyanocobalamin ($B_{12}$) in the fermented products, thereby increasing the nutritional value of the products. In addition, strains of lactic acid bacteria have been developed that produce low-calorie sweeteners, such as mannitol, sorbitol, and tagatose.[3]

## Flavor Compounds and Flavor Enhancers

Flavor compounds and enhancers include those that are associated directly with the desirable aroma and taste of foods and indirectly with the strengthening of some flavors.[5–7] Many microorganisms produce different types of flavor compounds, such as diacetyl (butter flavor by *Leuconostoc*), acetaldehyde (yogurt flavor by *Lactobacillus acidophilus*), some nitrogenous and sulfur-containing compounds (sharp cheese flavor by *Lactococcus lactis*), propionic acid (nutty flavor by dairy *Propionibacterium*), pyrazines (roasted nutty flavors by strains of *Bacillus subtilis* and *Lac. lactis*), and terpenes (fruity or flowery flavors by some yeasts and molds). Some natural flavors from plant sources are very costly because only limited amounts are available and the extraction process is very elaborate. By employing biotechnology, they can be produced economically by suitable microorganisms. Natural vanilla flavor (now obtained from plants), if produced by microorganisms, may cut the cost to only a 10th or less. Natural fruit flavors are extracted from fruits. Not only is it costly, but also large amounts of fruits are wasted. The possible production of many of these flavors by microorganisms through recombinant DNA technology is being studied.

Several flavor enhancers are now used to strengthen the basic flavors of foods. Monosodium glutamate (MSG, enhances meat flavor) is produced by several bacterial species, such as *Corynebacterium glutamicum* and *Micrococcus glutamicus*. Also, 5′ nucleotides, such as inosine monophosphate and guanosine monophosphate, give an illusion of greater viscosity and mouthfeel in foods such as soups. They can be produced from *Bac. subtilis*.

Several small peptides, such as lysylglycine, have strong salty tastes. They can be produced by recombinant DNA technology by microorganisms and used to replace NaCl. Sweet peptides, such as monellin and thaumatin from plant sources, can also be produced by microorganisms through gene cloning. At present, the dipeptide sweetener aspartame is produced synthetically, but a method to produce it by microorganisms has been developed. By metabolic engineering, strains of lactic acid bacteria have been developed that can produce large quantities of diacetyl (for the aroma of butter), acetaldehyde (for the aroma of yogurt), α-ketoglutarate (to produce cheese flavor), and other compounds.[3]

## Colors

Many bacteria, yeasts, and molds produce different color pigments. The possibility of using some of them, especially from those that are currently consumed by humans, is being studied.[8] Some of the common fermentative food-grade pigments include yellow, red, and orange pigments from

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*Monascus* sp.[9] (fungus), astaxanthin (pink-red) from *Xanthophyllomyces dendrorhous* (yeast), arpink red from *Penicillium oxalicum* (fungus), riboflavin from *Ashbya gossypii* (fungus), β-carotene from *Blakeslea trispora* (fungus). The pink-red color pigment astaxanthin gives the red color to salmon, trout, lobster, and crabs. Another red pigment, produced by *Monascus* sp., has been used for a long time in the Orient to make red rice wine. Because pigment production may involve multistep reactions, recombinant DNA techniques to produce some fruit colors by microorganisms may not be economical. However, they can be produced by the plant cell culture technique.

### *Exopolysaccharides (EPS)*

Different polysaccharides are used in food systems as stabilizers and texturizers. Although many of them are of plant origin, some are obtained from microbial sources.[10,11] Strains of many lactic acid bacteria, such as *Streptococcus thermophilus*, *Lab. rhamnosus*, *Lab. helveticus*, *Lab. casei*, and *Lac. lactis*, produce many different types of exopolysaccharides (EPS) that contain units of glucose, galactose, rhamnose, mannose, and other carbohydrates. Many of these strains are currently being used to produce fermented dairy products with better consistency and texture (in yogurt and buttermilk), to hold moisture in low fat–high moisture cheeses (in mozzarella cheese). Some examples of EPS produced by microorganisms include xanthan, gellan, alginate, cellulose, dextran, curdlan, succinoglycan, levan, galactopol, fucopol, etc.[12] Dextran, an EPS produced by *Leuconostoc mesenteroides* while growing in sucrose, is used as a stabilizer in ice cream and confectioneries. Xanthan gum, produced by *Xanthomonas campestris*, is also used as a stabilizer. Introducing lactose-hydrolyzing genes in *Xanthomonas* species can enable it to grow in whey to produce the stabilizer economically.

### *Organic Acids*

Production of lactic (by lactic acid bacteria), propionic (by propionic acid bacteria), and acetic (by acetic acid bacteria) acids and their different uses in foods are discussed in Chapters 12, 17, and 41. Several other organic acids and their salts are used in foods to improve taste (flavor and texture) and retain quality. Production of ascorbic acid by yeasts and its use as a vitamin supplement have also been discussed. Ascorbic acid is also used in some foods as a reducing agent to maintain color (to prevent color loss by oxidation). It also has an antibacterial action. Citric acid is used in many foods to improve taste and texture (in beverages) and stabilize color (in fruits). It also has some antibacterial property. Citric acid is produced by the mold *Aspergillus niger*.

### *Preservatives*

Bacterial cells of lactic acid bacteria, several organic acids produced by them, and their bacteriocins can be used to control spoilage and pathogenic bacteria in foods. This aspect is discussed in Chapter 17.

## Microbial Enzymes in Food Processing

Many enzymes are used in the processing of food as food additives.[13] About 80% of the total enzymes produced, on a dollar basis, is used by the food industries. Use of specific enzymes instead of microorganisms has several advantages. A specific substrate can be converted into a specific

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product by an enzyme through a single-step reaction. Thus, production of different metabolites by live cells from the same substrate can be avoided. In addition, a reaction step can be controlled and enhanced more easily by using purified enzymes. Finally, by using recombinant DNA technology, the efficiency of enzymes can be improved and, by immobilizing, they can be recycled. The main disadvantage of using enzymes is that if a substrate is converted to a product through many steps (such as glucose to lactic acid), microbial cells must be used for their efficient and economical production.

## *Enzymes Used*

Among the five classes of enzymes, three are predominantly used in food processing: hydrolases (hydrolyze C–C, C–O, C–N, etc., bonds), isomerases (isomerization and racemization), and oxidoreductases (oxygenation or hydrogenation). Some of these are listed in Table 18.1, and their uses are discussed here.

**Table 18.1 Some Microbial Enzymes Used in Food Processing**

| *Enzyme* | *Class*[a] | *Source* | *Substrate* | *Function/Use* |
|---|---|---|---|---|
| Alpha-Amylase | H | Bacteria, molds | Starch | Production of dextrins; brewing and baking |
| Catalase | OR | Molds | $H_2O_2$ | Removal of $H_2O_2$; milk, liquid eggs |
| Cellulase | H | Molds | Cellulose | Hydrolyze cellulose; ethanol production, juice extraction |
| Glucoamylase | H | Molds | Dextrins | Dextrins to glucose |
| D-Glucose isomerase | I | Bacteria | Glucose | Glucose to fructose; high-fructose corn syrup |
| D-Glucose oxidase | OR | Molds | D-Glucose, oxygen | Flavor and color of liquid egg, juice |
| Hemicellulase | H | Bacteria, molds | Hemicellulose | Juice clarification |
| Invertase | H | Yeasts | Sucrose | Production of invert sugar |
| Lactase | H | Molds, yeasts | Lactose | Glucose or galactose from whey; low-lactose milk |
| Lipases | H | Bacteria, molds | Lipids | Cheese ripening |
| Pectinases | H | Molds | Pectin | Clarification of wine, fruit juice, juice extraction |
| Proteinases | H | Bacteria, molds | Proteins | Meat tenderization, cheese making and ripening |

[a] H, hydrolases; I, isomerases; OR, oxidoreductases.

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### α-Amylase, Glucoamylase, and Glucose Isomerase

Together, these three enzymes are used to produce high-fructose corn syrup from starch. α-Amylase hydrolyzes starch at α-1 position randomly and produces oligosaccharides (containing three hexose units or more, for example, dextrins). Glucoamylase hydrolyzes dextrins to glucose units, which are then converted to fructose by glucose isomerase.

α-Amylase is also used in bread making to slow down staling (starch crystallization resulting from loss of water). Partial hydrolysis of starch by α-amylase can help reduce the water loss and extend the shelf life of bread.

### Catalase

Raw milk and liquid eggs can be preserved with $H_2O_2$ before pasteurization. However, the $H_2O_2$ needs to be hydrolyzed by adding catalase before heat processing of the products.

### Cellulase, Hemicellulase, and Pectinase

Because of their ability to hydrolyze respective substrates, the use of these enzymes in citrus juice extraction has increased juice yield. Normally, these insoluble polysaccharides trap juice during pressing. Also, they get into the juice and increase viscosity, causing problems during juice concentration. They also cloud the juice. By using these hydrolyzing enzymes, such problems can be reduced.

### Invertase

Invertase can be used to hydrolyze sucrose to invert sugars (mixture of glucose and fructose) and increase sweetness. It is used in chocolate processing.

### Lactase

Whey contains high amounts of lactose. Lactose can be concentrated from whey and treated with lactase to produce glucose and galactose. It can then be used to produce alcohol.

### Lipases

Lipases are produced by filamentous fungi, and commercially important lipase-producing fungi include *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp.[14] Lipases may be used to accelerate cheese flavor along with some proteases.

### Proteases

Different proteases are used in the processing of many foods. They are used to tenderize meat, extract fish proteins, separate and hydrolyze casein in cheese making (rennet), concentrate cheese flavor (ripening), and reduce bitter peptides in cheese (specific peptidases).

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## Enzyme Production by Recombinant DNA Technology

The enzymes that are currently used in food processing are obtained from bacteria, yeasts, molds, plants, and mammalian sources. They have been approved by regulatory agencies, and their sources have been included in the GRAS list (Table 18.2).[13] There are some disadvantages of obtaining enzymes from plant and animal sources. The supply of these enzymes can be limited and thus costly. Also, molds grow more slowly than bacteria or yeast, and some strains can produce mycotoxins. It would be more convenient and cost-effective if the enzymes now obtained from nonbacterial sources (including yeasts, as their genetic system is more complicated than bacteria) could be produced in bacteria. This can be hypothetically achieved through recombinant DNA technology.[15] However, in trying to do so, one has to recognize that the bacterial host strains need to be approved by regulatory agencies if they are not on the GRAS list. Also, regulatory approval will be necessary for the source if it is not currently on the GRAS list.

The technique is rapidly going through many improvements. In brief, it involves separating specific mRNA (while growing on a substrate) and using the mRNA to synthesize cDNA by employing the reverse transcriptase enzyme. The cDNA (double-stranded) is cloned in a suitable plasmid vector, which is then introduced by transformation into the cells of a suitable bacterial strain (e.g., *Esc. coli*). The transformants are then examined to determine the expression and efficiency of production of the enzyme. This method has been successfully used to produce rennin (of calf ) and cellulase (of molds) by bacteria. Rennin thus produced is used to make cheese. Yeast has also been used to produce recombinant proteins when *Esc. coli* was unable to express such proteins. The advantages of the yeast expression system is manyfold—primarily the yield is high because of high-density growth, stable production, cost-effectiveness, and can assist in protein folding, allowing the expression of active molecules.[15]

**Table 18.2 List of Enzymes from Recombinant Microorganisms that Have Either Received Approval from the FDA or Are Under Consideration**

| *Microorganisms* | *Enzymes* |
|---|---|
| *Aspergillus niger* | Phytase, chymosin, lipase |
| *Aspergillus* oryzae | Esterase–lipase, aspartic proteinase, glucose oxidase, laccase, lipase, pectin esterase, phospholipase A1 |
| *Bacillus licheniformis* | α-Amylase, pullulanase |
| *Bacillus subtilis* | α-Acetolactate, decarboxylase, α-amylase, maltogenic amylase, pullulanase |
| *Escherichia coli* K-12 | Chymosin |
| *Fusarium venenatum* | Xylanase |
| *Kluyveromyces marxianus* var. *lactis* | Chymosin |
| *Pseudomonas fluorescence* Biovar 1 | α-Amylase |
| *Trichoderma reesei* | Pectin lyase |

*Source:* Adapted from Olempska-Beer, Z.S. et al., *Regulatory Toxicol. Pharmacol.*, *45*, 144–158, 2006.

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## Immobilized Enzymes

Enzymes are biocatalysts and can be recycled. An enzyme is used only once when added to a substrate in liquid or solid food. In contrast, if the molecules of an enzyme are attached to a solid surface (immobilized), the enzyme can be exposed repeatedly to a specific substrate.[16] The major advantage is the economical use of an enzyme, especially if the enzyme is very costly.

Enzymes can be immobilized by several physical, chemical, or mechanical means. The techniques can be divided into four major categories (Figure 18.1).

### Adsorption on a Solid Support

This technique relies on the affinity of the support for enzyme molecules. The technique involves adding an enzyme solution to the support (such as ion-exchange resins) and washing away the unattached molecules. The association is very weak, and the molecules can be desorbed and removed.

### Covalent Bonding

The enzyme molecules are covalently bound to a solid surface (such as porous ceramics) by a chemical agent. The enzyme molecules are accessible to the substrate molecules. The enzymes are more stable.

### Entrapping

The enzyme molecules are enclosed in a polymeric gel (e.g., alginate) that has an opening for the substrate molecules to come in contact with the catalytic sites. The enzymes are added to the monomer before polymerization.

### Crosslinking

Crosslinking is achieved by making chemical connections between the enzyme molecules to form large aggregates that are insoluble. This is a very stable system.

There are several disadvantages in enzyme immobilization. Immobilization can reduce the activity of an enzyme. Substrate molecules may not be freely accessible to the immobilized

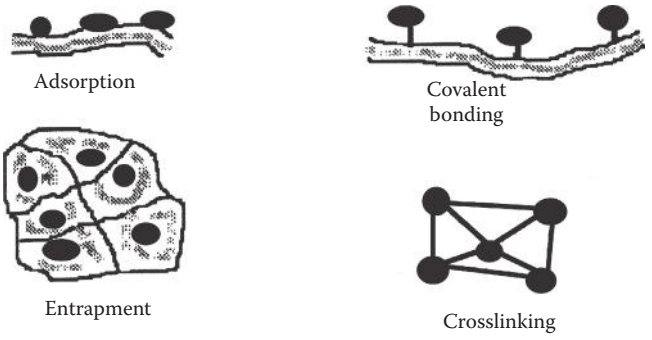



**Figure 18.1 Schematic presentation of four methods of immobilization of enzymes.**

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enzymes. The method may not be applicable if the substrate molecules are large. α-Amylase may not be a good candidate for immobilization because starch molecules, its substrate, are fairly large. However, glucose isomerase can be immobilized as its substrate is small glucose molecules. The supporting materials can be contaminated with microorganisms that are difficult to remove and can be a source of contamination in food. The materials to be used as support should not be made of substances that are unsafe and should be approved by regulatory agencies. Some of the immobilized enzymes currently used are glucose isomerase, β-galactosidase, and aminoacylase.

Microbial cells can also be immobilized by the methods listed previously, and the techniques have been studied in the production of some food ingredients and beverages. Examples include *Asp. niger* (for citric acid and gluconic acid), *Saccharomyces cerevisiae* (for alcoholic beverages), and *Lactobacillus* species (for lactic acid).

## Thermostable Enzymes

The term "thermostable enzymes" is generally used for those enzymes that can catalyze reactions above 60°C. There are several advantages of using thermostable enzymes in a process. The rate of an enzyme reaction doubles for every 10°C increase in temperature; thus, the production rate can be increased, or the amount of enzyme used can be reduced. At high temperatures, when an enzyme is used for a long time (as in the case of immobilized enzymes), the problems of microbial growth and contamination can be reduced.

At high temperatures, enzymes denature because of the unfolding of their three-dimensional structures. The stability of the three-dimensional structure of an enzyme is influenced by the ionic charges, hydrogen bonding, and hydrophobic interaction among the amino acids. Thus, the linear sequences of amino acids in an enzyme greatly influence its three-dimensional structure and stability. Studies have revealed that increases in both ion pairing and hydrogen bonding on the surface of an enzyme (on a three-dimensional structure) and increases in internal hydrophobicity increase the thermostability of an enzyme. For example, the enzyme tyrosinase from a thermolabile strain of *Neurospora* species denatures in 4 min at 60°C, but from a thermostable strain of the same species, it denatures in 70 min at 60°C. An analysis of the amino acid sequences revealed that at position 96, tyrosinase has an aspargine (uncharged) in the thermolabile strain, but aspartic acid (charged) in the thermostable strain. Thus, an extraionic charge (on the surface) increases the thermostability of this enzyme.

Several methods, such as chemical and recombinant DNA techniques, can be used to increase the thermostability of an enzyme.[17] Recombinant DNA technology can be used in two ways. If the enzyme is present in a thermostable form in a microorganism that is not on the GRAS list, the gene can be cloned in a suitable vector, which can then be introduced in a GRAS-listed microorganism and examined for expression and economical production. The other method is more complicated and involves determining the amino acid sequence of the enzyme and its three-dimensional structure (by computer modeling) to recognize the amino acids on the surface (or inside). The next step involves changing one or more amino acids on the surface to increase ionic or hydrogen bonding. This can be achieved by site-specific mutagenesis of base sequences of cDNA for the specific amino acid. The synthesized DNA can be incorporated in a vector and introduced in a desired microbial strain for expression of the enzyme and testing for its thermostability.

Several thermostable enzymes obtained from microorganisms on the GRAS list are currently being used. It is expected that in the future, their production by different methods and use in food will increase.

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### Enzymes in Food Waste Treatment

Food industries generate large volumes of both solid and liquid wastes. Waste disposal methods have used different physical, chemical, and some biological methods. Biological methods include anaerobic digestion and production of SCPs.[18] Because of an increase in regulatory restrictions in waste disposal, effective and economical alternative methods are being researched. The possibility of using enzymes to reduce wastes and convert the wastes to value-added products is being developed. The availability of specific enzymes at low costs has been a major incentive in their use for waste disposal.

Some of the enzymes used in food waste treatments are polysaccharidases (cellulase, pectinase, hemicellulase, chitinase, and amylase), lactase, and proteinases. Treatment of fruits with cellulase and pectinase has increased juice yield and improved separation of solids from the juice. The solids can be used as animal feed. Chitinases are used to depolymerize the shells of shellfish and the product used to produce SCPs. Amylases are used to treat starch-containing wastewater to produce glucose syrup for use in alcohol production by yeasts. Lactose in whey has been treated with lactase (β-galactosidase) to produce glucose and galactose, which are then used in alcohol production by yeast or to produce baker's yeasts. Proteases are used to treat wastewater from fish and meat-processing operations. Some of these products are used as fish food. In recent years, food processing wastes are now becoming the source of alternative energy, such as methane gas, ethanol, hydrogen gas, etc., resulting from microbial growth and fermentation.[19]

In the future, development of better and low-cost enzymes through recombinant DNA technology will increase their uses in food waste treatment.

## Conclusion

The materials discussed in this chapter briefly summarize some of the cell components, metabolic end products, and enzymes produced by food-grade and regulatory agency–approved microorganisms that are used in foods as additives to improve the nutritional and acceptance qualities of foods. Recent advances in genetic engineering and metabolic engineering of these bacteria have helped develop strains that can produce many unique products. As our knowledge on genome sequences and function of the genes of these strains increases, many new strains will be developed to produce other unique products. The future potential in this area is very high.

### QUESTIONS

1. What are the advantages and disadvantages of using microbial proteins as human food?
2. For each of the following, list one example from microbial origin that is used in food: amino acids, flavor compounds, flavor enhancers, color, polysaccharides (stabilizers), and nutraceuticals.
3. Briefly indicate how some lactic acid bacteria can be engineered to produce large quantities of diacetyl and acetaldehyde (see Chapter 12).
4. Name the microorganisms that are used to produce L(+)-lactic acid, acetic acid, and citric acid.
5. List the advantages and disadvantages of using microbial enzymes and microbial cells in food processing.
6. List five enzymes of microbial origin and discuss their specific uses in the food industry.
7. Discuss the advantages of using immobilized enzymes in the production of food ingredients. List three methods of immobilization.

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8. What are the advantages of using a thermostable enzyme in the production of food ingredients (or a food)? How will recombinant DNA technology help in producing thermostable enzymes?
9. Discuss how microbial enzymes can be helpful in the treatment of wastes from food production.
10. "The potential of producing many types of food additives from food-grade microorganisms is very high." Briefly comment on the possibilities (you may use materials from other chapters in the section).

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# MICROBIAL FOOD SPOILAGE

# IV

A food is considered spoiled when it loses its acceptance qualities. The factors considered in judging the acceptance qualities of a food include color, texture, flavor (smell and taste), shape, and absence of abnormalities. Loss of one or more normal characteristics in a food is considered to be a result of spoilage.

Food spoilage causes not only economic loss, but also loss of consumable foods. In the United States and some other countries, where foods are produced and procured from many countries much more than the need, spoilage up to a certain level is not considered serious. However, in many countries where food production is not efficient, food spoilage can adversely affect the availability of food. With the increase in world population, serious consideration needs to be given on not only increasing food production, but also reducing food spoilage, which, for certain produce in some countries, could reach 25% or more. The Food and Agriculture Organization (FAO) of the United Nations (UN) and the World Health Organization (WHO) estimates that each year one third of food produced for human consumption is wasted.

The acceptance qualities of a food can be lost because of infestation with insects and rodents, undesirable physical and chemical actions, and growth of microorganisms. An example of physical spoilage is dehydration of fresh vegetables (wilting). Chemical spoilage includes oxidation of fat, browning of fruits and vegetables, and autolytic degradation of some vegetables (by pectinases) and fishes (by proteinases). Microbial spoilage results either as a consequence of microbial growth in a food or because of the action of some microbial enzymes present in a food. In this section, food spoilage resulting from microbial growth and microbial enzymes is discussed under the following topics:

Chapter 19: Important Factors in Microbial Food Spoilage
Chapter 20: Spoilage of Specific Food Groups
Chapter 21: New Food Spoilage Bacteria in Refrigerated Foods
Chapter 22: Food Spoilage by Microbial Enzymes
Chapter 23: Indicators of Microbial Food Spoilage

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# Chapter 19

# Important Factors in Microbial Food Spoilage

## Introduction

Microbial food spoilage occurs as a consequence of either microbial growth in a food or release of microbial extracellular and intracellular (following cell lysis) enzymes in the food environment. Some of the detectable parameters associated with spoilage of different types of foods are changes in color, odor, and texture; formation of slime; accumulation of gas (or foam); and accumulation of liquid (exudate, purge). Spoilage by microbial growth occurs much faster than spoilage by microbial extracellular or intracellular enzymes in the absence of viable microbial cells. Between initial production (such as harvesting of plant foods and slaughtered animal foods) and final consumption, different methods are used to preserve the acceptance qualities of foods, which include the reduction of microbial numbers and growth. Yet, microorganisms grow and cause food spoilage, which for some foods could be relatively high. It is important to understand the factors associated with microbial food spoilage, both for recognizing the cause of an incidence and developing an effective means of control.

## Sequence of Events

Generally, for microbial food spoilage to occur, several events need to take place in sequence. Microorganisms have to get into the food from one or more sources; the food environment (pH, $A_W$, O-R potential, nutrients, and inhibitory agents) should favor growth of one or more types of these contaminating microorganisms; the food must be stored (or abused) at a temperature that enables one or more types to multiply; and finally, the food must be stored under conditions of growth for a sufficient length of time for the multiplying microbial types to attain the high numbers necessary to cause the detectable changes in a food. In a heat-treated food, the microorganisms associated with spoilage either survive the specific heat treatment (thermodurics) or get into the food following heating (as post-heat contaminants). Spoilage of a heated food by microbial



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enzymes, in the absence of viable microbial cells, can result from some heat-stable enzymes produced by microorganisms in the foods before heat treatment. In addition, the foods need to be stored at a temperature for a sufficient length of time for the catalytic activities of the enzymes to occur to produce the detectable changes.

## Significance of Microorganisms

### *Microbial Types*

Raw and most processed foods normally contain many types of molds, yeasts, and bacteria capable of multiplying and causing spoilage. (Viruses and parasites do not multiply in foods.) As multiplication is an important component in spoilage, bacteria (because of its shorter generation time), followed by yeasts, are in favorable positions over molds to cause rapid spoilage of foods. However, in foods where bacteria or yeasts do not grow favorably and the foods are stored for a relatively longer period of time, such as breads, hard cheeses, fermented dry sausages, and acidic fruits and vegetables, spoilage as a result of mold growth is more prevalent. Recent advances in anaerobic packaging of foods have also greatly reduced the spoilage of food by molds and, to some extent, by yeasts but not by anaerobic and facultative anaerobic bacteria. Thus, among the three microbial groups, the highest incidence of spoilage, especially rapid spoilage, of processed foods is caused by bacteria, followed by yeasts and molds.[1–5]

### *Microbial Numbers*

To produce detectable changes in color, odor, and texture of a food accompanied with slime formation or gas and liquid accumulation, microorganisms (mainly bacteria and yeasts) must multiply and attain certain levels, often referred to as the "spoilage detection level." Although they vary with the type of foods and microorganisms, bacteria and yeasts need to grow and reach approximately $10^7$ cells/g/mL, or/cm$^2$, of a food from the level present normally in a food. Depending on the specific nature of spoilage and microbial types, the spoilage detection level can range from $10^{6–8}$ cells/g/mL, or/cm$^2$. Spoilage associated with $H_2S$, some amines, and $H_2O_2$ formation can be detected at a lower microbial load, whereas formation of lactic acid may be detected at a higher microbial load. Slime formation, associated with accumulation of microbial cells, is generally detected at $\geq 10^8$ cells/g/mL, or/cm$^2$, of a food. It appears, then, that a food with relatively higher initial loads of spoilage bacteria (or yeasts) and a storage condition that favors rapid growth (shorter generation time) will spoil more rapidly than a food with a low initial load of microbes with a longer generation time. In a hypothetical example (Figure 19.1), the population reached the spoilage detection level within seven days with a high initial load (ca. $5 \times 10^5$/g) as opposed to 20 days with a low initial load (ca. $5 \times 10^5$/g) during storage at 12°C. However, when the product with a low initial load was stored at 4°C (to increase the generation time), it took approximately 55 days for the spoilage bacteria to reach the spoilage detection level. To reduce microbial spoilage of a food, one needs to aim at achieving both the low initial load and longer generation time of spoilage microorganisms during storage. It has to be recognized that the mere presence of $10^7$ cells/g/mL, or/cm$^2$, without growth (e.g., from a massive initial contamination) will not immediately cause a food to lose its acceptance quality, but such a food will spoil very rapidly following growth of the contaminants. Bioprocessed foods, in general, contain very high numbers of microorganisms ($10^{8–9}$ cells/g or /mL). However, under normal conditions, they are desirable types, and the

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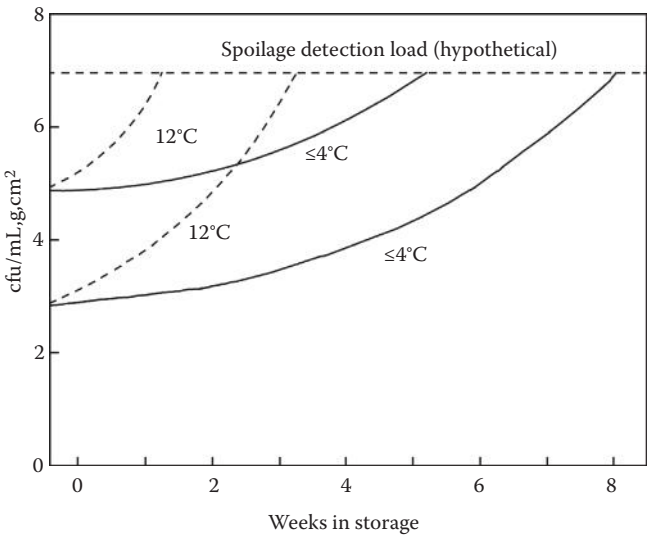



**Figure 19.1 Graphical illustration showing influence of initial bacterial levels and storage temperatures on shelf life of refrigerated product.**

fermented foods are not considered spoiled. Spoilage of these foods can occur because of growth of undesirable bacteria, such as slime formation and off-flavor in cottage cheese by *Alcaligenes* and *Pseudomonas* spp. In such products, selective methods should be used to determine the populations of undesirable bacteria or yeasts.[2,3,5]

## *Predominant Microorganisms*

The microbiological profile of a food is quite different from that of a pure culture growing in a laboratory medium. An unspoiled, nonsterile food generally contains many types of microorganisms, such as bacteria, yeasts, and molds (also viruses) from different genera, maybe more than one species from the same genus and even more than one strain from the same species. The population level of each type can vary greatly (Chapter 20). However, when the same food is spoiled, it is found to contain predominantly one or two types, and they may not even be present initially in the highest numbers in the unspoiled or fresh product. Among the different species initially present and capable of growing in a particular food, only those with the shortest generation time under the storage conditions attain the numbers rapidly and cause spoilage. In a study, a beef sample (pH 6.0) was found to initially contain approximately $10^3$ bacterial cells/g with relative levels of *Pseudomonas* spp. of 1%, *Acinetobacter* and *Moraxella* 11%, *Brochothrix thermosphacta* 13%, and others (*Micrococcus*, *Staphylococcus*, *Enterobacteriaceae*, lactic acid bacteria, etc.) 75%. Following aerobic storage at 2°C for 12 days, the population reached $6 \times 10^7$ cells/g with the relative levels of *Pseudomonas* spp. at 99% and all others at 1%. Many of the bacterial species present initially could grow at the storage condition of the meat, but *Pseudomonas* spp. had the shortest generation time. As a result, initially even they constituted only 1% of the total population; after 12 days, they became predominant (99%). If the same meat sample were stored at 2°C anaerobically (such as in a vacuum package) until the population had reached $10^7$/g, the predominant bacteria would have

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been, with most probability, facultative anaerobic *Lactobacillus* or *Leuconostoc*, or both, because of their growth advantages.[1]

In this context, it is important to recognize that the generation time of a microbial species, even under optimum conditions of growth, is much longer in a food than in a microbiological broth. Also, under the same storage conditions, the growth behavior of a microbial mixed population can be quite different in a food as compared to a broth. Because of this, the predominant types obtained following growing an initial mixed microbial population from a food in a broth and in the same food under identical conditions could be different. Although growing in a broth is convenient and, if properly designed, can provide valuable initial information, it is always better to have studies with specific foods.

## Food Spoilage Molds

Many species of molds are involved in food spoilage primarily on solid foods with very low water activity levels (0.7–0.8). They grow in a wide pH range of 3–8. The most common spoilage species are *Mucor*, *Rhizopus*, *Botrytis*, *Penicillium*, and *Aspergillus*. Some of these spoilage molds produce mycotoxins (toxigenic), such as *Penicillium* and *Aspergillus*. Fruits and vegetables are highly susceptible to mold spoilage. For example, *Botrytis* sp. is known to cause spoilage of fruits, such as raspberries, grapes, strawberries, kiwi fruit, pears, peaches, plums, and cherries, and vegetables, such as carrots, lettuce, peas, and beans, while *Mucor* and *Rhizopus* spp. affect apples, strawberries, etc.[6]

## Food Spoilage Yeasts

Yeasts generally cause spoilage in foods containing high sugar or salt content, such as maple syrup, fermented foods (pickles, sauerkrauts), and alcoholic beverages. In addition, they also grow in fruits and juices with a low pH and on the surface of meat and cheese. The major spoilage yeasts include *Saccharomyces* spp., *Zygosaccharomyces*, *Candida, and Dekkera/Brettanomyces*.[7]

## Some Important Food Spoilage Bacteria

Theoretically, any microorganism (including microorganisms used in food fermentation and pathogens) that can multiply in a food to reach a high level (spoilage detection level) is capable of causing it to spoil. Yet, in reality, bacterial species from only several genera have been implicated more with spoilage of most foods. This is dictated by the bacterial characteristics, food characteristics, and the storage conditions (Chapter 2). The influence of some of these factors in determining predominant spoilage bacteria in foods is briefly discussed.[2,3] Pathogens are not included here.

### *Psychrotrophic Bacteria*

As indicated before, psychrotrophic bacteria constitute the bacterial species capable of growing at 5°C and below but multiply quite rapidly at 10°C–25°C and even at higher temperatures.[5] Many foods are stored on ice (chilling) and in refrigerators, and some are expected to have a long shelf life (50 days or more). Between processing and consumption, they can be temperature abused to 10°C and higher. Psychrotrophic bacteria (also many yeasts and molds that are psychrotrophic)

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can cause spoilage in these foods. If the food is stored under aerobic conditions, psychrotrophic aerobes are the predominant spoilage bacteria. In foods stored under anaerobic conditions (also in the interior of a prepared food), anaerobic and facultative anaerobic bacteria predominate. If the food is given low-heat treatment and not exposed to post-heat contamination during storage at low temperatures, psychrotrophic thermoduric bacteria can cause it to spoil.

### *Some Important Psychrotrophic Aerobic Spoilage Bacteria*

These include *Pseudomonas fluorescens*, *Pse. fragi*, other *Pseudomonas* species, *Acinetobacter*, *Moraxella*, and *Flavobacterium*. (Some molds and yeasts are included in this group.)

### *Some Important Psychrotrophic Facultative Anaerobic Spoilage Bacteria*

These include *Bro. thermosphacta*, *Lactobacillus viridescens*, *Lab. sake*, *Lab. curvatus*, unidentified *Lactobacillus* spp., *Leuconostoc carnosum*, *Leu. gelidum*, *Leu. mesenteroides*, unidentified *Leuconostoc* spp., some *Enterococcus* spp., *Alcaligenes* spp., *Enterobacter* spp., *Serratia liquefaciens*, some *Hafnia* and *Proteus* spp., and *Shewanella* (previously *Alteromonas*) putrefaciens (and some microaerophilic yeasts).

### *Some Important Thermoduric Psychrotrophs*

These include facultative anaerobes, such as spores of *Bacillus coagulans* and *Bac. megaterium*; some strains of *Lab. viridescens*; and anaerobes, such as spores of *Clostridium laramie*, *Clo. estertheticum*, *Clo. algidicarnis*, *Clo. putrefaciens*, and unidentified *Clostridium* spp. The spores survive low-heat treatment. Following germination and outgrowth, the cells grow at low temperatures.

When a food is temperature abused above 5°C (such as during transport or display in stores), some true mesophiles (growth temperature range 15°C–45°C, optimum 25°C–40°C) can also grow. However, at 10°C–15°C, psychrotrophs will generally grow much faster than these mesophiles.

## *Thermophilic Bacteria*

By definition, the bacteria in this group grow between 40°C and 90°C with optimum growth at 55°C–65°C. Some high-heat-processed foods are kept warm between 50°C and 60°C for a long period of time (at delis, fast-food establishments, and restaurants). Spores of some thermophilic *Bacillus* and *Clostridium* spp. can be present in these heat-treated foods, which, at warm temperatures, germinate and multiply to cause spoilage. In addition, some thermoduric vegetative bacteria surviving low-heat processing (such as pasteurization) or thermophiles getting in food as post-heat contamination can also multiply in these warm foods, especially if the temperature is close to 50°C. These include some lactic acid bacteria, such as *Pediococcus acidilactici* and *Streptococcus thermophilus*, as well as some *Bacillus* and *Clostridium* spp. They can also survive and cause spoilage of foods that are cooked at low heat (60°C–65°C as for some processed meats) or kept warm for a long time.

## *Aciduric Bacteria*

Bacteria that can grow relatively rapidly in food at pH 4.6 or below are generally regarded as aciduric (or acidophilic). They are usually associated with spoilage of acidic food products, such as fruit juices,

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pickles, salsa, salad dressings, mayonnaise, and fermented sausages. Heterofermentative lactic acid bacteria (such as *Lab. fructivorans*, *Lab. fermentum*, and *Leu. mesenteroides*) and homofermentative lactic acid bacteria (such as *Lab. plantarum* and *Ped. acidilactici*) have been associated with such spoilage. (Yeasts and molds are aciduric and thus are also associated with spoilage of such foods.)

## Significance of Foods

### *Food Types*

Foods differ greatly in their susceptibility to spoilage by microorganisms. This is mainly because of their differences in intrinsic factors ($A_W$, pH, O-R potential, nutrient content, antimicrobial substances, and protective structures). A food with a lower $A_W$ (~0.90) or a lower pH (~5.3) is less susceptible to bacterial spoilage than one with $A_W$ of approximately 0.98 or a pH of approximately 6.4. However, molds and yeasts will probably grow equally well under both conditions. The influence of each of the intrinsic parameters on microbial growth has been described in Chapter 6. On the basis of susceptibility of spoilage, foods can be grouped as perishable (spoil quickly in days), semiperishable (have a relatively long shelf life of a few weeks or months), and nonperishable (have a very long shelf life of many months or years). In addition to intrinsic parameters, extrinsic parameters (storage conditions) play important roles in determining the ease of microbial spoilage of many foods.[3–5]

### *Food Nutrients*

Microbial growth in a food is associated with the metabolism of some food carbohydrates, proteinaceous and nonprotein nitrogenous (NPN) compounds, and lipids. The influences of major types of carbohydrates (polysaccharides, trisaccharides, disaccharides, monosaccharides, and sugar alcohols), proteinaceous compounds (proteins, peptides), NPN compounds (amino acids, urea, creatine, and trimethylamine oxide), and lipids (triglycerides, phospholipids, fatty acids, and sterols) present in foods on microbial spoilage are briefly discussed here. The metabolic pathways of some of these compounds by microorganisms have also been discussed in Chapter 8 and Chapter 12. It is evident from previous discussions that microorganisms differ greatly in their abilities to metabolize different food nutrients (such as the ability or inability to utilize cellulose and lactose as carbon sources, casein as a nitrogen source, and oxidation of oleic acid). Similarly, the same nutrient (substrate) can be utilized by different microorganisms by different metabolic pathways to produce different end products (e.g., glucose metabolized by homolactic and heterolactic acid bacteria). The same nutrient (substrate) can be degraded to produce different end products under aerobic and anaerobic metabolism (respiration and fermentation, respectively). Thus, glucose is metabolized (catabolized) by *Micrococcus* spp. aerobically to produce $CO_2$ and $H_2O$ and by *Lab. acidophilus* anaerobically to produce mainly lactic acid. *Saccharomyces cerevisiae* metabolizes glucose aerobically to $CO_2$ and $H_2O$ but anaerobically to ethanol and $CO_2$. Under specific conditions, some microorganisms can also synthesize (anabolism) polymeric compounds as end products, such as dextran (polymer of glucose) production by *Leu. mesenteroides* while metabolizing sucrose. Some microorganisms can also secrete extracellular enzymes to break down large molecular nutrients (polymers) in a food (such as the breakdown of starch by amylase produced by some molds). Finally, some microorganisms can synthesize pigments while growing in a food (such as *Micrococcus luteus* producing a yellow pigment).

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**Table 19.1 Some End Products from Microbial Metabolism of Food Nutrients**

| *Food Nutrient* | *End Products* |
|---|---|
| Carbohydrates | $CO_2$, $H_2$, $H_2O_2$, lactate, acetate, formate, succinate, butyrate, isobutyrate, isovalerate, ethanol, propanol, butanol, isobutanol, diacetyl, acetoin, butanediol, dextran, levans |
| Proteinaceous and NPN compounds | $CO_2$, $H_2$, $NH_2$, $H_2S$, amines, keto-acids, mercaptans, organic disulfides, putrescine, cadaverine, skatole |
| Lipids | Fatty acids, glycerol, hydroperoxides, carbonyl compounds (aldehydes, ketones), nitrogenous bases |

Thus, metabolism of food nutrients during growth of microorganisms in a food can adversely change its acceptance quality in several ways. Some of the changes are odor (resulting from production of volatile end products), color (pigment production or oxidation of natural color compounds, such as oxidation of meat myoglobin), texture (the breakdown of pectin by pectinases in vegetables, softening of the tissues in meat by proteinases, or thickening of milk by proteolytic enzymes), accumulation of gas (resulting from production of $CO_2$, $H_2$, or $H_2S$), formation of slime (resulting from production of dextran and different types of exopolysaccharides or too many microbial cells resulting in confluent growth), and accumulation of liquid (purge accumulation in fresh and processed meats resulting from breakdown of structures holding the water of hydration). Some of these changes also occur from the effect of microbial metabolites on food pH. Production of organic acids by microorganisms, causing a lowering of food pH, can reduce the water-holding ability of food (such as growth of some lactic acid bacteria in low-fat, high-pH-processed meat products). Similarly, production of basic compounds by microorganisms in a food can shift its pH to the alkaline side and reduce its acceptance quality (such as decarboxylation of amino acids in some low-heat-processed meat products with the production of amines, shifting the pH to basic and changing product color from light brown to pink in some processed meats; Chapter 21).

Table 19.1 lists some of the end products of microbial metabolism of food nutrients that are attributed to food spoilage. The end products vary with the nature of metabolism (e.g., aerobic respiration, anaerobic respiration, or fermentation; Chapters 8 and 12). It is evident that many of these metabolites are able to produce the changes associated with microbial food spoilage (change in odor, gas formation, or slime formation).[1–5]

## *Utilization of Food Nutrients*

Almost all foods contain some amounts of carbohydrates, proteinaceous and NPN compounds, and lipids that are available for use by microorganisms during growth. However, the characteristics of food spoilage differ greatly because of differences in the nature and the amount of a specific nutrient present in a food, the type of microorganisms growing in the food, and the nature of metabolism (respiration or fermentation). In general, for energy production, microorganisms prefer to use metabolizable monosaccharides, disaccharides, and large carbohydrates first; followed by NPN, small peptides, and large proteinaceous compounds; and, finally, lipids. However, again, metabolic characteristics depend on whether a particular species can use a specific carbohydrate (such as the ability or inability to utilize lactose) and the concentration it presents (limiting or high concentration). Also, with any nutrient, small molecules are used first before large molecules (polymers).

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If a food has carbohydrates that can be fermented by the contaminating microorganisms, then it will usually be metabolized first. If the metabolizable carbohydrates are present in sufficient quantities, then the metabolic pathway remains unchanged during rapid growth. However, if the carbohydrates are present in limited concentrations, then after the carbohydrates are used up the microorganisms usually start using NPN, small peptides, and other proteinaceous compounds. For example, yeasts growing in a fruit juice containing relatively high amounts of metabolizable carbohydrates (fructose, glucose, and sucrose) will produce either $CO_2$ and $H_2O$ (aerobically) or alcohol and $CO_2$ (anaerobically). However, *Pse. fluorescence* growing aerobically in fresh meat with limited amounts of glucose will first metabolize it and then start metabolizing free amino acids and other NPN compounds. If it is allowed to grow for a long time, it will produce extracellular proteinases to break down meat proteins to produce small peptides and amino acids for further metabolism. With time, it may even be able to produce lipases to break down meat lipids and use up some fatty acids. In a food (such as milk) containing large amounts of both carbohydrates (lactose) and proteins, a lactose-metabolizing microorganism will preferentially utilize the lactose and produce acid or acid and gas (*Lac. lactis* will produce lactic acid and *Leuconostoc* spp. will produce acid and gas), but a microorganism unable to utilize lactose will use the NPN and proteinaceous compounds for growth (*Pseudomonas* spp.). The spoilage patterns of these bacteria will be quite different.

In a mixed microbial population, as present normally in a food, the availability and amount of metabolizable carbohydrates greatly affect the spoilage pattern. Fresh meats, because of a low level of glucose, are susceptible to spoilage through microbial degradation of NPN and proteinaceous compounds. However, if a metabolizable carbohydrate (such as glucose, sucrose, or lactose) is added to meat, metabolism of carbohydrates will predominate. If lactic acid bacteria are present as natural microflora and the growth environment is favorable, they will produce enough acids to arrest the growth of many normal microflora that preferentially metabolize NPN and proteinaceous compounds (e.g., Gram-negative pyschrotroph). This is commonly known as the protein-sparing effect (proteins are not metabolized). In the formulation of many processed meat products, metabolizable simple carbohydrates (usually glucose) are used to produce the protein-sparing effect.[1,2]

## Microbial Growth in Succession

Intrinsic and extrinsic factors or environments of a food dictate which, among the mixed microbial species normally present, will multiply rapidly and become predominant to cause spoilage. However, as the predominant types grow, they produce metabolites and change the food environment. In the changed environment, some other species, initially present but previously unable to compete, may be in a favorable position to grow rapidly and again change the food environment further to enable a third type to grow rapidly. If sufficient time is given, the predominant microbial types and the nature of spoilage of a food can change. Sequential growth of *Lactococcus* spp., aciduric lactose-negative *Bacillus* sp., and Gram-negative rods (such as *Pseudomonas* spp.) in a milk sample can be used as a hypothetical example. Initially, rapid growth of *Lactococcus* spp. (able to metabolize lactose) under a favorable growth condition will reduce the pH from original 6.5 to 5.0 and reduce the growth rate of many other microbial species present. As the pH drops below 5.0, the generation time of *Lactococcus* spp. becomes longer. However, *Bacillus* sp., because of its aciduric nature, can then start multiplication, metabolize proteins, and increase the pH (say 5.8). In the high pH, the *Pseudomonas* spp. present initially can then grow by metabolizing NPN and proteinaceous compounds and increase the pH further by producing basic metabolites (amines, $NH_3$). This way, the predominant spoilage microorganisms and the metabolites associated with spoilage (e.g., nature of spoilage) of a food can change if a food is stored for a fairly long time.[3]

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## Conclusion

Growth of microorganisms in food to a high level causes detectable changes in the quality of food, which is generally termed as spoilage. Spoilage characteristics differ with the differences in microbial type and the food component being metabolized. Different aspects of food spoilage are discussed in this chapter. In Chapter 20, spoilage of specific food groups, the predominant microorganisms involved, and the nature of spoilage are discussed.

### QUESTIONS

1. Describe when a food is considered spoiled by microorganisms. You put a packet of lunchmeat in the refrigerator two to three weeks back and forgot about it. When you opened it, you thought it had a slightly sour odor. You asked your friends to smell it, but they said it smelled fine. What could be the reasons for this difference?
2. You opened a 2 L bottle of a soft drink and kept it in the refrigerator and forgot about it. After two weeks, you found some molds growing on the surface of the liquid. Describe the sequence of events that resulted in this spoilage.
3. Discuss the significance of microbial types and numbers in food spoilage.
4. A food is usually spoiled by limited (usually one or two) types of microorganisms among the many types initially present. Describe how this predominance is established.
5. At present, psychrotrophic bacteria seem to be the most important spoilage bacteria. Suggest the possible reasons.
6. How can psychrotrophic bacteria be arbitrarily grouped? Give examples of two bacterial species from each group. Also, give two examples each of thermoduric, thermophilic, and aciduric spoilage bacteria.
7. Define aerobic respiration, anaerobic respiration, and fermentation. List four metabolites (for each) associated with (a) odor and (b) gas formation from food nutrients.
8. Discuss the importance of preferential nutrient metabolism by microorganisms and the nature of food spoilage from this, using meat and milk as examples.

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# Chapter 20

# Spoilage of Specific Food Groups

## Introduction

The predominant microbial types normally expected to be present in different food groups have been listed in Chapters 2 and 4. Initially, a food produced under proper sanitary conditions generally contains microorganisms at a level (per g, mL, or $cm^2$) much lower than that at which spoilage is detected. Subsequently, growth of some of the microbial species among those initially present enables the microorganisms to reach the spoilage detection level. Many factors dictate which species will multiply relatively rapidly to become the predominant spoilage microorganisms. Along with microbial types, food types, and food environments (both intrinsic and extrinsic factors) have important roles in determining the predominant spoilage microflora in a food. These aspects have been discussed in Chapters 4 and 6. In this chapter, predominant microorganisms associated with the spoilage of different food groups are described. As the foods in each group have similarities in nutrient content and the environment, similar microbial types are generally associated with spoilage. Pathogens are not included.

## Fresh and Ready-to-Eat Meat Products

### *Raw Meat*

Fresh meats from food animals and birds contain a large group of potential spoilage bacteria that include species of *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Shewanella*, *Alcaligenes*, *Aeromonas*, *Escherichia*, *Enterobacter*, *Serratia*, *Hafnia*, *Proteus*, *Brochothrix*, *Micrococcus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, and *Clostridium*, as well as yeasts and molds.[1–3] The predominant spoilage flora in a meat is determined by nutrient availability, oxygen availability, storage temperature, pH, storage time of the product, and generation time of the microorganisms present in a given environment. Post-rigor meats are rich in nonprotein nitrogenous (NPN) compounds (ca. 13 mg/g amino acids and creatine), peptides, and proteins, but contain low



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concentrations of carbohydrates (ca. 1.3 mg/g glycogen, glucose, glucose-6-phosphate) with a pH of approximately 5.5 and $A_W$ more than 0.97. Dark-firm-dry (DFD) meats have almost no carbohydrates and a pH of 6.0 or above.

To delay microbial spoilage, fresh meats are stored at refrigerated temperatures (≤5°C) unless the facilities are not available. Thus, normally psychrotrophic bacteria are the most predominant types in raw meat spoilage. Under aerobic storage at low temperatures, growth of psychrotrophic aerobes and facultative anaerobes is favored. In retail-cut meats, because of a shorter generation time, *Pseudomonas* spp. grows rapidly, using glucose first and then amino acids; the metabolism of amino acids is accompanied by the production of malodorous methyl sulfides, esters, and acids. In meats with high pH or low glucose content, or both, *Acinetobacter* and *Moraxella*, which preferentially metabolize amino acids instead of glucose, can grow rapidly and produce undesirable odors. Spoilage by these strict aerobes in the form of off-odor is detected at a population of approximately $10^{7-8}$ cells/cm$^2$ and slime at approximately $10^{8-9}$ cells/cm$^2$. The oxygenated red color of myoglobin undergoes oxidation to produce gray or brown metmyoglobin. DFD meats are spoiled more rapidly inasmuch as the bacteria utilize amino acids immediately because of the absence of carbohydrates.

Refrigerated meat in a modified atmosphere, such as in a mixture of $CO_2$ and $O_2$, favors growth of facultative anaerobic *Brochothrix thermosphacta*, especially in meat with pH 6.0 or higher (DFD meat). It metabolizes glucose to acetic acid and acetoin and leucine and valine to isovaleric and isobutyric acids to produce off-odor (cheesy odor). Under anaerobic conditions, it metabolizes glucose to produce small amounts of lactic acid (which is not considered a cause of spoilage).

Psychrotrophic facultative anaerobes and anaerobes can grow in vacuum-packaged meat to produce different types of spoilage. *Lactobacillus curvatus* and *Lab. sake* metabolize glucose to produce lactic acid and the amino acids leucine and valine to isovaleric and isobutyric acids. These volatile fatty acids impart a cheesy odor in meat at a population level of more than $10^{7-8}$ cells/cm$^2$. This spoilage is not considered to be highly undesirable because, after opening the package, the odor disappears. However, when they metabolize cysteine and produce $H_2S$ gas, the products have an undesirable odor and color. Heterofermentative *Leuconostoc carnosum* and *Leu. gelidum* produce $CO_2$ and lactic acid, causing accumulation of gas and liquid in the package. *Shewanella putrefacience*, which can grow under both aerobic and anaerobic conditions, metabolizes amino acids (particularly cysteine) to produce methylsulfides and $H_2S$ in large quantities. Along with offensive odors, they adversely affect the normal color of meats. $H_2S$ oxidizes myoglobin to a form of metmyoglobin, causing a green discoloration. Facultative anaerobic *Enterobacter*, *Serratia*, *Proteus*, and *Hafnia* species metabolize amino acids while growing in meat to produce amines, ammonia, methylsulfides, and mercaptans and cause putrefaction. Some strains also produce $H_2S$ in small amounts to cause greening of the meat. Because amines and ammonia are produced, the pH of the meat usually changes to alkaline range, and meat can have a pinkish to red color. Psychrotrophic *Clostridium* spp., such as *Clostridium laramie*, have been found to cause spoilage associated with proteolysis and loss of texture of meat, accumulation of liquid in the bag, and offensive odor, with an $H_2S$ smell predominating. The color of the meat becomes unusually red initially and then changes to green (because of oxidation of myoglobin by $H_2S$). Some *Clostridium* spp., and probably *Enterococcus*, can cause spoilage of beef rounds and ham deep near the bone designated as bone sour or bone taint.

Comminuted meats spoil more rapidly than retail cuts because they have more surface area. Under aerobic storage, growth of aerobic bacteria (predominantly *Pseudomonas* spp.) causes changes in odor, texture, color, and sliminess. The inside is initially microaerophilic (because of dissolved oxygen from trapped air), which then changes to anaerobic, and growth of facultative

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bacteria predominates. In vacuum-packaged products, growth of lactic acid bacteria predominates in the initial stage. Heterofermentative lactic acid bacteria can cause accumulation of gas in the package. Some lactic acid bacteria also grow by utilizing amino acids. When the glucose is used up, Gram-negative facultative anaerobes grow and degrade amino acids to produce a putrid odor.

To reduce spoilage of fresh meats, the initial microbial level should be reduced. In addition, storage at low temperatures (close to 0°C to –1°C), modified atmosphere packaging, and vacuum packaging should be done. Several other methods to reduce initial microbial load and slow the growth rate of Gram-negative rods are being either used or tested. These include the addition of small amounts of organic acids to lower the pH of meat (slightly above pH 5.0), drying of meat surfaces (to reduce $A_W$), and a combination of the factors given previously, including lower storage temperature.

## *Ready-to-Eat Meat Products*

This group includes high-heat-processed and low-heat-processed uncured and cured meat products. High-heat-processed cured and uncured meats are given heat treatment to make them commercially sterile. Thus, they may only have some thermophilic spores surviving, which will not germinate unless the products are temperature abused. This aspect has been explained along with microbial growth in the canned products and canning (see Chapters 4 and 33).

Low-heat-processed uncured meats, such as roasts, are given heat treatment at an internal temperature of 140°F–160°F (60°C–71°C). Generally, the surface of the meats (and thus most of the microorganisms) is exposed to the final temperature for one hour or more, depending on the size of the meat (which could be more than 10 lb [4.6 kg]). Under this condition, only the spores of *Bacillus* and *Clostridium* spp. and some extremely thermoduric vegetative species (*Lab. viridescens*, some *Enterococcus*, *Micrococcus* inside the product) can survive. However, the products, even when cooked in bags and not cut into portions, are opened and handled before final vacuum packaging and refrigerated storage.[1,3] Many types of microorganisms can enter as post-heat contaminants into the products from equipment, personnel, water, and air. In some situations, spices and other ingredients are added to the products after heating, which, in turn, can be the source of microbial contamination of the products. Some products are sliced before vacuum packaging, which increases the chances of contamination on the surface area of the product from the equipment and environment. Psychrotrophic facultative anaerobic and anaerobic bacteria have been implicated in the spoilage of these products. In vacuum-packaged roast beef, roast turkey, and roast chicken, heterofermentative *Lactobacillus* spp. and *Leuconostoc* spp., as post-heat contaminants, have been involved in spoilage with the accumulation of large quantities of gas ($CO_2$) and liquid (resulting from acid production) inside the bag without causing much flavor, color, or texture change. Gas production and purge accumulation in vacuum-packaged roast beef by psychrotrophic *Clostridium* spp., along with off-flavor and color changing from brown to pink to red (after four weeks), have been detected. In spoiled sliced roast beef, the brown color of roast beef changes to pink (in one week), and the beef has a putrid odor (after six weeks). *Proteus* and *Hafnia* spp. are involved in spoilage of this product (Chapter 21).

Low-heat-processed cured meats include a wide variety of products, such as franks, bologna, ham, and luncheon meats made from beef, pork, and poultry. Meats are mixed with different types of additives to improve color, texture, flavor, shelf life, and safety. Some of these additives are nitrite, salt, dextrose, phosphate, sorbate, erythorbate, nonfat dry milk, soy proteins, carrageenan, and different types of spices. Some of the reduced-fat products have very low fat (≤2%) as compared to products normally with ≥30% fat (some regular franks). Some of the products, especially

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low-fat products, have pH values as high as 6.8 (compared to pH < 6.0 in other products) because of the addition of phosphate and other ingredients and higher moisture. They are cooked at an internal temperature of 150°F–160°F (65°C–71°C). Depending on the size of the products, the surface can be exposed to the final temperature for a longer time than the center. As the products are made from ground or chopped meats, microorganisms are distributed throughout the products (as compared with mainly on the surfaces of a roast or retail cuts, but preformed roasts, steaks, etc. made from chopped meat have microorganisms throughout the products). Thus, the thermodurics surviving cooking are present throughout the products. The products, following cooking, are extensively handled before they are again vacuum packaged or packed with modified atmospheres of $CO_2$ or $CO_2 + N_2$ or sold unpackaged. Depending on the post-heat treatment processing steps (slicing, portioning, and skinning) involved, the products can be contaminated with microorganisms from equipment, personnel, air, and water. Some of the microorganisms establish themselves in the processing environment, especially in places difficult to sanitize (or dead spots). In products such as franks, they contaminate only the surface; but in sliced products, they are distributed over the slices following cutting.

Vacuum-packaged and gas-packaged products, during storage, can be spoiled by psychrotrophic *Lactobacillus* spp. (such as homofermentative *Lab. sake*, *Lab. curvatus*, heterofermentative *Lab. viridescens*) and *Leuconostoc* spp. (such as *Leu. carnosum*, *Leu. gelidum*, and *Leu. mesenteroides*). In vacuum-packaged products, the package becomes loose because of growth. The products, depending on bacterial types, show a cloudy appearance, a large accumulation of gas ($CO_2$) and liquid, slime formation resulting from bacterial cells, and dextran production by *Leuconostoc* spp. in products containing sucrose (or honey) and a slight acidic odor. In some products, growth of *Serratia* spp. (*Serratia liquefaciens*) causes amino acid breakdown, which then causes an ammonia-like odor (diaper smell). Low-fat-containing, vacuum-packaged turkey rolls (portions or sliced) develop a pink color after approximately five weeks, probably because of growth of some lactic acid bacteria. Under aerobic conditions of storage (unpackaged or permeable-film wrapped), some *Lactobacillus* spp. rapidly produce $H_2O_2$, which can oxidize nitrosohemochrome (formed by heating nitrosomyoglobin) to brown metmyoglobin or to oxidized porphyrins, some of which are green in color. Refrigerated luncheon meat can develop brown to yellow spots with fluorescence from the growth of *Enterococcus casseliflavus* (Chapter 21). Yeasts and molds can grow inside if a package, because of even undetectable damage, allows air to leak in.

Unpackaged cooked products that do not have carbohydrates can become putrid from the growth of and protein degradation by proteolytic Gram-negative bacteria. If the products are stored for long times, yeasts and molds can also grow, causing off-flavor, discoloration, and sliminess. Because of growth of $H_2O_2$-producing lactic acid bacteria, they can have green to gray discolorations.

## Eggs and Egg Products

### *Shell Eggs*

The pores in the eggshell and inner membrane do not prevent entrance of bacteria and hyphae of molds, especially when the pore size increases during storage. The presence of moisture enhances the entrance of motile bacteria. The albumen (egg white) and yolk have approximately 0.5%–1.0% carbohydrate and are high in proteins but low in nonprotein nitrogen. During storage, the pH can shift to the alkaline side (pH 9–10). In addition, lysozyme (causes lysis of bacterial cell wall

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mucopeptide), conalbumin (chelates iron), antivitamin proteins (avidin binds riboflavin), and protease inhibitors in eggs inhibit microbial growth. The most predominant spoilage of shell eggs is caused by Gram-negative motile rods from several genera that include *Pseudomonas*, *Proteus*, *Alcaligenes*, *Aeromonas*, and the coliform group. The different types of spoilage are designated as rot. Some examples are green rot, causing greening of albumen because of growth of *Pseudomonas fluorescens*; black rot, causing muddy discoloration of yolk because of $H_2S$ production by *Proteus vulgaris*; and red rot by *Ser. mercescens*, caused by production of red pigment. On some occasions, molds from genera *Penicillium*, *Alternaria*, and *Mucor* can grow inside eggs, especially when the eggs are spoiled, and produce different types of fungal rots.[1]

### *Egg Products*

Liquid eggs consisting of whole eggs or yolks or whites only are generally pasteurized or frozen, or both, to prevent microbial growth. If the liquid products are held at room temperature following breaking and prior to pasteurization, spoilage bacteria can grow and cause off-flavor (putrid), sourness, or fish flavor (resulting from formation of trimethylamine). Pasteurized eggs at refrigerated temperatures have a limited shelf life unless additional preservatives are added. The predominant bacteria in pasteurized products are some Gram-positive bacteria that survive pasteurization, but spoilage is mainly caused by psychrotrophic Gram-negative bacteria getting into products after heat treatment. Dried eggs are not susceptible to microbial spoilage because of low $A_W$.

## Fish, Crustaceans, and Mollusks

### *Fish*

Fish harvested from both fresh and saltwater are susceptible to spoilage through autolytic enzyme actions, oxidation of unsaturated fatty acids, and microbial growth. Protein hydrolysis by autolytic enzymes (proteinases) is predominant if the fish are not gutted following catch. Oxidation of unsaturated fatty acids is also high in fatty fish. Microbial spoilage is determined by the microbial types, their level, fish environment, fish types, methods used for harvest, and subsequent handling. These aspects have been discussed in Chapter 4. Fish tissues have high levels of NPN compounds (free amino acids, trimethylamine oxide, and creatinine), peptides, and proteins, but almost no carbohydrates; the pH is generally above 6.0. Gram-negative aerobic rods, such as *Pseudomonas* spp., *Acinetobacter*, *Moraxella*, and *Flavobacterium*, and facultative anaerobic rods, such as *Shewanella*, *Alcaligenes*, *Vibrio*, and coliforms, are the major spoilage bacteria. However, because of the relatively shorter generation time, spoilage by psychrotrophic *Pseudomonas* spp. predominates under aerobic storage at both refrigerated and slightly higher temperatures. In fish stored under vacuum or $CO_2$, lactic acid bacteria (including *Enterococcus*) can become predominant.[4]

Gram-negative rods initially metabolize the NPN compounds by decay (oxidation), followed by putrefaction to produce different types of volatile compounds, such as $NH_3$, trimethylamine ($N{:}CH_3$ from reduction of trimethylamine oxide), histamine (from histidine, the cause of Scombroid poisoning), putrescine, cadaverine, indoles, $H_2S$, mercaptans, dimethyl sulfide (especially by *She. putrefaciens*), and volatile fatty acids (acetic, isobutyric, and isovaleric acids). Proteolytic bacterial species also produce extracellular proteinases that hydrolyze fish proteins and supply peptides and amino acids for further metabolism by spoilage bacteria. The volatile compounds produce different types of off-odors, namely, stale, fishy (because of trimethylamine), and

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putrid. Bacterial growth is also associated with slime production, discoloration of gills and eyes (in whole fish), and loss of muscle texture (soft because of proteolysis).

In fish stored by vacuum or by $CO_2$ packaging, growth of aerobic spoilage bacteria is prevented. However, anaerobic and facultative anaerobic bacteria can grow, including lactic acid bacteria. Under refrigeration, products have relatively long shelf life as a result of slower growth of spoilage bacteria. Salted fish, especially lightly salted fish, are susceptible to spoilage by halophilic bacteria, such as *Vibrio* (at lower temperatures) and *Micrococcus* (at higher temperatures). Smoked fish, especially with lower $A_W$, inhibit the growth of most bacteria. However, molds can grow on the surface. Minced fish flesh, surimi, and seafood analogs prepared from fish tissues generally have high initial bacterial levels as a result of extensive processing (ca. $10^{5-6}$/g). The types include those present in fish and those that get in during processing. These products, such as fresh fish, can be spoiled rapidly by Gram-negative rods unless frozen quickly or used soon after thawing. Canned fish (tuna, salmon, and sardines) are given heat treatment to produce commercially sterile products. They can be spoiled by thermophilic spore formers unless proper preservation and storage conditions are used.[1,4]

## Crustaceans

Microbial spoilage of shrimp is more prevalent than that of crabs and lobsters. Whereas crabs and lobsters remain alive until they are processed, shrimps die during harvest. The flesh of crustaceans is rich in NPN compounds (amino acids, especially arginine, trimethylamine oxide), contains approximately 0.5% glycogen, and has a pH above 6.0. The predominant microflora are *Pseudomonas* and several Gram-negative rods. If other necessary factors are present, the nature of spoilage is quite similar to that in fresh fish. Microbial spoilage of shrimp is dominated by odor changes resulting from production of volatile metabolites of NPN compounds (from decay and putrefaction), slime production, and loss of texture (soft) and color. If the shrimp are processed and frozen rapidly, the spoilage can be minimized. Lobsters are frozen following processing or sold live and thus are not generally exposed to spoilage conditions. Crabs, lobsters, and shrimp are also cooked to extend their shelf life. However, they are subsequently exposed to conditions that cause post-heat contamination and then stored at low temperatures (refrigerated and frozen). Blue crabs are steamed under pressure, and the meat is picked and marketed as fresh crabmeat. To extend shelf life (and safety), the meat is also heat processed (85°C for 1 min) and stored at refrigerated temperatures. Under refrigerated conditions, they have a limited shelf life because of growth of surviving bacteria and post-heat contaminants.

## Mollusks

As compared with fish and crustaceans, oyster, clam, and scallop meats are lower in NPN compounds but higher in carbohydrates (glycogen, 3.5%–5.5%) with pH normally above 6.0. The mollusks are kept alive until processed (shucked); thus, microbiological spoilage occurs only after processing. The resident microflora are predominantly *Pseudomonas* and several other Gram-negative rods. During refrigerated storage, microorganisms metabolize both NPN compounds and carbohydrates. Carbohydrates can be metabolized to produce organic acids by lactic acid bacteria (*Lactobacillus* spp.), enterococci, and coliforms, thereby lowering the pH. Breakdown of nitrogenous compounds primarily by *Pseudomonas* and *Vibrio*, especially at refrigerated temperatures, results in production of $NH_3$, amines, and volatile fatty acids.

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## Milk and Milk Products

### *Raw Milk*

Raw milk contains many types of microorganisms, including pathogens and spoilage coming from different sources.[5] The average composition of cow's milk is protein 3.2%, carbohydrates 4.8%, lipids 3.9%, and minerals 0.9%. Besides casein and lactalbumin, it has free amino acids, which can provide a good N source (and some C source, if necessary). As the main carbohydrate is lactose, those microorganisms with lactose-hydrolyzing enzymes (phospho-β-galactosidase or β-galactosidase) have an advantage over those unable to metabolize lactose. Milk fat can be hydrolyzed by microbial lipases, with the release of small-molecular volatile fatty acids (butyric, capric, and caproic acids).

Microbial spoilage of raw milk can potentially occur from the metabolism of lactose, proteinaceous compounds, fatty acids (unsaturated), and the hydrolysis of triglycerides. If the milk is refrigerated immediately following milking and stored for days, the spoilage will be predominantly caused by Gram-negative psychrotrophic rods, such as *Pseudomonas*, *Alcaligenes*, *Flavobacterium* spp., and some coliforms. *Pseudomonas* and related species, being lactose negative, metabolize proteinaceous compounds to change the normal flavor of milk to bitter, fruity, or unclean. They also produce heat-stable lipases (producing a rancid flavor) and heat-stable proteinases that have important implications; this is discussed in Chapter 22. The growth of lactose-positive coliforms produces lactic acid, acetic acid, formic acid, $CO_2$, and $H_2$ (by mixed-acid fermentation) and causes curdling, foaming, and souring of milk. Some *Alcaligenes* spp. (*Alc. faecalis*) and coliforms can also cause ropiness (sliminess) by producing viscous exopolysaccharides. However, if the raw milk is not refrigerated soon, growth of mesophiles, such as species of *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Micrococcus*, and coliforms, along with *Pseudomonas*, *Proteus*, and spore formers, such as *Bacillus* and *Clostridium*, predominates.[6] But lactose-hydrolyzing species, such as *Lactococcus* spp. and *Lactobacillus* spp., generally predominate, producing enough acid to lower the pH considerably and prevent or reduce growth of others. In such situations, curdling of milk and sour flavor are the predominant spoilages. If other microorganisms also grow, gas formation, proteolysis, and lipolysis become evident. Yeast and mold growth, under normal conditions, is generally not expected.

### *Pasteurized Milk*

Raw milk is pasteurized before it is sold for consumption as liquid milk. Thermoduric bacteria (*Micrococcus*, *Enterococcus*, some *Lactobacillus*, *Streptococcus*, *Corynebacterium*, and spores of *Bacillus* and *Clostridium*) survive the process. In addition, coliforms, *Pseudomonas*, *Alcaligenes*, *Flavobacterium*, and similar types can enter as postpasteurization contaminants. Pasteurized milk, under refrigerated storage, has a limited shelf life, mainly because of growth of these psychrotrophic contaminants. Their spoilage pattern is the same as described for raw milk spoilage. Flavor defects from their growth are detectable when the population reaches $\geq 10^6$ cells/mL. Growth of psychrotrophic *Bacillus* spp., such as *Bacillus cereus*, has been implicated in the spoilage of pasteurized refrigerated milk, especially when the levels of postpasteurization contaminants are low. Spores of psychrotrophic *Bacillus* spp., surviving pasteurization, germinate and multiply to cause a defect known as bitty. They produce the enzyme lecithinase, which hydrolyzes phospholipids of the fat globule membrane, causing aggregation of fat globules that adhere to the container

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surfaces. Production of rennin-like enzymes by psychrotrophic *Bacillus* spp. and others can cause sweet curdling of milk at a pH higher than that required for acid curdling.[7]

Ultrahigh temperature-treated (UHT) milk (150°C for a few seconds) is essentially a commercially sterile product that can only contain viable spores of some thermophilic bacteria. The UHT milk is not susceptible to spoilage at ambient storage temperatures but can be spoiled if exposed to high temperatures (40°C or above as observed with canned foods).

### Concentrated Liquid Products

Evaporated milk, condensed milk, and sweetened condensed milk are the principal types of concentrated dairy products susceptible to limited microbial spoilage during storage. All these products are subjected to sufficient heat treatments to kill vegetative microorganisms as well as spores of molds and some bacteria.

Evaporated milk is condensed whole milk with 7.5% milk fat and 25% total solids. It is packaged in hermetically sealed cans and heated to obtain commercial sterility. Under proper processing conditions, only thermophilic spores of spoilage bacteria can survive, and exposure to high storage temperatures (43°C or higher) can trigger their germination and subsequent growth. Under such conditions, *Bacillus* species, such as *Bac. coagulans*, can cause coagulation of milk (flakes, clots, or a solid curd).

Condensed milk is generally condensed whole milk and has 10%–12% fat and 36% total solids. The milk is initially given a low-heat treatment, close to pasteurization temperature, and then subjected to evaporation under partial vacuum (at ca. 50°C). Thus, it can have thermoduric microorganisms that subsequently can grow and cause spoilage. Other microorganisms can also get into the product during the condensing process. Even at refrigerated temperatures, this product has a limited shelf life, as does pasteurized milk.

Sweetened condensed milk contains approximately 8.5% fat, 28% total solids, and 42% sucrose. The whole milk is initially heated to a high temperature (80°C–100°C) and then condensed at approximately 60°C under vacuum and put into containers. Because of a low $A_W$, it is susceptible to spoilage from the growth of osmophilic yeasts (such as *Torula* spp.), causing gas formation. If the containers have enough headspace and oxygen, molds (e.g., *Penicillium* and *Aspergillus*) can grow on the surface.

### Butter

Butter contains 80% milk fat and can be salted or unsalted. The microbiological quality of butter depends on the quality of cream and the sanitary conditions used in the processing. Growth of bacteria (*Pseudomonas* spp.), yeasts (*Candida* spp.), and molds (*Geotrichum candidum*) on the surface causes flavor defects (putrid, rancid, or fishy) and surface discoloration. In unsalted butter, coliforms, *Enterococcus*, and *Pseudomonas* can grow favorably in the water phase (which has nutrients from milk) and produce flavor defects.

## Vegetables and Fruits

### Vegetables

Fresh vegetables contain microorganisms coming from soil, water, air, and other environmental sources, and can include some plant pathogens. They are fairly rich in carbohydrates (5% or more),

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**Figure 20.1 (See color insert.) Tomato spoilage by mold (*Mucor* spp.).**

low in proteins (ca. 1%–2%), and, except for tomatoes, have high pH. Microorganisms grow more rapidly in damaged or cut vegetables. The presence of air, high humidity, and higher temperatures during storage increases the chances of spoilage. The most common spoilage is caused by different types of molds, some of those from genera *Penicillium*, *Phytopthora*, *Alternaria*, *Botrytis*, and *Aspergillus* (Figure 20.1). Among the bacterial genera, species from *Pseudomonas*, *Erwinia*, *Bacillus*, and *Clostridium* are important.[8]

Microbial vegetable spoilage is generally described by a common term, "rot," along with the changes in the appearance, such as black rot, gray rot, pink rot, soft rot, and stem-end rot. In addition to changes in color, microbial rot causes off-odor and loss of texture. This is more apparent in ready-to-eat cut salads and sliced vegetables and fruits that are refrigerated for a long time (3–4 weeks) and can be temperature abused.

Refrigeration, vacuum or modified atmosphere packaging, freezing, drying, heat treatment, and chemical preservatives are used to reduce microbial spoilage of vegetables. Spoilage of canned vegetables, vegetable juices, and fermented vegetables are discussed later in this chapter.

## *Fruits*

Fresh fruits are high in carbohydrates (generally 10% or more), very low in proteins (≤1.0%), and have a pH of 4.5 or below. Thus, microbial spoilage of fruits and fruit products is confined to molds, yeasts, and aciduric bacteria (lactic acid bacteria, *Acetobacter*, *Gluconobactor*). Like fresh vegetables, fresh fruits are susceptible to rot by different types of molds from genera *Penicillium*, *Aspergillus*, *Alternaria*, *Botrytis*, *Rhizopus*, and others.[9] According to the changes in appearance, the mold spoilage is designated as black rot, gray rot, soft rot, brown rot, or others. Yeasts from genera *Saccharomyces*, *Candida*, *Torulopsis*, and *Hansenula* are associated with fermentation of some fruits, such as apples, strawberries, citrus fruits, and dates.[10] Bacterial spoilage associated with the souring of berries and figs has been attributed to the growth of lactic acid and acetic acid bacteria.

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To reduce spoilage, fruits and fruit products are preserved by refrigeration, freezing, drying, reducing $A_W$, and heat treatment. Spoilage of fruit juices, jams, and wine is discussed later.

## Soft Drinks, Fruit Juices and Preserves, and Vegetable Juices

Carbonated and noncarbonated soft drinks, fruit juices, and preserved and concentrated fruit juices and drinks are low-pH products (pH 2.5–4.0). The carbohydrate (sucrose, glucose, and fructose) content ranges from 5% to 15% in juices and drinks but is 40%–60% in concentrates and preserves. High sugar content reduces the $A_W$ of these products, which, in the concentrates and preserves, can be approximately 0.9. Carbonated beverages also have low O-R potential.

Among the microorganisms that can be present in these products, only aciduric molds, yeasts, and bacteria (*Lactobacillus*, *Leuconostoc*, and *Acetobacter*) are able to cause spoilage if appropriate preservation methods are not used.[11] In carbonated beverages, some yeast species from genera *Torulopsis*, *Candida*, *Pichia*, *Hansenula*, and *Saccharomyces* can grow and make the products turbid. Some *Lactobacillus* and *Leuconostoc* species can also grow to cause cloudiness, and *Leuconostoc* can cause ropiness (resulting from production of dextrans) of the products. Noncarbonated beverages can be similarly spoiled by yeasts, *Lactobacillus*, and *Leuconostoc* spp. In addition, if there is enough dissolved oxygen, molds (*Penicillium*, *Aspergillus*, *Mucor*, and *Fusarium*) and *Acetobacter* can grow; the latter produces acetic acid to give a vinegar-like flavor. Fruit juices are susceptible to spoilage by molds, yeasts, *Lactobacillus*, *Leuconostoc*, and *Acetobacter* spp. However, a particular type of juice may be susceptible to spoilage by one or another type of microorganism. Molds and *Acetobacter* can grow if enough dissolved oxygen is available. Yeasts can cause both oxidation (producing $CO_2$ and $H_2O$) and fermentation (producing alcohol and $CO_2$) of the products. *Acetobacter* can use alcohol to produce acetic acid. Heterofermentative *Lab. fermentum* and *Leu. mesenteroides* can ferment carbohydrates to lactate, ethanol, acetate, $CO_2$, diacetyl, and acetoin. In addition, *Leu. mesenteroides* and some strains of *Lab. plantarum* can produce slime as a result of production of dextran and other exopolysaccharides. In fruit drinks, *Lactobacillus* and *Leuconostoc* spp. can also convert citric and malic acids (additives) to lactic and acetic acids and reduce the sour taste (flat flavor). Because concentrated fruit drinks and fruit preserves have a low $A_W$ (0.9), only osmophilic yeasts can grow; molds also can grow if oxygen is available. A new group of bacterial species, *Alicyclobacillus* (e.g., *Alicyclobacillus acidoterrestris*), has been recognized recently to cause spoilage of low-pH fruits and vegetable juices.

To prevent growth of these potential spoilage microorganisms, several additional preservation methods are used for these products, which include heat treatment, freezing, refrigeration, and addition of specific chemical preservatives.

Tomato juice has a pH of approximately 4.3. It is generally given high heat treatment to kill vegetative microorganisms. However, bacterial spores can survive. Flat sour spoilage of tomato juice resulting from germination and growth of *Bac. coagulans* is documented. Most other vegetable juices have pH values between 5.0 and 5.8, and many have growth factors for lactic acid bacteria. These products are susceptible to spoilage because of the growth of many types of microorganisms. Effective preservation methods are used to control their growth.

## Cereals and Their Products

Some of the products susceptible to microbial spoilage include high-moisture cereal grains, germinated or sprouted seeds and grains, refrigerated dough, breads, soft pastas, and pastries.[1]

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## Grains and Seeds

Grains and seeds normally have 10%–12% moisture, which lowers the $A_W$ to approximately ≤0.6 and thus inhibits microbial growth. In cereal grains, during harvesting, processing, and storage, if the $A_W$ increases above 0.6, some molds can grow. Some species of storage fungi from genera *Aspergillus*, *Penicillium*, and *Rhizopus* can cause spoilage of high-moisture grains. Many types of lentils, beans, and seeds (alfalfa) are germinated and used as ready-to-eat products. During storage, Gram-positive and Gram-negative bacteria, yeasts, and molds can grow and produce off-flavor.

## Refrigerated Dough

Refrigerated dough (for biscuits, rolls, and pizzas) is susceptible to spoilage (gas formation) from the growth of psychrotrophic heterolactic acid bacterial species of *Lactobacillus* and *Leuconostoc*. Rapid $CO_2$ production can blow the containers, especially when the storage temperature increases to 10°C and above.

## Breads

The $A_W$ of breads is normally low enough (0.75–0.9) to prevent growth of bacteria. However, some molds (bread molds: *Rhizopus stolonifer*) can grow, especially if moisture is released because of starch crystallization during storage. Molds are killed during baking; however, spores can get in from air and equipment following baking. When breads are frozen, they may contain ice crystals in the bags. Following thawing, some portions can absorb enough moisture for yeasts and bacteria to grow and cause spoilage (sour taste, off-flavor). A specific type of bread spoilage, designated as ropiness and characterized by a soft, stringy, brown mass with fruity odor, is caused by the growth of some mucoid variants of *Bac. subtilis*. The spores, coming from flour or equipment, survive baking and then germinate and grow inside within 1–2 days. They also produce extracellular amylases and proteases and break down the bread structure. High moisture inside the bread, slow cooling, and pH above 5.0 favor ropiness.

## Pastas

Pastas can be spoiled by microorganisms before drying because of improper manufacturing practices. Dry pastas do not favor microbial growth. However, soft pastas can be spoiled by bacteria, yeasts, and molds. Anaerobic packing and refrigeration storage can prevent mold growth and slow down growth of yeasts and psychrotrophic anaerobic and facultative anaerobic bacteria. Suitable preservatives can be used to prevent their growth.

## Pastries

Pastries include cakes and baked shells filled with custard, cream, or sauces. They can be spoiled by microorganisms coming with the ingredients that are added after baking, such as icing, nuts, toppings, and cream. Most products, because of low $A_W$, allow only molds to grow. However, some materials used as fillings may have high $A_W$, which allows for bacterial growth.

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## Liquid Sweeteners and Confectioneries

Liquid sweeteners include honey, sugar syrups, maple syrups, corn syrups, and molasses. Confectionery products include soft-centered fondant, cream, jellies, chocolate, and Turkish delight. Most of these products have an $A_W$ of 0.8 or below and are normally not susceptible to bacterial spoilage. Under aerobic conditions, some xerophilic molds can produce visible spoilage. However, osmophilic yeasts from genera *Zygosaccharomyces* (*Zygosaccharomyces rouxii*), *Saccharomyces* (*Saccharomyces cerevisiae*), *Torulopsis* (*Torulopsis holmii*), and *Candida* (*Candida valida*) can ferment these products. To prevent growth of yeasts in some of these products with slightly higher $A_W$ (such as in maple syrup), chemical preservatives are added.

## Mayonnaise, Salad Dressings, and Condiments

These products normally contain some molds, yeasts, spores of *Bacillus* and *Clostridium*, and aciduric bacteria, such as *Lactobacillus* and related species. Because of low pH, acid-sensitive bacteria may not survive long. Mayonnaise, with 65% or more edible oil and approximately 0.5% acetic acid, has an $A_W$ of approximately 0.92 and a pH of 3.6–4.0. Salad dressings generally contain 30% or more edible oil, 0.9%–1.2% acetic acid, an $A_W$ of 0.92, and a pH of 3.2–3.9. The major factors controlling microbial growth are undissociated acetic acid, low pH, and relatively low $A_W$. However, some aciduric microorganisms can grow to cause spoilage. Molds can grow only on the surface exposed to air. Some microaerophilic and facultative anaerobic yeasts and heterolactic *Lactobacillus* spp. (especially those that can grow at an $A_W$ of 0.92) can also multiply to produce $CO_2$. *Lab. fructivorans* hydrolyzes sucrose present in the products and produces gas ($CO_2$), especially from rapid metabolism of fructose that is released from sucrose. Strains of *Saccharomyces bailii* have also been implicated in gas spoilage ($CO_2$ and alcohol) of these products. *Lab. fructivorans* cells usually die rapidly after multiplication and are difficult to isolate unless specific methods are used. This can lead to a wrong assumption regarding the microorganisms that cause spoilage of these products.

Low-calorie salad dressing, in which oil and acetic acid are added in much lower concentrations, has high pH and $A_W$ values. Many microorganisms can grow in these products. To enhance their shelf life (and safety), refrigerated storage is recommended.

Yeasts, *Lactobacillus* spp., and *Leuconostoc* spp. can also cause gassy spoilage of ketchup, salsa, sauces, and prepared mustard. Some *Bacillus* spp. have also been implicated in gassy spoilage of mustard preparations. To control growth of spoilage microorganisms, additional preservation methods, especially chemical preservatives, are used.[12,13]

## Fermented Foods

Desirable microorganisms are used directly or indirectly to produce many types of fermented foods and beverages from meat, fish, milk, vegetables, fruits, cereal grains, and others (Chapter 15). The desirable microorganisms are present in very high numbers and the products contain either high levels of organic acids or alcohol. In addition, the products have a low pH, and some have a low $A_W$ (e.g., dry salami). Generally, these products have a longer shelf life. However, under certain conditions, they are susceptible to microbial spoilage. This aspect is discussed briefly.[1]

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## Fermented Meat Products

Fermented meat products normally have a pH between 4.5 and 5.0 and an $A_W$ between 0.73 and 0.93 (Chapter 15). During fermentation, if the acid production of homofermentative lactic acid bacteria is slow, undesirable bacteria can grow. *Clostridium*, *Bacillus*, and other mesophilic bacteria have been reported to cause spoilage in such conditions. Products with a pH lower than 5.0 but an $A_W$ of 0.92 or above and vacuum packaged can be spoiled by heterofermentative *Leuconostoc* and *Lactobacillus* spp., with the accumulation of gas and liquid inside the package and creamy white growth of the bacterial cells. If they are not vacuum packaged and have a low $A_W$ (0.72–0.90), yeasts and molds can grow on the surface, resulting in slime formation, discoloration, and undesirable flavor of the products.

## Fermented Dairy Products

Cultured buttermilk, yogurt, and cheese are a few of the large number of fermented dairy products generally produced by inoculating milk with specific starter-culture bacteria (Chapter 15). They differ in their acidity, $A_W$, and storage stability. Buttermilk generally has approximately 0.8% lactic acid and a pH of 4.8. Yeasts can grow and cause spoilage by producing gas. Some strains of starter cultures can produce exopolysaccharides to give a slimy texture (which is desirable in some products).

Plain yogurt generally has a pH of 4.5 or lower (with ca. 1% lactic acid) and is not spoiled by undesirable bacteria. However, the product can develop a bitter flavor because of production of bitter peptides by some strains of *Lab. delbrueckii* ssp. *bulgaricus* used as a starter culture. During storage, the starter bacteria can continue to produce lactic acid, causing an objectionable sharp acid taste. Both starters can produce exopolysaccharides, causing sliminess of the product. Yeasts (especially in fruit yogurts) may grow in the acid environment and produce $CO_2$ as well as yeasty and fruity off-flavors. In blended yogurts, many of these changes are not detected because of added ingredients. Some species of molds can grow on the surface if yogurt is stored for a long time.

Microbial cheese spoilage is greatly influenced by $A_W$ and pH. Unripened cottage cheese with high moisture content and low acidity is susceptible to spoilage by Gram-negative bacteria, predominantly psychrotrophic rods, and yeasts and molds. *Alcaligenes* and *Pseudomonas* spp. are frequently involved, causing a slimy texture and an unclean, putrid flavor. Some unripened ethnic cheeses (such as Mexican-style) are vacuum packaged and stored at refrigerated temperature for a shelf life of approximately 30 days or more (see Chapter 21). They are occasionally spoiled by heterofermentative *Leuconostoc* spp., characterized by gas ($CO_2$) and liquid accumulation in the bag. Gassiness in some cheeses with high pH, low salt, and relatively high $A_W$ (such as Gouda, Emmentaler, and provolone) can also occur from the growth of some *Clostridium* (e.g., *Clo. tyrobutyricum*). Their spores survive pasteurization of milk, germinate, and grow in the anaerobic environment to produce $CO_2$, $H_2$, and butyrate from the metabolism of lactate. Hard-ripened cheese, such as Cheddar, can have a bitter taste resulting from rapid production of bitter peptides during ripening. Fast acid-producing strains of *Lactococcus lactis* used as starters are generally associated with this defect. Sharp-ripened cheeses can also have large amounts of biologically active amines (e.g., histamine and tyramine), produced from the decarboxylation of the respective amino acids by decarboxylase. Decarboxylase enzymes can be present in some starter strains or in secondary microflora of the cheeses (*Enterococcus*, some coliforms). Lysis of the cells releases the enzymes during the ripening process, causing decarboxylation of amino acids and accumulation of these amines. Hard and semihard cheeses are generally susceptible to spoilage from mold growth on the

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surface and produce undesirable color and flavor defects in the products. Anaerobic packaging greatly reduces this problem.

### Fermented Vegetable and Fruit Products

Many types of vegetables are fermented, among which cucumber pickles and sauerkraut are produced in large volumes. In salt stock pickles containing approximately 15% salt, yeasts and halophilic bacteria can grow, especially if the acidity is not sufficient. Dill pickles with low salt (<5%) can have a bloating defect from $CO_2$ production by yeasts, heterofermentative lactic acid bacteria, and coliforms, especially if the desirable bacteria associated with fermentation do not grow properly. *Candida*, *Torulopsis*, and *Saccharomyces* spp. are often the causative yeasts. Sweet and sour pickles preserved with sugar and vinegar can be spoiled from the growth of yeasts and lactic acid bacteria, especially when the acid level is not sufficient. Sauerkraut can be spoiled from the growth of yeasts and molds if the air is not excluded during fermentation of cabbage (see Chapter 15). Failure of lactic acid bacteria to grow rapidly and in proper sequence can lead to low acid production. Under this condition, coliforms and other Gram-negative bacteria can multiply to produce undesirable flavor, texture, and color defects. Olives are fermented for a long time and are susceptible to many types of spoilage. The most common problem is gassiness (bloating) resulting from $CO_2$ production by heterofermentative lactic acid bacteria, coliforms, and yeasts. Softening of texture can be caused by pectinases of yeasts (*Rhodotorula* spp.).

### Fermented Beverages

Wines can support the growth of film yeasts and acetic acid bacteria (*Acetobacter* and *Gluconobacter*) under aerobic conditions. Film yeasts oxidize alcohol and organic acids and form a surface pellicle, whereas acetic acid bacteria oxidize alcohol to acetic acid and $CO_2$. Under anaerobic conditions, several lactic acid bacteria (e.g., *Lactobacillus*, *Leuconostoc*, and *Pediococcus* spp.) can grow in wine. Some heterofermentative *Lactobacillus* spp. can ferment glucose and fructose and increase the acidity of wine, producing a defect called tourne spoilage (by *Lab. brevis*, *Lab. buchneri*). They can also produce cloudiness and a mousy odor. Some *Leuconostoc* spp. can produce sliminess and cloudiness. *Oenococcus oenos* can convert malic acid to lactic acid and $CO_2$ and reduce the acidity of wine. Sometimes, this malolactic fermentation is used advantageously to reduce the sourness of wine.

Beer spoilage can be caused by some lactic acid bacteria and yeasts. Growth of *Pediococcus* spp. causes an increase in acidity and cloudiness. Some *Lactobacillus* spp. can also multiply and cause turbidity. *Acetobacter* and *Gluconobacter*, in the presence of air, can produce cloudiness and sliminess as well as make the beer sour. Wild yeasts (yeasts other than those used in fermentation) can grow in beer, causing off-flavor.[14]

## Canned Foods

Canned foods are heat treated to kill microorganisms present, and the extent of heat treatment is predominantly dependent on the pH of a food (Chapter 4). High-pH (pH 4.6 and above; also called low-acid) foods are heated to destroy most heat-resistant spores of the pathogenic bacteria *Clo. botulinum* to ensure that a product is free of any pathogen.[1,15] However, spores of some spoilage bacteria, which have greater heat resistance than do spores of *Clo. botulinum*, can survive.

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**Figure 20.2 Canned tomato paste with bulged top indicating formation of gas resulting from the growth of spore-forming aciduric thermophilic spoilage bacteria.**

Thus, these products are called commercially sterile (instead of sterile, which means free of any living organism) foods. The spores that survive the heat treatment designed to destroy *Clo. botulinum* spores are thermophilic spores and can germinate at 43°C and above. However, once germinated, some can outgrow at temperatures as low as 30°C. The other food group, designated as a low-pH or high-acid food with a pH below 4.6, is heat treated to kill all vegetative cells and some spores. Although low pH inhibits germination of spores and subsequent growth of *Clo. botulinum*, spores of some aciduric thermophilic spoilage bacteria can germinate and grow when the products are stored at higher temperatures, even for a short time, which induces germination (Figure 20.2). Some spores of thermoduric mesophilic spoilage bacteria (including pathogenic) can also survive heating in these products, but they are inhibited to germinate by the low pH.

Canned food spoilage is both a result of nonmicrobial (chemical and enzymatic reactions) and microbial reasons. Production of hydrogen (hydrogen swell), $CO_2$, browning, corrosion of cans from chemical reactions, and liquification, gelation, and discoloration resulting from enzymatic reactions are some examples of nonmicrobial spoilage. Microbial spoilage happens because of three main reasons: (1) inadequate cooling after heating or high-temperature storage, allowing germination and growth of thermophilic spore formers; (2) inadequate heating, resulting in survival and growth of mesophilic microorganisms (vegetative cells and spores); and (3) leakage (can be microscopic) in the cans, allowing microbial contamination from outside following heat treatment and their growth.

## *Thermophilic Spore Formers*

Thermophilic spore formers can cause three types of spoilage of low-acid (high-pH) foods (such as corn, beans, and peas) when the cans are temperature abused at 43°C and above even for a short duration.

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### Flat Sour Spoilage

The cans do not swell, but the products become acidic because of germination and growth of facultative anaerobic *Bac. stearothermophilus*. Germination occurs at high temperatures (43°C and above), but growth can take place at lower temperatures (30°C and above). The organism ferments carbohydrates to produce acids without gas but with some off-flavor and cloudiness.

### Thermophilic Anaerobe (TA) Spoilage

This spoilage is caused by the growth of anaerobic *Clo. thermosaccharolyticum* with the production of large quantities of $H_2$ and $CO_2$ gas and swelling of cans with a sour and cheesy odor. Following germination in the thermophilic range (43°C and above), the cells can grow at lower temperatures (30°C and above).

### Sulfide Stinker Spoilage

This spoilage is caused by the Gram-negative anaerobic spore former *Desulfotomaculum nigrificans*. The spoilage is characterized by a flat container but with darkened products with the odor of rotten eggs resulting from $H_2S$ produced by the bacterium. $H_2S$, produced from the sulfur-containing amino acids, dissolves in the liquid and reacts with iron to form the black color of iron sulfide. Both germination and growth occur in the thermophilic range (43°C and above).

## Spoilage Resulting from Insufficient Heating

Insufficient heat treatment results in the survival of mainly spores of *Clostridium* and some *Bacillus* spp. Following processing, they can germinate and grow to cause spoilage. The most important concern is the growth of *Clo. botulinum* and production of toxins.

Spoilage can be from either the breakdown of carbohydrates or proteins. Several *Clostridium* spp., such as *Clo. butyricum* and *Clo. pasteurianum*, ferment carbohydrates to produce volatile acids and $H_2$ and $CO_2$ gas, causing swelling of cans. Proteolytic species, *Clo. sporogenes* and *Clo. putrefacience* (also proteolytic *Clo. botulinum*), metabolize proteins and produce foul-smelling $H_2S$, mercaptans, indole, skatole, and ammonia as well as $CO_2$ and $H_2$ (causing swelling of cans).

Spores of aerobic *Bacillus* spp., surviving inadequate heating, do not grow in cans. However, spores of some facultative anaerobic *Bacillus* spp., such as *Bac. subtilis* and *Bac. coagulans*, grow with the production of acid and gas ($CO_2$).

## Spoilage Resulting from Container Leakage

Damaged and leaky containers allow different types of microorganisms to get inside from the environment after heating. They can grow in the food and cause different types of spoilage, depending on the microbial types. Contamination with pathogens makes the product unsafe.

# Conclusion

This chapter discusses why different food types or food groups differ in microbial spoilage characteristics. The predominant nutrients being metabolized and the predominant microorganisms

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growing rapidly in an environment determine the nature of the end products that are produced, which, in turn, are associated with spoilage characteristics. An understanding of these parameters is important in the firsthand determination of the causes of spoilage. This will be useful to investigate the cause, identify it, and take necessary corrective measures. These aspects are discussed further in Chapter 20, using some real situations of refrigerated food products.

### QUESTIONS

1. List the important psychrotrophic spoilage bacteria of raw meats and describe the metabolic pattern and corresponding spoilage associated with each microbial genus.
2. Briefly discuss the following: (a) sources of microorganisms in low-heat-processed meat products; (b) predominant spoilage bacteria in low-heat-processed, vacuum-packaged meat products stored at ≤4°C; and (c) factors associated with the greening of unpackaged and packaged meat and meat products during refrigerated storage.
3. List four microbial inhibitors in shell eggs and predominant spoilage microflora of eggs and egg products.
4. Briefly explain the following: (a) metabolism of nutrients by predominant spoilage bacteria while growing in fish tissues; (b) post-heat contamination and spoilage of crabmeat; and (c) metabolism of nutrients by spoilage bacteria in shucked oysters.
5. List the predominant microflora of pasteurized milk and their sources. Discuss the reasons for the limited shelf life of pasteurized milk.
6. Discuss the predisposing factors associated with microbial spoilage of UHP-treated milk, sweetened condensed milk, and butter.
7. List bacteria, yeasts, and molds (two of each) associated with the spoilage of vegetables and fruits. Discuss the major differences in spoilage by bacteria of these two groups of food. How are ready-to-eat vegetables, fruits, and germinated seeds and beans spoiled by bacteria?
8. Discuss the influence of intrinsic factors associated with microbial spoilage of fruit drinks, fruit juices, and vegetable juices and the nature of spoilage of these products caused by the predominant microflora.
9. Jams, jellies, peanut butter, and salsa are not spoiled at room temperature as long as the bottles/jars are not opened. The labels on the product, however, suggest to refrigerate the products after opening. What could be the reasons?
10. List the similarities and differences in the microbial spoilage of (a) breads, (b) pastries, (c) soft pastas, and (d) confectioneries.
11. List the microorganisms involved and the predisposing causes for the following spoilage conditions: (a) gassy defect in mayonnaise, (b) gassy defect of prepared mustard, (c) gassy defect in unripened soft cheese (such as Mexican-style cheese) and Gouda cheese, (d) bitter taste in yogurt and Cheddar cheese, (e) tourne spoilage of wine, (f) malolactic fermentation in wine, and (g) sour beer.
12. List the major causes of spoilage of canned foods. Describe the factors, causative bacterial species, and the nature of spoilage of canned foods produced by thermophilic spore formers.

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# Chapter 21

# New Food Spoilage Bacteria in Refrigerated Foods

## Microorganisms that Grow in Refrigerated Foods (Psychrotrophs)

In food microbiology, the terms psychrophiles and psychrotrophs are used to identify those microorganisms that can grow in food stored at low temperatures, namely, chilling and refrigeration, which can range between –1°C and 7°C. A present-day domestic refrigerator is expected to maintain a temperature of approximately 40°F (4.4°C), whereas commercial refrigeration can be lower or higher than this, depending on the particular food stored and shelf life expected. Highly perishable foods are either refrigerated or chilled on ice. Thus, psychrotrophs can multiply in these foods, provided other conditions for growth are not restricting.[1,2]

There is some confusion about the definitions of the two terms, particularly in relation to their importance in food microbiology. The terms "psychrophile" and "psychrophilic" are quite specific and includes those microorganisms that grow optimally at approximately 12°C–15°C and have a growth temperature range between ≤–5°C and 22°C. These microorganisms can grow in refrigerated and chilled foods. The definition of psychrotrophs is, however, not clear-cut. Originally, in 1960, this term was introduced to include those microorganisms that grow at 0°C–5°C, irrespective of their optimum or the range of growth temperatures. Rather, they seem to grow best at 25°C–30°C and might not grow above 35°C. Thus, they appear to be a subgroup of mesophiles (growth temperature of mesophiles: optimum 30°C–40°C and range 5°C–45°C) but not a subgroup of psychrophiles. This group includes Gram-positive and Gram-negative aerobic, anaerobic, and facultative anaerobic, motile and nonmotile, spore formers and non–spore formers, and coccus and rod-shaped bacteria, as well as many yeasts and molds. Later in 1976, the International Dairy Foundation defined psychrotrophs as those microorganisms that grow at 7°C, irrespective of their optimum and range of growth temperatures. However, this does not indicate whether it includes only the subgroup of mesophiles with the ability to grow at or below 7°C or both psychrophiles and the subgroup of mesophiles that grow at the lower temperature range. More recently, the term psychrotrophs includes mesophilic subgroups that can grow at 40°F or below (≤4.4°C, refrigerated temperatures).[1,2]



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Several studies have revealed that there are some mesophilic pathogens (e.g., *Yersinia enterocolitica* and *Listeria monocytogenes*) and spoilage bacteria (e.g., *Leuconostoc* spp., several *Lactobacillus* spp., and *Serratia* spp.) that can grow in vacuum- and modified-air (MA) packaged foods at 0°C–1°C. Spoilage of vacuum-packaged meats by the psychrophilic *Clostridium* spp. with a growth range between –2°C and 20°C was also reported. It is important to have understandable communication among the people associated with food production, regulations, sanitation, academic and research activities, and others without creating confusion by using the designation "psychrotrophic spoilage" for the mesophilic subgroup and "psychrophilic spoilage" for the second group. For easier understanding, it is probably not unscientific to use the term "psychrotrophs" for the microorganism that can grow (in food) at refrigerated or chilled storage temperature (≤40°F or 4.4°C). This includes both the mesophile subgroup that can grow at lower temperature and psychrophiles. As both groups are important in spoilage and foodborne diseases and the methods used to detect and control them do not differentiate the two groups, a single terminology can be used for both. The same system has been used in food microbiology for the term "thermoduric," the microorganisms that survive pasteurization (or low-heat treatment), and can include thermophiles, mesophiles, and psychrophiles (*Clostridium laramie*). In this book, the term psychrotroph is used to include microorganisms that can grow in food stored at refrigeration temperatures irrespective of their being psychrophilic or mesophilic.[1,2]

## Popularity of Refrigerated Foods

The demand for refrigerated foods has increased dramatically in countries where refrigeration systems are economically available for food processors, retailers, and consumers.[3,4] Three major factors can be attributed to this increase. The first is the need and desire of consumers for convenient foods. The changes in socioeconomic pattern, such as an increase in two-income families, single-parent households, singles, the elderly, and college students living away from home, have created a demand for convenient foods. Second, there is an increase in awareness and belief that harshly processed and harshly preserved foods, as well as foods with high fat, cholesterol, and sodium, are not beneficial for a long and healthy life. Health-conscious consumers are interested in foods that are natural and fresh. These two factors have created a market for healthy foods that can be of high quality (restaurant quality), convenient (e.g., take the least time to prepare at home), and economically affordable. Finally, the technologies necessary for economical production of such foods and subsequent handling until they are consumed are available, especially in developed countries. As a result, different types of refrigerated foods, including new-generation refrigerated and chilled foods, sous vide foods, many of which are "ready-to-eat," "heat-'n'-eat," and "microwave-'n'-eat" types, are being commercially produced. Many of the new-generation foods are given low-heat treatment to no heat treatment, contain little or no preservatives, and many are vacuum-packaged or packaged with modified atmosphere (MA) (with 100% $CO_2$ or a mixture of $CO_2$ and nitrogen without or with some oxygen). They are expected to have a shelf life of 20–60 days or more. Vacuum- or MA-packaged refrigerated unprocessed or raw foods are also expected to have a much longer shelf life than the same foods stored in the presence of air. Sous vide foods are fresh foods (e.g., vegetables, meat, or fish) that are vacuum packaged, cooked at low heat, and stored in a refrigerator or over ice. They are warmed in the package before serving and eating, and are expected to have a three-week shelf life. Fresh and ready-to-eat cut fruits and vegetables are packaged with air and have a shelf life of approximately two weeks at refrigerated or higher temperatures.

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Consumers prefer refrigerated foods because of their better taste, texture, quality, and convenience. They also perceive these foods to be relatively fresh, nutritious, and close to natural as compared with frozen, canned, or dried foods and fast foods. The popularity of these refrigerated or chilled foods has convinced food experts that this is not a fad—it is here to stay, and the demand will continue to increase in the future.[3,4]

## Microbiological Problems

Vacuum- or MA-packaged refrigerated (≤5°C) foods are expected to inhibit the growth of aerobic, most mesophilic, and thermophilic microorganisms. However, if the product contains dissolved or trapped oxygen and the modified air contains oxygen, aerobic and microaerophilic microorganisms capable of growing at refrigerated temperatures can also grow as long as the oxygen is available. Similar possibilities also exist if the packaging material is relatively permeable to atmospheric oxygen.[5]

Even under ideal conditions (no oxygen and refrigeration at ≤5°C), the vacuum-packaged unheated foods normally harbor both anaerobic and facultative anaerobic spoilage and pathogenic bacteria that can multiply during refrigerated storage. Heat-processed products are usually given a low-heat treatment that, depending on a product, varies from 60°C to 74°C (140°F–170°F) for a specified time. At this temperature, pathogenic and spoilage bacterial spores and cells of some thermoduric bacteria can survive. Some of these survivors can be anaerobic and facultative anaerobic and can multiply at refrigerated temperatures. Many of these heat-processed products are, however, handled extensively following heating and before final repackaging under vacuum or MA. Pathogenic and spoilage bacteria that can grow during storage can also get into the products during this time as post-heat contaminants. Thus, these products have the potential of harboring pathogens and spoilage bacteria that can multiply under vacuum or MA at refrigerated temperatures. As these products are expected to have a long shelf life, some up to 100 days, even a very low population ($\leq 10^1$/g) of psychrotrophic anaerobic and facultative anaerobic bacteria can multiply and reach a level that causes spoilage of the food or makes it unsafe. These products are often temperature abused during transportation, in display cases at the retail store, and at the homes of consumers. Depending on the time and temperature of abuse, the product temperature can go to 10°C–15°C for a considerable period. The shelf life and safety of the products can be drastically reduced because of accelerated growth rates of psychrotrophs as well as by some mesophiles (anaerobic and facultative anaerobic) that do not grow at ≤5°C but can at the abusive temperature. It has been suspected that even a few hours (4–6 h) at 12°C–15°C can reduce the shelf life of such products by 8–10 days.

There is concern among regulatory agencies regarding the safety of these products because the incidences of foodborne diseases following consumption of pasteurized refrigerated foods have been recorded (e.g., *Lis. monocytogenes* in ready-to-eat processed meat products). Food processors are also encountering spoilage of these products in higher frequencies (see examples later). To overcome the microbiological problems in these foods, the National Food Processors Association has recommended several guidelines. These include selecting good-quality raw materials, installing good sanitary procedures, incorporating hazard analysis critical control points (HACCP) at all phases between production and consumption of these foods (from field to table), and, where possible (Chapter 33), applying the highest permissible heat treatment to a product at the final step and eliminating or minimizing post-heat treatment contamination. Other recommendations are to incorporate multiple barriers or hurdles (Chapter 41) along with refrigeration and vacuum or

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MA packaging. Some of these can be incorporated into the product formulation to reduce the pH and $A_W$. In addition, acceptable and suitable preservatives (e.g., biopreservatives; see Chapter 17) can be included to combat microbial survival and growth. Some packaged products, such as processed meats, can also be given a hydrostatic pressure processing treatment (Chapter 40).

Many of the food processors are following one or more of these recommendations, especially good sanitation. This helps reduce the initial microbial population of the products considerably. Yet incidences of spoilage of (and foodborne diseases from) vacuum- or MA-packaged refrigerated products are not infrequent and, at times with some processors, spoilage occurs at epidemic proportions both in raw and low-heat-processed products. Many of the bacteria frequently isolated from such spoiled products are being recognized either as new species or species that were not of major concern before. Some of these are *Clo. laramie*, *Clo. estertheticum*, *Clo. algidicarnis*, *Carnobacterium* spp., *Leuconostoc carnosum*, *Leu. gelidum*, *Lactobacillus sake*, *Lab. curvatus*, atypical or unidentifiable lactobacilli and leuconostocs, *Brochothrix thermosphacta*, *Enterococcus* spp., *Serratia liquefaciens*, *Hafnia* spp., *Proteus* spp., and some other *Enterobacteriaceae*. Except for spores of *Clostridium* spp., and some thermodurics (some lactobacilli and enterococci), cells of the others are sensitive to heat treatment (pasteurization) given to the processed products. Thus, they are getting in heated products as post-heat contaminants on the product surfaces (so are some pathogens). There is a growing speculation and belief among scientists that although these bacterial species have become major causes of spoilage of vacuum- or MA-packaged refrigerated foods, most are neither new nor variants of the existing species. They are present in the environment but probably as minor flora. Three reasons could have helped them become major spoilage organisms: the changes in the environment and food, such as efficient vacuum (or compositions used in MA) packaging and oxygen barrier systems; higher pH in some meat products (low fat, high phosphate); and long storage times at low temperatures—these might have controlled the growth of traditional bacteria associated with food spoilage before but are not efficient for these nontraditional bacteria. A second reason could be that in order to produce a safer food with a long shelf life, food processors are using "super sanitation," which has efficiently eliminated the traditional microorganisms associated with spoilage before but, at the same time, has enabled the minor flora to establish in the environment and contaminate the products. Finally, processors might be introducing equipment that are highly efficient in producing a large volume of products but may also be the microbiologist's nightmare for efficient cleaning and sanitation. As a result, the equipment harboring the microorganisms in dead spots remains unaffected by sanitation and serves as a source of inoculation to the products. Some of the examples used here may justify these assumptions.[5]

## Incidence of Spoilage of Vacuum-Packaged Refrigerated Food

### *Spoilage of Unprocessed (Fresh) Beef by* Clostridium *Species*

Large-scale spoilage of vacuum-packaged, refrigerated, unprocessed beef has been recognized recently.[5–9] The spoilage is characterized by accumulation of large quantities of $H_2S$-smelling gas and purge (liquid) in the bag within two weeks at 4°C (39.2°F) and loss of texture (soft) and color (cherry red) of meat, which, in 10–12 weeks, changes to greenish (Figure 21.1a). Enumeration by plating revealed approximately $10^8$/mL leuconostocs colony-forming units (CFUs) in the purge. However, examination of a little purge under a phase-contrast microscope revealed a large number of medium-to-large, thick, motile rods and some cells with large terminal spores (drumstick-shaped) as well as some leuconostoc-like cells (lenticular, small chains). The rods and the lenticular

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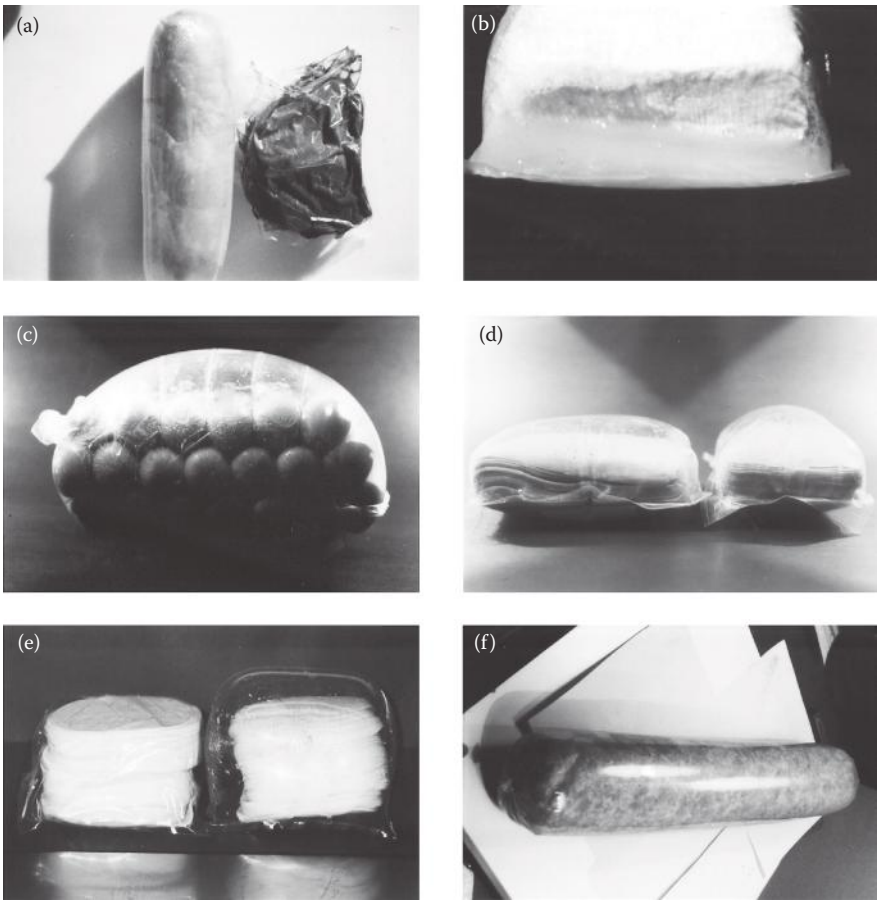



**Figure 21.1 Bacterial spoilage of vacuum-packaged refrigerated foods. (a) Spoilage of beef by *Clostridium laramie*: left, a spoiled sample showing gas accumulation; right, an unspoiled sample. (b) Spoiled tofu by a *Clostridium* sp. showing accumulation of liquid and gas. (c) Spoiled frankfurters and (d) spoiled luncheon meat predominantly by *Leuconostoc* spp. showing gas accumulation. (e) Spoiled sliced turkey roll by heterofermentative lactobacilli, leuconostocs, and *Serratia liquefaciens*: right, gas accumulation (and pink discoloration); left, an unspoiled sample. (f) Spoilage of ground beef chub by heterofermentative lactobacilli showing gas accumulation.**

cells were Gram-positive. The rod-shaped bacterium was suspected to be a *Clostridium* species but could not be cultured in agar and broth media recommended for *Clostridium* spp. Later, it was purified by heating the purge (from the package of 12-week-old spoiled meat containing spores) at 80°C (176°F) for 10 min and culturing the material at 15°C in a broth under anaerobic conditions. Biochemical studies revealed it to be a new species, and it was designated *Clo. laramie* NK1 (ATCC 51254). The cells are extremely sensitive to oxygen and grow optimally under a good anaerobic environment at 12°C–15°C (53.6°F–59°F) with a growth temperature range of –2°C to 22°C. The species can sporulate and germinate at 2°C (35.6°F).

Inoculation of raw beef with these spores followed by vacuum packaging and refrigeration storage caused the samples to develop similar characteristics, and the purge had the *Clostridium* cells. A limited in-plant sampling was done from the slaughtering area to the conveyor systems

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carrying the primal cuts for vacuum packaging (in a plant having the specific problem). Three of the 25 samples were positive for *Clo. laramie*, and all three were from the conveyor system at the vacuum-packaging area. The conveyor system used by this plant consisted of small links. In general, the microbiological quality of the meat was very good (~$10^3$/g aerobic plate counts, APCs). It was suspected that the sanitation specifically designed to keep the microbial load low was good but was unable to kill the spores, especially in the inaccessible joints (dead spots) and similar places in the links of the conveyor system. Thus, the fabricated meats were constantly inoculated with the spores (and cells), which, in the absence of competition, because of low associated bacteria, germinated or grew (or both) to cause large-scale spoilage (along with psychrotrophic *Leuconostoc* spp.). Subsequent studies with spoiled meat samples from several beef processors having similar problems in the United States have revealed that this incidence is not isolated, and more than one strain of *Clo. laramie* is involved in spoilage.[5–8]

A similar spoilage of vacuum-packaged refrigerated beef in Europe was also reported to be caused by a similar *Clostridium* sp. and named *Clo. estertheticum*. This species also could not be enumerated by agar-plating methods and was first detected microscopically.[7] Other clostridial species that are involved in the spoilage of vacuum-packaged meat include *Clo. algidicarnis*, *Clo. algidixylanolyticum*, *Clo. frigidicarnis*, and *Clo. gasigenes*.[9] In recent years, spoilage of vacuum-packaged refrigerated raw beef by *Clostridium* spp. has been reported from Canada, Brazil, and New Zealand.

## *Spoilage of Roasted Beef by* Clostridium *Species*

*Clostridium* spp. have been isolated from at least two separate incidents of large-scale spoilage of vacuum-packaged refrigerated roasted beef produced commercially. The meats (10–12 lbs [4.5–5.5 kg] each) were roasted ≤160°F (71°C) (internal temperature) and then vacuum-packaged and refrigerated.[6,8] When the samples were received in the laboratory, they had large quantities of gas and purge, but because of the spices used in the meat, only a strong spicy smell was evident. Phase-contrast microscopy of the purge revealed large numbers of motile *Clostridium* cells, some with typical terminal spores. Colonies of this bacterium were not obtained by isolation in specific agar media, but large numbers of leuconostoc colonies (~$10^8$/mL of purge) were obtained. The spore-containing purge was heated and cultured to purify the *Clostridium* spp. Inoculation of the pure culture to rare roasted beef, followed by vacuum packaging and refrigeration, showed the formation of $H_2S$ gas, a change of meat color from light brown to pink, and accumulation of pink to red purge within 2–3 weeks. Examination of the purge under a phase-contrast microscope revealed the presence of *Clostridium* cells and spores. Some of these isolates seem to differ in some biochemical and physiological patterns when compared with *Clo. laramie* isolated from the spoiled raw beef (see the section on spoilage of unprocessed (fresh) beef by *Clostridium* spp.).[5,7]

## *Spoilage of Pork Chops by* Clostridium algidicarnis

This new species is associated with spoilage of cooked, vacuum-packaged pork meat products during storage at 4°C.[10] It produces an offensive spoilage odor.

## *Spoilage of Tofu by* Clostridium *Species*

A vacuum-packaged soybean milk curd product (tofu) during refrigerated storage revealed gas and liquid accumulation in the bag and off-flavor (Figure 21.1b).[8] Microscopic examination of the

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liquid showed the presence of large numbers of motile rods, many with terminal oval spores. The strain was isolated in pure form and found to have some biochemical and physiological characteristics different from those of *Clo. laramie* NK 1 because it could be grown in agar medium under anaerobic conditions. However, it is psychrophilic and does not grow at 25°C or above.

## *Spoilage of Unripened Soft Cheese by* Leuconostoc *Species*

Spanish-style soft cheese, made by coagulating pasteurized milk with lactic acid and rennet and collecting the curd and vacuum packaging, showed gas formation and liquid accumulation in the package within 30 days at refrigerated temperature. (The expected shelf life is 50+ days.)[8] The curd texture was soft and gassy. Examination of the liquid under a phase-contrast microscope revealed short chains of leuconostoc-like cells. Enumeration in pH 5.0 plating media revealed $10^{8-9}$ CFUs/g product. Purified cells from the colonies were Gram-positive, heterofermentative, and biochemically identified as *Leuconostoc* spp. The bacteria possibly got in the product during extensive handling of the product following pasteurization of milk. Even when they are initially present at a low level, during long storage they can multiply, form gas, and produce the defect. Any temperature abuse accelerates the spoilage.

## *Spoilage of Low-Heat-Processed Meat Products by* Leuconostoc *Species*

Many types of commercially processed, vacuum-packaged refrigerated meat products, following spoilage, were collected from local supermarkets. The products were restructured ham steak, chopped and formed ham, chopped sliced ham, chunked and formed turkey ham, cooked luncheon meat (sliced), several brands of frankfurters (beef, chicken, and turkey), wieners, cooked and smoked Polish sausage, summer sausage, and hot-link sausage. The common symptoms were accumulation of large quantities of gas and cloudy purge (Figure 21.1c,d).[5–8] The products did not have any disagreeable odor. The pH ranged from 5.0 to 6.0. Some were low-fat products. Phase-contrast microscopy of the purge revealed predominantly the presence of leuconostoc-like cells. Colony enumeration of the purge revealed lactic acid bacteria counts of approximately $10^{8-9}$/mL but lower APCs. *Bro. thermosphacta*, when present, was $<10^3$/mL. Biochemical tests revealed that the predominant lactic acid bacteria were *Leu. carnosum* and *Leu. mesenteroides*, and many could produce bacteriocins. It was suspected that bacteriocin-producing leuconostocs, by inhibiting the growth of other Gram-positive bacteriocin-sensitive contaminants, grew preferentially and produced $CO_2$ and acid and were responsible for gross distension (or bloating) of the packs and release of liquid (purge). Most likely, the leuconostocs contaminated the products as post-heat contaminants from the equipment and other sources during handling before vacuum packaging.[11]

## *Ammonia Odor in Turkey Roll*

Low-fat (2%), high-pH (6.5) vacuum-packaged turkey breast rolls showed gas and liquid accumulation with a strong ammonia odor (diaper smell) when opened.[8] Examination of the liquid under a phase-contrast microscope showed predominantly leuconostoc-like cells as well as motile single or small-chain rods and some lactobacilli-like cells. Purification and Gram staining revealed the leuconostoc- and lactobacilli-like cells to be Gram-positive and the motile rods to be Gram-negative. APCs and lactic acid bacteria counts of liquid were approximately $10^8$/mL. Biochemical analysis revealed the Gram-negative species to be *Ser. liquifaciens*, and the Gram-positive bacteria were *Leu. mesenteroides* and *Lab. sake*. It was suspected that these predominant bacterial types

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contaminated the products after heat treatment and before vacuum packaging. *Leuconostoc* sp. produced gas ($CO_2$) and acid, and *Lab. sake* also produced acid but not gas because it is homofermentative. But the pH did not drop owing to the high phosphate content used in this low-fat product. Subsequently, *Serratia* sp. grew and caused deamination of amino acids, thus releasing ammonia. The products also had a slight pink color, which could be a result of partial reduction of metmyoglobin (see the section on pink discoloration of sliced, chopped, and formed roast beef and the section on gas distension and pink discoloration of sliced turkey rolls).

## Yellow Discoloration of Luncheon Meat

Vacuum-packaged cooked luncheon meat, prepared from chopped ham, developed yellow-colored spots within 3–4 weeks of storage at 40°F (4.4°C).[12] Microbiological analysis indicated the presence of high numbers of *Enterococcus faecium* ssp. *casseliflavus*, which was able to survive at 71.1°C for 20 min. Thus, the species probably survived cooking. The yellow discoloration was suspected to be a result of the production of a carotenoid substance by the bacterial species.

## Gray Discoloration of Turkey Luncheon Meat

Turkey luncheon meat slices prepared mainly from dark meat developed gray spots or patches within 2–3 days during aerobic storage at refrigeration temperatures.[8] The causative bacteria was isolated and identified to be an $H_2O_2$-producing strain of *Lactobacillus* species. The product formulation contained approximately 1% lactate. It is suspected that the bacterial strain under aerobic conditions of growth utilized lactate to produce $H_2O_2$ (lactate + $O_2$ → pyruvate + $H_2O_2$, reaction catalyzed by L-lactate oxidase). $H_2O_2$ then oxidized the myoglobin to produce a white-gray color. The $H_2O_2$-producing strain could come as a post-heat contaminant or survive low-heat treatment as some lactobacilli are thermoduric. Under vacuum storage, the strain does not produce $H_2O_2$ to cause discoloration.

## Pink Discoloration of Sliced, Chopped, and Formed Roast Beef

Vacuum-packaged chopped and formed low-fat roast beef slices during refrigerated storage for 1–2 weeks developed pink to red patches over the normal brown color.[8] Initially, the fresh product had a pH of 5.8–6.0 and a slight off-odor. Within 4–5 weeks at refrigerated temperatures, the slices developed a strong fishy to putrid odor; no $H_2S$ was detected, and the pH remained at approximately 6.0. The meat color changed to deep pink to red with a dark-red colored purge, but there was a very small amount of gas accumulation. Microbiological enumeration revealed approximately $9 \times 10^9$/mL purge of APC and $1 \times 10^9$/mL purge of lactic acid bacteria (leuconostocs or lactobacilli). Examination of the purge under a phase-contrast microscope revealed leuconostoc- and lactobacilli-like cells as well as motile rods. Streaking on xylose-lysine iron agar plates helped isolate three types of colonies of Gram-negative rods, which were biochemically identified as *Hafnia alvei*, *Proteus vulgaris*, and *Ser. liquifaciens*. Inoculation of the three isolates in roast beef, followed by vacuum packaging and storage at refrigerated temperatures, revealed that all three Gram-negative isolates could change the brown color of roasts to pink or red (like rare roast beef) with a red-colored purge, but *Pro. vulgaris* changed the meat color to cherry red. The packages inoculated with *Ser. liquifaciens* also had slight gas. All three Gram-negative isolates produced small amounts of $H_2S$. Analysis of absorption spectra of the extract revealed that the color change occurs because of the partial reduction of metmyoglobin.

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The lactic acid bacteria as well as the Gram-negative species isolated from these samples were heat-sensitive psychotrophic and facultative anaerobic. They definitely entered the product as post-heat contaminants, indicating poor sanitation in the production facilities. The absence of large amounts of gas in this product, as opposed to some products described before, could be a result of the presence of very little metabolizable carbohydrate. The reason for the pH to remain unchanged (~pH 6.0) during deamination of amino acids could be the use of high amounts of phosphate in the formulation to bind water.

## Gas Distension and Pink Discoloration of Sliced Turkey Rolls

Sliced low-fat turkey rolls (initial pH 6.5) were packaged by modified atmosphere (drawing vacuum and then flushing a mixture of 30% $CO_2$ and 70% $N_2$ in small amounts) so that the packages remained slightly loose. The product was expected to have an eight-week shelf life at refrigerated temperatures. By five weeks, many packages developed gross distension with gas, little to moderate amounts of purge, and pinkish-red discoloration from the normal gray-white color of the breast meat (Figure 21.1e).[8] The intensity of the color was more inside the slices than at the rim. The gas had an off-odor (like wet socks), but no $H_2S$ was detected. Soon after opening a bag, the color of the meat slices changed to normal. Vacuum packaging the meat slices again changed the color in 8–12 hours to pinkish red. Addition of ascorbic acid to the normal colored meat slices gave the similar pinkish-red color quickly (in 1–2 hours). Examination of the liquid under a phase-contrast microscope revealed leuconostoc- and lactobacilli-like cells as well as motile rods. Colony enumeration revealed the presence of lactic acid bacteria at approximately $10^{7-8}$/g and *Enterobacteriaceae* at approximately $10^6$/g. While lactic acid bacteria were predominantly *Leu. carnosum* (heterofermentative) and *Lab. curvatus* (homofermentative), *Enterobacteriaceae* were *Ser. liquifaciens*. Inoculation studies revealed that *Ser. liquifaciens* was important for the off-flavor. The pink color was a result of formation of reduced metmyoglobin; however, it is not clear how it formed. It seems that psychrotrophic Gram-positive and Gram-negative bacteria, entering the product as post-heat contaminants, grew during storage and reduced the O-R potential of the products. This changed the metmyoglobin from a reversible oxidized to a reduced state and the color from gray-white to pink. In air (oxygen), metmyoglobin reversed from a reduced to an oxidized state, changing the color from pink to gray-white. Absorption spectrum studies with a spectrophotometer of extracted materials also supported the conclusion that the pink color was the result of a reduced form of metmyoglobin. A large amount of gas was produced by *Leu. carnosum* from the metabolism of glucose used in the formulation. Because the product had fairly large amounts of phosphate, pH did not change, even from the growth of lactic acid bacteria; thus, there was only a small amount of purge present in the bags.

Examination of the production facility revealed that the cooked products were contaminated by these bacteria mainly from the slicer and the conveyors between the slicer and the packaging machine. However, the initial contamination level was very low (<10/g). High numbers were reached during storage. It was also revealed by the processor that during distribution from the warehouse to stores in a van, the product temperature could be as high as 50°F–55°F (10°C–12°C) for 6–8 hours; this could also result in rapid growth of psychrotrophs, causing reduction in shelf life.

## Gas Distension (Bloating) of Ground Beef Chubs

Vacuum-packaged, low-fat ground beef chubs were found to accumulate large quantities of gas within a few days of refrigerated storage (Figure 21.1f). Initially (within one week of storage), there

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was a slight off-odor (no $H_2S$) in the gas or meat; the meat pH was 5.6, and microbiological analysis revealed large numbers ($1–4 \times 10^7$/g) of psychrotrophic lactic acid bacteria, mainly *Leu. mesenteroides* and two atypical *Lactobacillus* spp. (as determined from biochemical and physiological reaction patterns). APCs were $\leq 1 \times 10^5$/g, and Gram-negatives were $\leq 10^3$/g. The most predominant *Lactobacillus* sp. is heterofermentative and has atypical morphology, and its biochemical profile (by API system) does not match that of any known species. Following storage of the meat for approximately six weeks at refrigerated temperatures, psychrotrophic lactic acid bacterial counts increased to approximately $1.5 \times 10^8$/g (with APC ~ $2.7 \times 10^7$/g), the pH increased to 6.2, the gas had slight $H_2S$, and the meat had a strong putrid odor. It was suspected that initial gas formation was a result of the metabolism of carbohydrates in meat by heterofermentative psychrotrophic lactic acid bacteria. During extended storage, lactic acid bacteria as well as other bacteria (especially Gram-negative) assimilated amino acids, including sulfur-containing amino acids, and produced the offensive odor.

## Egg Odor in Refrigerated Fresh Chicken Meat Products

Skinless and boneless chicken breast meat were diced and marinated (the mixture contained different ingredients, including 1.2% salt and 1.8% sodium lactate), vacuum packaged, and stored at 30°F (–1.1°C). The pH of the product was 6.0–6.2. Within seven days, the products developed a mild egg odor that increased in intensity during the expected storage life of 28 days.

Vacuum-packaged samples (four days and 24 days old) were received in ice pack containers and analyzed within 24 hours. Duplicate samples were tested for each microbial group examined. There was no gas or exudate accumulation in the bags, but in the 24-day-old samples the bags were slightly loose. Following opening, the fresh samples had a chicken odor, but the old samples had a distinct egg odor and were positive for the presence of $H_2S$ (tested with lead acetate). The pH of both groups of products was 6.0. The bacterial groups enumerated were APCs (35°C for two days) and psychrotrophic counts (10°C for seven days), both with plate count agar; lactic acid bacteria (35°C for two days) in MRS-agar (adjusted to pH 5.0); coliforms (35°C for one day) in violet red bile agar; and *Enterobacteriaceae* (35°C for two days) in violet red bile glucose agar media. Aliquots from appropriate dilutions were pour-plated in duplicate for each dilution.

**Table 21.1 Change in CFUs/g of Several Bacterial Groups in a Refrigerated Chicken Meat Product During Storage**

| | *CFUs/g*[a] | |
|---|---|---|
| *Bacterial Groups* | *Fresh (4 days)*[b] | *Old (24 days)* |
| Aerobic plate count | $5.9 \times 10^4$ | $5.8 \times 10^5$ |
| Lactic acid bacteria | $4.9 \times 10^4$ | $3.5 \times 10^4$ |
| Psychrotrophic bacteria | $7.0 \times 10^4$ | $4.5 \times 10^5$ |
| Coliforms | $4.0 \times 10^3$ | $7.0 \times 10^2$ |
| *Enterobacteriaceae* | $5.6 \times 10^3$ | $1.5 \times 10^2$ |

[a] Average of two samples.
[b] Fresh samples, but not the old samples, had approximately $6 \times 10^2$ Proteus.

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The results (Table 21.1) revealed that in the four-day-old products the levels of five bacterial groups were not unexpectedly high for a diced meat product. Similar levels for APC were also obtained in one-day-old products, which were tested in the processor's laboratory. After 24 days at 30°F (–1.1°C), both APCs and psychrotroph counts increased but remained below the usually accepted spoilage detection level (ca. $\geq 10^7$/g). However, levels of coliforms and *Enterobacteriaceae* decreased during storage. Interestingly, fresh samples had approximately $6 \times 10^2$/g *Proteus*, but the 24-day-old samples did not have any in a $10^{-1}$ dilution. It was suspected that cells of *Enterobacteriaceae*, including *Proteus* sp., were killed during storage by the combined action of 1.8% lactate, 1.2% salt, and 30°F (–1.1°C). Their proteolytic enzymes (exocellular and endocellular) were able to break down proteins and sulfur-containing amino acids to produce a small amount of $H_2S$ associated with the egg odor. To overcome such problems, an important objective will be to keep the level of *Enterobacteriaceae*, as well as the level of Gram-negative bacteria, very low.

## Off-Odor in Frozen Chicken Meat Product

Skinless and boneless chicken thigh meat was diced and marinated (the mixture contained different ingredients, including 3% sodium lactate and 1.8% salt). The products (pH 6.3) were vacuum packaged and frozen to –20°C. The product, expected to have a shelf life of 90 days or more, is thawed by customers immediately before use. When thawed during storage, the products gave an off-odor, which increased in intensity with storage period.

Vacuum-packaged meat samples consisting of frozen unmarinated and marinated and frozen samples stored for 25 days and 100 days were enumerated for the levels of several bacterial groups by the methods described in the section on egg odor in refrigerated fresh chicken meat products. The packages of the marinated products were loose, and the thawed meat had an off-flavor, which was distinct in the 100-day stored products. The results (Table 21.2) revealed that unmarinated products had low levels of four bacterial groups. In contrast, the 25-day stored products had high levels of all four groups. In the 100-day stored products, the counts of all four bacterial groups dropped by two to three log cycles.

It was suspected that the unmarinated product was heavily contaminated during the process involving marination. Many of these were Gram-negatives (psychrotrophs, coliforms, *Enterobacteriaceae*). During frozen storage, in the presence of high lactate and salt, many of them were killed. Some of their enzymes were able to catalyze reactions slowly at –20°C and caused slight proteolysis (and maybe lipolysis), producing flavor compounds that could be identified after

**Table 21.2 Change in CFUs/g of Several Bacterial Groups in Frozen Chicken Meat Product During Storage**

| *Meat Types* | *Days at –20°C* | *CFUs/g*[a] | | | |
|---|---|---|---|---|---|
| | | *APC* | *Psychrotrophs* | *Coliforms* | *Enterobacteriaceae* |
| Unprocessed | 7 | $1 \times 10^4$ | $3 \times 10^4$ | $5 \times 10^1$ | $2.8 \times 10^3$ |
| Processed | 25 | $1 \times 10^7$ | $1.9 \times 10^7$ | $1.1 \times 10^4$ | $1 \times 10^5$ |
| Processed | 100 | $4 \times 10^4$ | $3.2 \times 10^4$ | $1.4 \times 10^2$ | $1.1 \times 10^2$ |

[a] Average of two or more samples.

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the meat was thawed (see Chapter 22). To overcome the problem, it will be important to adopt good sanitary practices and proper temperature control during processing and before freezing the product.

### *Gas and Slime Development in Vacuum-Packaged Smoked Salmon Products*

Processors have reported different types of spoilage in vacuum-packaged refrigerated smoked salmon hot dog and deli-type products. The hot dog packages had large amounts of gas and very thick, slimy, yellow-whitish purge. The deli-type packages had very little gas but large volumes of cloudy purge. Examination revealed that $H_2S$ was absent in the gas, and pH of the purge was approximately 4.8 for both types of samples. Phase-contrast microscopy of the purge revealed the presence of coccoid to lenticular cells in small chains in the hot dogs but different-sized rods in the deli samples. Microbiological enumeration of the purge revealed the presence of lactic acid bacterial CFUs more than $10^{10}$/mL. Biochemical analysis revealed the predominant coccoid and lenticular isolates in hot dogs to be *Leu. mesenteroides* ssp. *dextranicum* and the predominant rod-shaped isolates in deli-type products to be *Lab. sake*. Both bacterial isolates were killed at 71°C in five minutes (the products were processed at 72°C, internal temperature) but were able to multiply at 4°C.

It was suggested that both lactic acid bacterial strains were present in the packaged products as post-heat treatment contaminants. The ingredients had both glucose and brown sugar in fairly high concentrations, which facilitate both psychrotrophic facultative anaerobic strains to multiply at refrigerated storage temperatures. The heterofermentative *Leuconostoc* species produced gas and dextran (from sugar). Both reduced the original product pH of 5.6 to 4.8, causing the loss of bound water to produce purge in the bags. The recommendations are to replace the sugar in the formulation, use proper sanitation to reduce post-heat contamination load, maintain the storage temperature below 4°C, and reduce temperature abuse.

## Conclusion

The examples in this chapter describe how products are contaminated with different types of spoilage bacteria, many as post-heat contaminants. Complicated machineries, handling of large volumes of products, and a desire for a low initial microbial load by good sanitation have probably selected out those bacteria that can establish in the facilities and contaminate the products. In the absence of competition and being psychrotrophic and anaerobic or facultative anaerobic, even from a low initial contamination level, the bacteria can multiply and reach high levels during long storage to cause spoilage. In the absence of effective preservatives as well as because of temperature abuse during storage, transport, and display, they can grow more rapidly and cause rapid spoilage of the products. In some situations, poor sanitation (contamination with Gram-negatives) of the facilities was observed. Table 21.3 summarizes some general characteristics of predominant spoilage bacteria in vacuum-packaged refrigerated processed foods. It is also important to recognize that because processed products are stored at refrigerated or frozen temperatures for a long time (60–100 days or more), some enzymes of the contaminating microorganisms can cause spoilage even in the absence of live cells. This is discussed in Chapter 22.

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**Table 21.3 Spoilage Characteristics of Some Predominant Bacteria in Vacuum-Packaged Refrigerated Foods**

| *Spoilage Characteristics*[a] | *Cell Morphology*[b] | *Most Likely Bacteria*[c] |
|---|---|---|
| Large volume of gas and purge, no $H_2S$, not much off-odor, purge pH ≤ 5.5 | Coccoid to lenticular, small chains, Gram +ve | *Leuconostoc* spp. |
| Little gas and purge, no $H_2S$, not much off-odor, purge pH ≤ 5 | Rods, some in chains, nonmotile, Gram +ve | Homofermentative *Lactobacillus* spp. |
| Large gas, little purge, $H_2S$ +/–, off-odor, purge pH ≤ 6 | Rods, some in chains, nonmotile, Gram +ve | Heterofermentative *Lactobacillus* spp. |
| Little gas and purge, $NH_3$ odor, purge pH ≥ 7.5 | Small rods, motile, Gram –ve | *Serratia* spp. |
| Little gas and purge, putrid odor, purge pH ≥ 8.0 | Small rods, motile, Gram –ve | *Hafnia*, other *Enterobacteriaceae* |
| Large volume of gas and purge, $H_2S$ odor, purge pH ≥ 7.5 | Thick rods of different sizes, motile, some with terminal spores, Gram +ve | Psychrotrophic *Clostridium* spp. |

[a] There are some characteristics commonly found in the early stage of spoilage of a product. However, a sample can be spoiled by more than one type, especially in the early stages, and can show mixed characteristics.

[b] Morphology is examined with a drop of purge under a phase-contrast microscope. With practice, an almost presumptive positive identification can be made in minutes.

[c] Except for spores of *Clostridium* spp., all are sensitive to low-heat treatments (pasteurization). Their presence in heated products results mostly from post-heat contamination and on the product surface.

## QUESTIONS

1. Define psychrophilic and psychrotrophic microorganisms. Discuss why psychrotrophs are important in food spoilage.
2. Briefly discuss the reasons for the current popularity of refrigerated foods.
3. What are the possible causes of microbiological problems in refrigerated foods?
4. List six bacterial genera or species that are currently designated as new spoilage bacteria. Explain the possible reasons for their becoming important in the spoilage of refrigerated foods.
5. A processor recently started producing a new vacuum-packaged, low-heat processed, low-fat meat product with an expected shelf life of 60 days at 40°F (4.4°C). He found that approximately 10% of the product was getting spoiled within 30–40 days. The characteristics were accumulation of gas and purge (cloudy) with an acidic and cheesy odor. He asks you to help him determine the causes and resolve the problem. Explain briefly, in a stepwise fashion, how you plan to proceed in order to understand the problem (causes, sources, sequence of events, etc.) and solve it.
6. Answer questions 3–6 in Chapter 3 with the current understanding from this chapter on the pathogen problems in the listed foods. Suggest their route of contamination of the respective products and the sequences that led to the problem in each case.

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# Chapter 22

# Food Spoilage by Microbial Enzymes

## Introduction

The metabolism of food nutrients, principally carbohydrates, nitrogenous compounds, and lipids, by spoilage bacteria enables the cells to increase in number as well as produce metabolites that can adversely reduce the acceptance quality of a food. A food is considered spoiled when the changes are detectable and the microbial population has reached approximately $10^{7-9}$/mL/g or/cm$^2$. The changes are brought about by the catalytic actions of a large number of microbial enzymes. Most microbial enzymes are intracellular and act on the nutrients that can be transported inside the cells through several transport mechanisms. A bacterial cell contains many enzymes, many of which are intracellular, and some are extracellular. Intracellular enzymes are involved in the metabolism of small nutrient molecules of food that are transported inside the cells. Many intracellular enzymes can also act on intracellular large molecules, such as endonucleases, mucopeptidase, lipases, and proteinases. Extracellular enzymes, after synthesis, either remain bound to the cell surface or are excreted into the food environment. Many of the latter group can hydrolyze large nutrient molecules of food (e.g., polysaccharides, proteins, and lipids) to small molecules before they are transported into the cells (see Chapters 8 and 12).

Most foods have some amounts of low-molecular-weight, metabolizable carbohydrates (monosaccharides and disaccharides and their derivatives, such as glucose-6-phosphate), nitrogenous compounds (small peptides, amino acids, nucleosides, nucleotides, urea, creatinine, and trimethylamine oxide), free fatty acids, and some organic acids (lactic, citric, and malic acids). Many spoilage microorganisms, particularly spoilage bacteria, are able to utilize the low-molecular-weight food components to reach a population of $10^{7-9}$/g/mL or/cm$^2$ food and cause detectable food spoilage. Thus, the supply of extra nutrients from the hydrolysis of the macromolecules of foods by bacterial extracellular enzymes is not necessary for the onset of spoilage of many foods. In fact, studies with *Pseudomonas* spp. and *Bacillus* spp. show that in the presence of low-molecular-weight nitrogenous compounds, the synthesis of extracellular proteinases is repressed. When the supply of the small molecules is used up, the mechanism is derepressed, leading to the synthesis



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and excretion of extracellular proteinases. The proteinases hydrolyze the large protein molecules of food to produce small peptides and amino acids for transport and endogenous metabolism in the cells, which, in turn, intensify the spoilage. In general, microbial food spoilage from the metabolism of low-molecular-weight nutrients occurs at the early stage of microbial growth; spoilage from the breakdown of macromolecules by extracellular enzymes appears late in the sequence of events.

After microbial cells die normally or are killed by nonthermal treatments so that the intracellular and extracellular enzymes are not inactivated or destroyed, the enzymes can cause food spoilage even in the absence of viable cells or growth of microorganisms. Many microbial cells in a food that are subjected to freezing (then thawing), drying (then rehydration), modified atmosphere or vacuum packaging, refrigeration, high hydrostatic pressure, an electric pulse field, or high-intensity light, or are exposed to some preservatives, may die and undergo autolysis to release intracellular enzymes. If these foods are stored for a long time under conditions that favor catalysis of one or more intracellular and extracellular enzymes, they can undergo spoilage. Ripening (not spoilage) of cheddar cheese (at low temperatures and low $A_W$) is a good example; wherein following death, bacterial intracellular enzymes are involved in the breakdown of milk nutrients, not to cause spoilage but to impart desirable product characteristics. In most foods, the initial microbial population is generally low and spoilage by microbial enzymes in the absence of growth may not be of practical significance. However, if a product is heavily contaminated with a high initial microbial load (in the absence of growth) and then subjected to a nonthermal treatment that kills microorganisms but does not inactivate enzymes, spoilage of the food by microbial enzymes can occur (Chapter 21). In thermally processed foods, several heat-stable enzymes of the microorganisms retain their activity even after the producer cells are killed. During subsequent storage of the food under favorable conditions, these enzymes can break down the food nutrients to cause spoilage. Among the heat-stable enzymes, some extracellular proteinases, lipases, and phospholipases of several psychrotrophic bacteria found in food cause spoilage of thermally processed dairy products.[1–3]

## Characteristics of Heat-Stable Enzymes of Psychrotrophic Bacteria

Raw milk, between production and pasteurization in commercial operations, is usually stored for 1–2 weeks at refrigerated temperatures (<7°C). Psychrotrophic bacteria in the raw milk, coming from water, equipment, and the environment, can multiply during storage. Depending on initial numbers, bacterial species and strains, temperature and time of storage, and the extent of temperature abuse, the population can reach the level of producing sufficient amounts of extracellular heat-stable enzymes. Even though raw milk may not be spoiled, the heat-stable enzymes produced can cause spoilage of heat-treated dairy products manufactured from this raw milk. Heating, such as pasteurization and ultrahigh temperature treatments (UHT), kills psychrotrophic bacteria but does not inactivate heat-stable enzymes.

In raw milk, heat-stable proteinases, lipases, and, to some extent, phospholipases produced by several psychrotrophic Gram-negative bacteria are considered to be of major economic importance because of the spoilage potential of the products.[4,5] Species from genera *Pseudomonas* (*Pseudomonas fluorescens*, *Pse. fragi*), *Aeromonas*, *Flavobacterium*, *Shewanella* (*Alteromonas*), *Serratia*, and *Acinetobacter* produce heat-stable extracellular proteinases, and *Pseudomonas*, *Alcaligenes*, *Shewanella*, *Acinetobacter*, and *Serratia* produce heat-stable lipases. *Pseudomonas* spp. also produce heat-stable phospholipases. Many species from these genera are normally present in

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raw milk, meat, fish, and other food products. During refrigerated storage, psychrotrophs are able to grow and produce heat-stable enzymes in foods.[6,7]

Production of heat-stable proteinases and lipases in milk by some of these bacteria, especially *Pseudomonas* spp., and the characteristics of these enzymes have been well studied. The results show that different species and strains produce proteinases and lipases that differ in molecular weight and activity. Thus, a highly active proteinase or lipase produced by a *Pseudomonas* strain can produce extensive proteolysis or lipolysis, even at $10^{5-6}$ cells/mL milk. But another strain may need to reach to $10^9$ cells/mL to produce similar changes. The enzymes can be produced in raw milk at refrigerated temperatures (1°C–7°C) in sufficient quantities to hydrolyze proteins and lipids in detectable levels within 3–7 days. The catalytic activity of the proteinases is highest between pH 6.0 and 7.0 with a pH range between 5.0 and 9.0. Pasteurization of milk (at 63°C for 30 minutes or 71°C for 15 seconds) results in a loss of 6%–36% activity and, even after heating at 121°C for 10 minutes, some activity of the proteinases is retained. UHT treatment (140°C–150°C for 1–5 seconds) fails to completely inactivate proteinases produced by some species and strains of *Pseudomonas* and other psychrotrophs. UHT-treated milk can thus be spoiled by the residual activity of the proteinases during storage. Lipases are only partially inactivated by pasteurization or by heating (in cream) at 90°C for two minutes. They are generally inactivated by UHT treatment. Lipases from some *Pseudomonas* strains retain sufficient activity even after heating at 100°C for 10 minutes.

Heat-stable proteinases of psychrotrophic bacteria differ in their substrate specificity and rate of substrate degradation. Proteinases of *Pseudomonas* spp. preferentially degrade casein of milk by a different mechanism. Proteinases from some species or strains initially degrade β-casein, whereas proteinases from other species or strains initially degrade k-casein. With time, they can also degrade other casein fractions. Proteinases from psychrotrophic species or strains of *Flavobacterium*, *Aeromonas*, and *Serratia* also showed initial differences in the degradation of β- and k-caseins. α-Casein is degraded last by all strains.

Lipases of psychrotrophic bacteria differ in their specificity toward lipids. A lipase from a strain of *Pse. fragi* specifically hydrolyzes fatty acids from positions 1 and 3 of the triglycerides, whereas another lipase from a second strain hydrolyzes only at position 1. Similar specificities have been observed with extracellular lipases from *Pse. fluorescens*. Some lipases of psychrotrophic bacteria equally hydrolyze all three fatty acids.

Among the phospholipases produced by psychrotrophic Gram-negative bacteria, phospholipase C from *Pseudomonas* spp. has relatively high heat stability. It is not destroyed by pasteurization. Phospholipase C from *Pse. fluorescens* is more active against phosphatidylethanolamine than other phospholipids.

## Spoilage of Foods with Heat-Stable Microbial Enzymes

The presence of heat-stable extracellular enzymes of psychrotrophic bacteria in raw milk can cause spoilage of dairy products made from it. In addition, when these dairy products are used as ingredients to make other food products, the action of heat-stable enzymes can also reduce their acceptance qualities. Several examples are used here to emphasize the spoilage potential of these enzymes.[8,9]

### *Pasteurized Milk*

Heat-stable proteinases and lipases of psychrotrophic bacteria are not inactivated by pasteurization and can cause proteolysis of casein and lipolysis of milk lipids to produce flavor defects. However,

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under normal short-term refrigerated storage, these defects may not be enough to detect. In addition, psychrotrophic bacteria contaminating milk after pasteurization can also multiply during storage and cause spoilage, especially when the milk is either stored for a long time or temperature abused. In that event, it is difficult to differentiate the role of bacterial growth and heat-stable enzymes in spoilage.

### Ultrahigh Temperature (UHT)-Treated Milk Products

UHT-treated milks, heated at 140°C–150°C for 1 to 5 seconds, are considered commercially sterile products with a shelf life of three months at 20°C. Spoilage of these products during storage at 20°C has been observed in the form of bitter flavor, sediments, and gel formation, resulting from the action of heat-stable proteinases, and rancid flavor from the action of heat-stable lipases. Generally, the changes produced by proteinases are more predominant than those associated with lipases. However, in the presence of heat-stable phospholipases, the lipolysis caused by lipases can be detected. It is speculated that phospholipases degrade the phospholipids in the membrane of fat globules and increase the susceptibility of fat to lipases.[7,9]

The time of spoilage of UHT-treated milk by heat-stable proteinases is dependent on the numbers and strains of *Pseudomonas* species growing in the raw milk. In a controlled study, UHT-treated milk was prepared from raw milk inoculated with a *Pse. fluorescens* strain and grown to $8 \times 10^5$ to $5 \times 10^7$ cells/mL. During storage at 20°C, the product with $5 \times 10^7$ cells/mL gelled in 10–14 days; the product with $8 \times 10^6$ cells/mL gelled in 8–10 weeks; and the product with $8 \times 10^5$ cells/mL did not gel in 20 weeks but had sediments. Raw milk whose caseins have been highly degraded by proteolytic enzymes may even be unstable to high heat treatment and thus cannot be used for the manufacture of UHT-treated milk.

### Cheeses

Proteolytic activity by the extracellular proteinases of psychrotrophic bacteria in raw milk was reported to reduce cheese yield and increase the levels of nitrogenous compounds in whey. Depending on the proteolysis of caseins, the loss in cheese yield can be as high as 5%. The loss was directly related to the storage time of the raw milk and psychrotrophic counts. In addition, the heat-stable proteolytic enzymes were associated with increased proteolysis of cheeses (especially soft cheeses, such as cottage cheese), lower flavor quality, and higher texture problems in cheddar cheese. Lipases have also been implicated in the development of off-flavor in cheese.

### Cultured Dairy Products

Buttermilk and yogurt, made from milk that has had substantial growth of psychrotrophic bacteria before heat treatment, generally have poor texture and rapidly develop off-flavor during storage, even though they have low pH and are stored at approximately 10°C. These defects are attributed to bacterial heat-stable proteinases.

### Cream and Butter

Cream and butter are more susceptible to spoilage by heat-stable lipases than by proteinases. Extracellular lipases of psychrotrophic bacteria preferentially partition with the cream phase of milk, increasing their concentrations in cream. Lipases are responsible for off-flavor development

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in cream. A cream that has undergone lipolysis foams excessively and takes a longer time to churn during processing of butter. The butter prepared from such cream is susceptible to rancidity more quickly.

Butter containing residual heat-stable bacterial lipases undergoes rapid lipid hydrolysis even during storage at –10°C. Lipases that preferentially release short-chain fatty acids (C4–C8), with and without long-chain fatty acids, cause the most off-flavor in butter.

### *Milk Powder*

The activity of heat-stable bacterial proteinases and lipases present in raw and pasteurized milk is not denatured during the manufacture of spray-dried milk powder. The low $A_W$ prevents these enzymes from degrading the proteins and lipids in dry milk. Powdered whole milk, nonfat milk, and whey are used as ingredients in a wide variety of foods that can have high amounts of proteins and lipids and are expected to have a long shelf life. Bakery products, ice cream, desserts, processed meat products, chocolate, cheese products, and condensed dairy products are some of the foods in which dry milks are used. These products can develop off-flavor and texture defects from the action of heat-stable proteinases and lipases during storage.

## Spoilage of Foods by Microbial Enzymes at Low Temperatures

Limited studies have shown that bacterial extracellular proteinases are not directly associated with development of off-flavor in fish and meat. The initial off-flavors associated with degradation of nitrogenous compounds develop from the bacterial metabolism of nonprotein nitrogenous (NPN) compounds present in these products. However, the bacterial extracellular proteinases once produced are able to act on tissue proteins and cause texture defects (such as slime formation). This probably occurs when the NPN compounds have been used up and the population has reached approximately $10^8$/g or/cm$^2$ of the products. Protein hydrolysis by bacterial proteinases favors the development of a higher degree of putrefactive changes. Some proteinases of *Pse. fragi* reduce oxymyoglobin and discolor meat. The enzymes probably hydrolyze the globin part of the polypeptide chain of the myoglobin, which alters the reactivity of the heme group, leading to discoloration of meat. Muscle lipids can also be hydrolyzed by bacterial lipases, causing flavor defects in meat and fish. However, because lipids are susceptible to rancidity caused by lipases of the flesh and by autooxidation, the contribution of bacterial lipases is probably very little.

The heat stability of the bacterial proteinases and lipases associated with flavor and texture defects in raw meat and fish is not known. However, these enzymes are produced by the same psychrotrophic bacteria, namely, *Pseudomonas*, *Aeromonas*, and similar species that are known to produce extracellular heat-stable enzymes.[8,9] It can be assumed that some of these enzymes are heat stable. It will be important to determine whether these enzymes are potentially able to cause spoilage of low-heat-processed (pasteurized) meat products, some of which are expected to have a shelf life of 100 days.

In recent years, some specialty raw chicken meat products that were stored at refrigerated temperatures (–1°C to 0°C for 28 days) or at frozen temperatures (–20°C up to 90 days) were reported to develop distinct flavor defects before the expiration dates. Microbiological analysis of the freshly prepared products and the products with flavor defects during storage led to the suspicion that bacterial enzymes active at low temperatures might be associated with the flavor defects. The enzymes could be the exoenzymes produced by the bacteria in a product while it was still fresh,

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which acted on specific food components during long storage and produced the flavor components in detectable amounts. At low temperatures, bacterial cells can also die, lyse, and release intracellular enzymes. Some of these enzymes can be active at low temperatures to produce a defect that can be detected during long storage. It is likely that, in the future, commercial production of many such value-added specialty products with a long shelf life will increase. It will be important to recognize the problem and determine the cause to overcome such product defects. These aspects are discussed with actual incidents in Chapter 21.

## Conclusion

Enzymes of microorganisms either secreted outside or released from inside following lysis of the cells can cause spoilage of specific foods even in the absence of live cells or cell growth. Some of these enzymes can be resistant to physical and chemical treatments given to food and can be active at the storage conditions of the food. As our likings for the refrigerated foods increase and the foods are stored for a longer time, problems associated with these enzymes will increase. There is a need to determine the presence of live cells as well as their enzymes to predict the shelf life of foods. In Chapter 23, some of the methods currently used to determine shelf life as well as some new approaches are discussed.

### QUESTIONS

1. Discuss the role of intracellular enzymes of spoilage bacteria in food spoilage.
2. Describe the role of extracellular enzymes of spoilage bacteria in food spoilage.
3. Briefly discuss the production and characteristics of heat-stable enzymes by psychrotrophic Gram-negative bacteria. List three bacterial genera that produce heat-stable proteinases and lipases.
4. Briefly describe the problems associated with heat-stable microbial enzymes in UHT milk, cottage cheese, cheddar cheese, and cream.
5. Explain possible spoilage problems from using nonfat dry milk and cream made from a batch of raw milk in which a *Pse. flourescens* grew to $5 \times 10^6$ cells/mL; nonfat dry milk is used to make yogurt and cream to make sour cream.
6. What are the possibilities for heat-stable enzymes of spoilage bacteria to cause spoilage of a low-heat-processed, vacuum-packaged, refrigerated meat product with a shelf life of 15 weeks?
7. Nowadays, many foods are stored under refrigerated or frozen temperatures (≤–20°C). Discuss possible spoilage problems in these foods because of microbial enzymes in the absence of microbial growth.

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# Chapter 23

# Indicators of Microbial Food Spoilage

## Introduction

Microorganisms cause food spoilage in two ways. The first, and the more important one, is through the growth and active metabolism of food components by live cells. The second is produced, even in the absence of live cells, by extracellular and intracellular enzymes that react with food components and change their functional properties, leading to spoilage. The loss of food by microbial spoilage has economic consequences for producers, processors, and consumers. With the increase in world population, loss of food by microbial (and nonmicrobial) spoilage means that less food is available for the hungry mouth. World population in 2011 was about 7 billion, and it is expected to reach 8 billion in 2025 and 9.1 billion in 2050. To fight world hunger, efforts should be directed not only toward increasing food production, but also to minimizing spoilage so that enough food is available for consumption.[1] Many preservation methods have been devised to reduce microbial spoilage and are discussed in Part VI. Under certain methods of preservation, both raw and partially processed (semipreserved, perishable, nonsterile) foods are susceptible to microbial spoilage. This is more evident in foods that are expected to have a long shelf life. To reduce loss of raw and partially processed foods by microbial spoilage, two points are important. One is to predict how long a food, following production, will stay acceptable under the conditions of storage normally used for that food; that is, what will be the expected shelf life under normal conditions of handling and storage. The other is to determine the current status, with respect to spoilage, of a food that has been stored for some time. This information needs to be available well before a food develops obvious detectable spoilage and therefore becomes unacceptable.

Many criteria have been evaluated for their efficiency as indicators predicting expected shelf life as well as to estimating stages of microbial food spoilage. These criteria or indicators can be grouped as sensory, microbiological, and chemical (specific microbial metabolites). Sensory tests (e.g., changes in color, odor, flavor, texture, and general appearance), although easy and quick to perform, have several drawbacks as indicators, especially if used alone. Changes in texture and flavor generally appear at the advanced stages of spoilage. Odor changes can be masked by the spices



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used in many products. Odor changes from volatile metabolites may not be detected in a product that is exposed to air as compared with the same product in an airtight package. Color changes, such as in meat exposed to air, may not be associated with microbial growth. Finally, individuals differ greatly in their perception of organoleptic criteria. However, sensory criteria can be used advantageously along with microbiological or chemical criteria or both.

Studies by many researchers have clearly revealed that a single microbiological or chemical test is not effective in predicting either the shelf life of a product or its spoilage status. The contributing factors in microbial spoilage of a food include the type of product, its composition, methods used during processing, contamination during processing, nature of the packaging, temperature and time of storage, and possible temperature abuse. Because these factors differ with products, it may be rational to select indicators on the basis of a product or a group of similar products. Some factors to be considered in selecting a microbial or chemical indicator for a product (or several similar types of products) are the following:

1. In a good fresh product, it can be present in low numbers (microbial) or absent (chemical).
2. Under normal conditions of storage (temperature, time, packaging), it should increase (microbial or chemical) in quantity to reach a very high level.
3. When spoilage occurs under normal storage conditions, it should be the predominant causative agent (microbial or chemical).
4. It can be detected rapidly (microbial or chemical).
5. It can be used reliably to predict shelf life and spoilage status (microbial or chemical).
6. It should have a good relationship with the sensory criteria of spoilage of the particular product (microbial or chemical).

Different microbial groups and their metabolites (chemicals) have been evaluated for their suitability as indicators of food spoilage. As bacteria are the most predominant microbial group in food spoilage, the effectiveness of some bacteria and metabolites as indicators is briefly discussed. In addition, the effectiveness of testing microbial heat-stable enzymes in predicting shelf life of products susceptible to spoilage by them is also discussed.

## Microbiological Criteria

### *Enumeration of Colony-Forming Units (CFUs)*

Previous discussions indicated that the initial levels and types of spoilage microorganisms differ with the products or, more accurately, with the sources and intrinsic and extrinsic environments of the products. It is rational to select the microorganisms predominantly involved in spoilage of a food (or a food group) as the indicators of spoilage for that food. As an example, refrigerated ground meat, during aerobic storage, is normally spoiled by Gram-negative psychrotrophic aerobic rods, most importantly by *Pseudomonas* spp. Thus, the population level of psychrotrophic Gram-negative rods should be the most appropriate indicator of spoilage for this product (or for raw meats stored under the same conditions), both for predicting shelf life of the product and estimating the status of spoilage during storage. Total aerobic plate count (TAPC), which measures mesophilic populations, may not be a good indicator for this product as many mesophiles do not multiply at psychrotrophic temperatures and, conversely, some psychrotrophic bacteria do not multiply at 35°C in the two days used to enumerate APCs in meats. However, TAPC (also

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standard plate count or SPC for dairy products) has special importance in food microbiology. In fresh products, it indicates the effectiveness of sanitary procedures used during processing and handling and before storage of the product. A high TAPC or SPC in a food product, such as chicken salad or pasteurized milk, is viewed with suspicion, both for stability and safety. Thus, it is good to include TAPC or SPC along with the method suitable to detect the load of an appropriate spoilage indicator group for a food, based on its specific type and storage conditions.

Some of the specific microbial groups that can be used as spoilage indicators in different foods (or food types) are listed here. Details of the procedure can be found in books on microbiological examination of foods (also see Chapter 42).

1. *Refrigerated raw (fresh) meats stored aerobically*. Enumeration of CFUs/g or/$cm^2$ of psychrotrophic aerobes, especially Gram-negative aerobes. Data can be available in two to seven days, depending on the agar media and indicator, plating methods (pour or surface), and incubation temperature (10°C–25°C) used.
2. *Refrigerated raw (fresh) meats stored anaerobically (vacuum packaged)*. Enumeration of CFUs/g or/$cm^2$ of psychrotrophic lactic acid bacteria (by plating in a suitable agar medium adjusted to pH 5.0 with lactic acid) as well as psychrotrophic Enterobacteriaceae (in violet red bile glucose agar medium) and probably gram-negative bacteria. Depending on incubation temperature, data can be available in two to seven days. The plates may be incubated in a $CO_2$ environment for lactic acid bacteria. The products can also be tested for psychrotrophic *Clostridium* spp., such as *Clo. laramie*, by specific methods (strict anaerobic conditions).
3. *Refrigerated low-heat-processed vacuum-packaged meat products*. Enumeration of CFUs/g or/$cm^2$ of psychrotrophic lactic acid bacteria (by plating in an agar medium adjusted to pH 5.0) as well as psychrotrophic Enterobacteriaceae (in violet red bile glucose agar medium) and gram-negative bacteria. Depending on incubation temperature, data can be available in two to seven days. The plates for lactic acid bacteria can be incubated in a $CO_2$ environment. The products can be tested for psychrotrophic *Clostridium* spp., such as *Clostridium laramie*, by specific methods.
4. *Raw milk.* SPC, psychrotrophic gram-negative bacteria, thermoduric bacteria.
5. *Pasteurized milk*. SPC, psychrotrophic bacteria (gram-negative and gram-positive).
6. *Butter*. Lipolytic microorganisms.
7. *Cottage cheese*. Psychrotrophic, especially gram-negative bacteria.
8. *Fishery products (raw)*. Psychrotrophic gram-negative bacteria.
9. *Beverages*. Aciduric bacteria, yeasts, and molds.
10. *Salad dressing and mayonnaise*. *Lactobacillus* spp. (especially *Lactobacillus fructivorans*) and yeasts.

The major disadvantage of microbiological enumeration methods is that it takes several days for population levels of the indicator microorganisms from enumeration of CFUs to become available. To overcome this problem, several indirect methods that indicate the probable population of microorganisms in foods have been devised. One such method is to determine lipopolysaccharides (LPS) present in a food. LPS is specifically found in gram-negative bacteria. Thus, by measuring LPS concentration (with a proper standard curve), the level of gram-negative bacteria in a food can be estimated. However, this method is not applicable for spoilage by gram-positive bacteria. Several other indirect methods studied are measurement of ATP (ATP concentrations increase with high numbers of viable cells), impedance or conductivity (electric conductivity decreases with increase in cell numbers), and dye reduction time (the higher the population, the faster the reduction). For each method, appropriate standard curves are used to determine bacterial levels.

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However, each method has specific advantages and disadvantages. Also, all methods are not applicable in all food systems (see Chapter 42).

### *Phase-Contrast Microscopy*

Small and easy-to-use phase-contrast microscopes are available to rapidly identify microbial types (morphology, motility, spore, and cell arrangement) present in a food. However, the population has to reach a fairly high level (~$10^{5-6}$/mL) before it can be viewed under a phase-contrast microscope. Also, food particles can interfere with the identification. With practice, it can be a very quick and easy method to get initial ideas about the predominant microbial types (Chapter 21). It is also possible to do quick and direct enumeration of cells by a suitable counting device (e.g., Petroff Hauser counter). The results can be interpreted in several ways. If a desired level is set (specification level), such as a spoilage-detection level, one can interpret the result as less than the level (desirable), very close to the level (should be used immediately), or above the level (and disposed of). For a nonliquid food, a known amount of food can be suspended in sterile water in a 1:1 dilution, mixed well, and one to two drops of supernatant fluid can be used on a microscopic slide or a counter for viewing or counting.

## Chemical Criteria

As microorganisms (particularly bacteria) grow in foods, they produce many types of metabolic byproducts associated with the spoilage characteristics. If a method is developed that is sensitive enough to measure a specific metabolite in very low concentrations and long before spoilage becomes obvious, then the results can be used to determine the spoilage status of a food. Methods studied thus far to measure microbial metabolites include $H_2S$ production, $NH_3$ production by colorimetric or titration methods, production of volatile reducing substances, $CO_2$ production, diacetyl and acetoin production, and indole production. However, different metabolites are produced by different species and strains of bacteria, and the results are not consistent; they cannot be used for different types of products.

Change in food pH, especially in meat and meat products, resulting from microbial growth has also been used to determine the spoilage status of a food. In normal meats, with a pH of approximately 5.5, metabolism of amino acids by some spoilage bacteria generates $NH_3$, amines, such as histamine, cadaverine, putrescine, and other basic compounds.[2] This shifts the pH to the basic side (as high as pH 8.0). In contrast, metabolism of carbohydrates (present or added) by some bacteria produces acids and reduces the pH further to the acidic side. Thus, measurement of pH of a stored meat product can also give some indication of its type of spoilage bacteria and the spoilage status (see Table 21.3). As the pH increases, the proteins become more hydrated, that is, the water-holding capacity (WHC) increases, and, when pressed, this meat has less extract-release volume (ERV); in contrast, when the pH shifts toward the acidic side, the WHC is lower and ERV is higher. However, many low-fat processed meat products are formulated with high phosphate and generally have a pH close to 7.0 (to increase WHC). The buffering action of phosphate may not allow pH to shift to the basic or acidic side from the microbial metabolism of amino acids and carbohydrates, respectively. In these products, pH measurement (or WHC or ERV measurements) may not be good indicators of spoilage status.

None of the microbiological and chemical criteria studied fulfills all the factors necessary for a good indicator that will indicate the expected shelf life of a fresh product as well as its spoilage status

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during storage. More emphasis needs to be given to develop suitable indicators to reduce the loss of food by microbial spoilage. In the future, biosensors (Chapter 42) may be developed that could be effective for indicating changes in specific metabolites by a group of bacteria with similar characteristics, which are considered important spoilage bacteria in a food group.[3] Commercially available electric nose (EN) biosensors, such as Sensor*fresh*Q™, LibraNose, FreshSense, etc. have been used to monitor microbial spoilage of fresh food, including meat, fish, and poultry. These devices detect volatile byproducts, such as $H_2S$, $NH_3$, $CO_2$, diacetyl, and acetoin, resulting from microbial metabolism.

## Molecular Methods in Spoilage Microbe Detection

Inherent drawbacks in conventional methods led to the application of modern molecular techniques in detection and identification of microorganisms or microbial community in food products. Because spoilage in products, such as raw meat, poultry, or meat products, are associated with multiple organisms, molecular tools allow analysis of microbial community. Methods, such as randomly amplified polymorphic DNA (RAPD)-PCR, real-time PCR, pulsed field gel electrophoresis (PFGE), rep-PCR, multilocus sequence typing (MLST), and whole genome sequencing, have been used on various meat products[4] (see Chapter 42).

## Assay of Heat-Stable Enzymes

### *Heat-Stable Proteinases in Milk*

Proteinases of some psychrotrophic bacteria, such as *Pseudomonas fluorescens* strain B52, even when present as low as 1 ng/mL, in raw milk can reduce the acceptance quality of UHT-treated milk during normal storage.[5,6] Because of this, it is very important that sensitive assay methods be used in their estimation to predict the shelf life of dairy products. Some of the earlier methods, such as UV absorbance, Folin–Ciocalteu reagent reaction, and gel diffusion assay, are probably not sensitive enough for this purpose. Several new methods, such as the use of trinitrobenzene sulfonic acid (TNBS) and fluorescamine reagents, are quite sensitive and are being tested to assay proteinases in milk. In the TNBS method, the reagent reacts with free amino groups and, under the experimental conditions, develops color that can be colorimetrically measured to determine the amount of free amino acids present because of proteolysis. Fluorescamine reacts with amino acids to form fluorescent compounds at pH 9.0 and can thus be fluorimetrically measured to determine protein hydrolysis. Other methods, such as enzyme-linked immunosorbant assay (ELISA) and luciferase inactivation assay are extremely sensitive and need further development before they can be used reliably.

### *Heat-Stable Lipases in Milk*

Because natural lipases are present in milk, the measurement of lipases produced specifically by psychrotrophic bacteria creates some difficulties. However, it can be overcome by heating the milk, which destroys milk lipases but not the bacterial heat-stable lipases. Assay methods that measure release of free fatty acids (FFAs) as a result of the hydrolysis of milk fat by the lipases can be titrated to determine the potential of lipolysis of the lipases. As milk contains FFAs naturally, this method may not be accurate. Methods in which esterases of chromogenic and fluorogenic

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compounds react with lipases to produce color or fluorescent products have also been developed, but they have limitations. Recently, a rapid and sensitive sandwich ELISA method was tested to determine lipases of *Pseudomonas* spp. An antibody produced against a *Pse. fluorescens* strain and linked to horseradish peroxidases reacted with lipases from many strains of *Pseudomonas* spp. The reliability and sensitivity of this technique are being studied.

## Conclusion

Food spoilage causes not only economic loss but also unavailability of food, especially in places of food shortage and with high populations, such as in some developing countries. To reduce both, efficient techniques need to predict storage potential of foods, especially because the consumption of processed foods is going to increase. The methods now used have limitations and are not highly efficient. New methods that are now being studied could be more effective in the future. One of these methods is the development of biosensors, which could detect important bacterial metabolites at very low concentrations. Another method is to determine bacterial load rapidly by PCR. However, this technique cannot differentiate between live and dead cells. Recent approaches involve the use of molecular typing methods that allow analysis of the microbial community to assess the microbial quality of products. As with lactic acid bacteria at present, if, in the future, the genome sequences of important spoilage bacteria become available, better and specific biosensors and PCR techniques can be developed to detect spoilage potential of a food at an early stage.

### QUESTIONS

1. List the factors to be considered in selecting indicators of food spoilage.
2. List two spoilage aspects that a suitable food spoilage indicator should indicate to prevent loss of a food by spoilage. Explain why different indicators need to be selected for different groups of foods.
3. Suggest and justify suitable microbial spoilage indicators for (a) tray-packed raw sausage; (b) vacuum-packaged, low-fat, refrigerated ground turkey; (c) fish fillet kept over ice in air; (d) salsa in a bottle stored at room temperature; (e) ready-to-eat baby carrots, ready-to-eat salads, and sprouts of seeds packaged aerobically in bags and stored at approximately 15°C; and (f) cut fruits in aerobically packaged containers at 15°C (consult Chapter 20).
4. List four chemical methods that can be used to detect spoilage of fresh meat stored at refrigerated temperature under aerobic conditions.
5. Explain how pH changes in fresh meats can be used as an indicator of spoilage. How is it related to WHC and ERV?
6. What promises does genome-based nanotechnology hold for the future to detect microbial spoilage potential of a food?

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# V MICROBIAL FOODBORNE DISEASES

Human illness from the consumption of foods contaminated with factors other than poisons or chemical toxic agents was recognized long before the understanding of the role of pathogens in foodborne diseases. Dietary guidelines in the ancient civilizations, such as eating foods after cooking well and serving warm, and not eating spoiled foods, were probably invoked to protect people at least partly from the danger of microbial foodborne diseases. In the Middle Ages, several mass food poisoning incidents in Europe from the consumption of grains infested with toxin-producing fungi were recorded. Concerns about the possible role of foods of animal origin in human diseases led to the introduction of proper hygienic methods in the handling of fresh meat. However, not until Pasteur's discovery of the role of microorganisms in foods was their involvement in foodborne diseases understood. This helped in searching for and isolating pathogens by suitable techniques from foods incriminated in foodborne diseases. *Salmonella* and *Staphylococcus aureus*, because of the high incidence of salmonellosis and staphylococcal poisoning, and *Clostridium botulinum*, because of the high fatality rate from botulism, were isolated from foods incriminated with foodborne disease before the 20th century (Chapter 1). Subsequently, many other pathogenic bacteria, toxin-producing molds, parasites, and pathogenic viruses were recognized as causative agents in human foodborne illnesses. The involvement of a few new pathogenic bacteria and viruses has been recognized only relatively recently. Even now, the exact role of many bacteria and viruses in foodborne diseases is not well understood, and several of them are designated as opportunistic pathogens. The changes in food production, processing, marketing, and consumption, together with our knowledge about the characteristics of these microorganisms and the development of efficient techniques for their detection, have enabled us to identify the role of some new pathogens or emerging pathogens in foodborne diseases. History suggests there will probably always be new pathogens, and, thus, as we develop methods to control the existing pathogens, we have to remain alert for the new ones. Furthermore, in recent years, global political instability has raised concerns about food bioterrorism with intentional administration of pathogenic microbes or toxins in food to disrupt the economy and to harm people. In this section, an overview of the microorganisms commonly associated with foodborne diseases is presented. The diseases caused by the foodborne pathogens have been arbitrarily divided into three groups; the basis of division is not always clear cut. Also included are some parasites and fish and shellfish toxins that can cause foodborne diseases. The aspects of new foodborne pathogens and the importance of indicator

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bacteria on establishing sanitary quality in food production have been included. The following topics are discussed in this section:

Chapter 24: Important Facts in Foodborne Diseases
Chapter 25: Foodborne Intoxications
Chapter 26: Foodborne Bacterial Infections
Chapter 27: Foodborne Toxico-Infections
Chapter 28: Opportunistic Bacterial Pathogens, Molds and Mycotoxins, Viruses, Parasites, and Fish and Shellfish Toxins
Chapter 29: New and Emerging Foodborne Pathogens
Chapter 30: Indicators of Bacterial Pathogens

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# Chapter 24

# Important Facts in Foodborne Diseases

## Introduction

The objective of this topic is to recognize the causes of foodborne diseases, the role of microorganisms and several other agents in foodborne diseases, and the importance of predisposing factors in the occurrence of a foodborne disease. This information will help not only to understand the epidemiology of foodborne diseases but also to develop means of controlling them. Foodborne pathogens/toxins are not only responsible for gastrointestinal disorders, such as diarrhea and vomiting, but also several systemic and chronic diseases, such as abortion, stillbirth, central nervous system disorders, meningitis, encephalitis, arthritis, and allergic diseases, which are summarized in Table 24.1. The causes of foodborne diseases can be broadly divided into three groups:[1,2]

1. From the consumption of food and water containing viable pathogenic microorganisms or their preformed toxins.
2. From the ingestion of pathogenic parasites, fish, and shellfish containing algal toxins and their preformed toxins through food.
3. For reasons other than viable pathogens or their toxins.

Some of the factors included in group 3 are

1. Ingestion of toxins naturally present in many foods. This includes certain mushrooms, some fruits and vegetables, and some seafoods.
2. Toxins formed in some foods. Examples are some biological amines (e.g., histamine) that form in some fish, cheeses, and fermented meat products as a result of breakdown of proteins by bacterial proteases.
3. The presence of toxic chemicals in contaminated food and water, such as heavy metals and some pesticides.



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**Table 24.1 Diseases Caused by Foodborne Pathogens**

| *Disease or Clinical Symptoms* | *Pathogens/Toxins Involved* |
|---|---|
| Vomiting, diarrhea, dysentery | *Staphylococcus*, *Bacillus*, *Cronobacter*, *Salmonella*, *Shigella*, *Vibrio*, *Norovirus*, Rota virus, Entamoeba; *Cryptosporidium*; *Cyclospora*; *Giardia*; *Cystoisospora*; *Taenia* |
| Arthritis (reactive arthritis, Reiter's syndrome, rheumatoid arthritis) | *Campylobacter, Salmonella, Shigella, Yersinia* |
| Hemorrhagic uremic syndrome (HUS), kidney disease | Shiga-toxin producing *Esc. coli* (STEC); *Shigella* spp. |
| Hepatitis and jaundice | Hepatitis A virus (HAV), Hepatitis E virus (HEV) |
| Guillain-Barre syndrome (GBS) | *Campylobacter* |
| CNS/meningitis/encephalitis | *Listeria*, bovine spongiform encephalopathy (BSE) |
| Miscarriage, stillbirth, neonatal infection | *Listeria, Toxoplasma* |
| Paralysis | *Clostridium botulinum*, fish and shellfish toxins, *Campylobacter* |
| Malignancies and autoimmune diseases | Mycotoxin |
| Allergic response | Fish and shellfish toxins |

4. Allergy to some normal components of a food. There are individuals who are allergic to gluten in cereals and develop digestive disorders (celiac disease) following consumption of food containing gluten.
5. Genetic inability to metabolize normal food components. The inability of some individuals to hydrolyze lactose in the small intestine because of the lack of production of enzyme lactase results in digestive disorders (lactose intolerance).
6. Nutritional disorders, such as rickets from calcium deficiency.
7. Indigestion from overeating or other reasons.

## Epidemiological Aspects

### *Investigation of a Foodborne Disease*

In general, various regulatory agencies at the local, state, and national levels are empowered with the responsibility of investigating the cause of a reported foodborne disease (see Appendix B). In the United States, the regulatory agencies involved in the investigation of a foodborne disease include local health departments; state health, food, and agriculture departments; the federal Food and Drug Administration (FDA); U.S. Department of Agriculture (USDA); Centers for Disease Control and Prevention (CDC); and several others. Initially, a medical doctor, suspecting a patient or patients to have a foodborne disease, informs the local or state health officials about the incidence. These agencies, following a preliminary investigation and recognizing the cause to

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be of food origin, report the incidence to the appropriate federal agencies, which then conduct an epidemiological investigation. The investigation at the state and federal levels involves examination of the suspected food(s), environmental samples and materials obtained from the patient(s) for pathogens, microbial and nonmicrobial toxins, and chemicals. Results of these tests provide direct evidence of the association of an agent (e.g., pathogen, toxin, parasite, chemicals) with the disease. In addition to testing suspected samples, both the sick and other people who consumed the same food from the same source are interviewed to establish an indirect association to the most likely food(s) with the disease. This information is collected, recorded, and reported by the CDC based in Atlanta, Georgia (see the section on current trends).

## *Foodborne Disease Outbreak*

In the United States, the federal regulatory agencies define a foodborne disease as an outbreak in which two or more people become sick with a similar illness (symptoms) from the consumption of the same food(s) from the same source, and the epidemiological investigations implicate, either directly or indirectly, the same food(s) from the same source as the cause of the illness. However, in the case of botulism, because of its high fatality rate, even when only one person has the illness, it is considered an outbreak. For chemical poisoning, a single case is also considered an outbreak.

## *Incidence of Foodborne Disease Outbreak*

The incidence of foodborne illnesses in most of the developed countries is lower than in many developing countries. The major reasons for the low incidence are the implementation of necessary regulations in the production and handling of foods, good sanitary and hygienic practices, and the availability of necessary facilities to reduce abuse. In the United States from 2007 to 2010, an average of 915 foodborne disease outbreaks were reported each year, involving 18,460 individuals (Table 24.2). However, the causative agents involved in 33% of the outbreaks are not known. This shows that even for the reported incidents, only some could be confirmed from the direct evidence. It is suspected that even in the developed countries, only a small fraction of the total incidences are reported. In many instances an individual will not go to a doctor, and even if a

**Table 24.2 Foodborne Disease Outbreaks from 2007–2010 in the United States**

| | *2007* | | *2008* | | *2009* | | *2010* | |
|---|---|---|---|---|---|---|---|---|
| *Agents* | *Outbreaks* | *Cases* | *Outbreaks* | *Cases* | *Outbreaks* | *Cases* | *Outbreaks* | *Cases* |
| Bacterial | 320 | 7115 | 265 | 8643 | 229 | 6279 | 251 | 6848 |
| Viral | 324 | 8087 | 359 | 9233 | 198 | 3984 | 299 | 5841 |
| Chemicals and toxins | 49 | 210 | 36 | 258 | 22 | 124 | 21 | 85 |
| Parasites | 5 | 65 | 6 | 106 | 1 | 8 | 1 | 5 |
| Unknown etiologic agents | 363 | 5122 | 350 | 4262 | 212 | 3013 | 263 | 2441 |
| Total | 1097 | 21,244 | 1034 | 23,152 | 675 | 13,759 | 852 | 15,685 |

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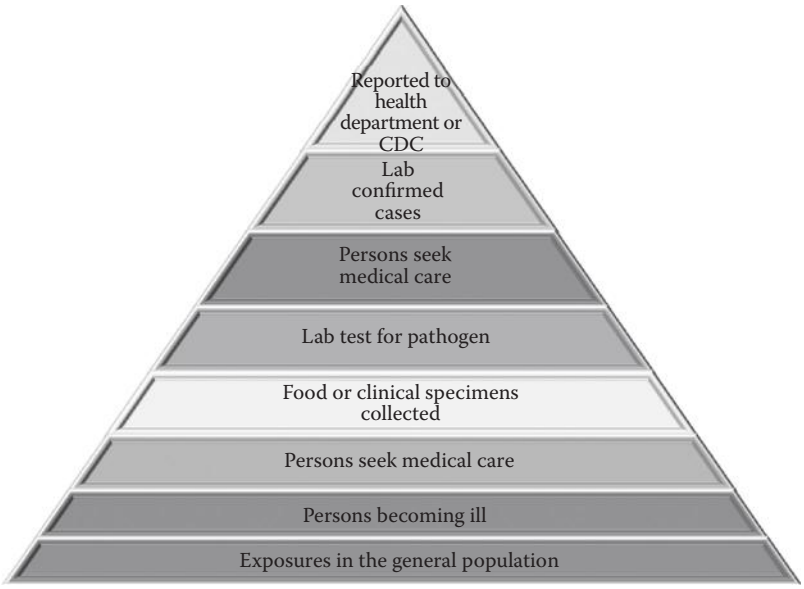



**Figure 24.1 Reporting of true burden of foodborne diseases. (Adapted from CDC, FoodNet Surveillance—Burden of Illness Pyramid, http://www.cdc.gov/FoodNet/surveillance_pages/burden_pyramid.htm.)**

person sees a doctor, the incidence may not be reported to the regulatory agencies; thus, the true burden of foodborne disease remains unknown (see CDC illustration in Figure 24.1). The World Health Organization (WHO) estimated that worldwide, in 2005, 1.4 million people died from diarrheal diseases from contaminated water and food.[2] In 2012, it was estimated that the global mortality rate resulting from foodborne disease are 0.26 to 15.65 deaths per 100,000 populations.[3] In the United States, it is estimated that foodborne pathogens are responsible for 48 million illnesses, 128,000 hospitalizations, and 3000 deaths. Of these, 9.4 million cases are caused by 31 known pathogens of bacterial, viral, and parasitic origin. Viruses were responsible for 5.5 million cases (59%), bacteria for 3.6 million cases (39%), and parasites for 0.2 million (2%) cases. Among the pathogens, most of the illnesses are caused by norovirus (58%), followed by nontyphoidal *Salmonella* (11%), *Clostridium perfringens* (10%), *Campylobacter* (9%), and others.[1,4] Even now, the occurrence of foodborne diseases caused by the pathogenic microorganism far exceeds all others combined. In recent years, foodborne diseases of microbial origin have become the number one food safety concern among U.S. consumers and regulatory agencies. This trend is probably true in most other developed and developing countries.

## *Cost of Foodborne Diseases*

Foodborne illnesses can be fatal as well as cause suffering, discomfort, and debilitation among the survivors. The economic losses from various factors could be very high. The factors include medical treatment, death, lawsuits, lost wages and productivity, loss of business, recall and destruction of products, and investigation of the outbreaks. In the United States, the annual cost of foodborne diseases is estimated to be over $78 billion. Costs associated with six major pathogens, including *Campylobacter*, *Escherichia coli* (O157:H7 and Shiga toxin producing *Esc. coli*), *Listeria*

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*monocytogenes*, nontyphoidal *Salmonella*, *Toxoplasma gondii*, and Norovirus were estimated, based on 2010 outbreak data, to be about $15 billion.[5]

## Predominant Etiological Agents

As indicated previously, gastrointestinal disorders can be caused by the consumption of food and water containing pathogenic microorganisms and their toxins; pathogenic algae and parasites and their toxins; toxic chemicals, either natural or as contaminants; and by other factors. Among these, the largest number of outbreaks, the total number of cases, and the number of deaths are caused by pathogenic bacteria and their toxins. Data presented in Table 24.2 show that, on average, the United States experiences 915 outbreaks each year based on the data collected from 2007 to 2010, and the majority of outbreaks were caused by bacterial and viral pathogens. Among the bacterial pathogens, nontyphoidal *Salmonella enterica* is responsible for the majority of foodborne bacterial illnesses (1.02 million/year) followed by *Clostridium perfringens*, *Campylobacter* spp, *Staphylococcus aureus*, Shiga-toxin producing *Esc. coli* (STEC), *Shigella* spp., *Yersinia enterocolitica*, and so forth (Table 24.3). Among the viral pathogens, norovirus is responsible for 5.4 million foodborne illnesses each year followed by sapovirus, rotavirus, and Hepatitis A virus. The top three foodborne

**Table 24.3 Estimated Yearly Cases of Foodborne Diseases and Related Deaths in the United States**

| *Pathogens* | *Cases* | *Hospitalizations* | *Deaths* | *% Deaths* |
|---|---|---|---|---|
| **Bacteria** | | | | |
| *Bacillus cereus* | 63,400 | 20 | 0 | 0 |
| *Brucella* spp. | 839 | 55 | 1 | 0.3 |
| *Campylobacter* spp. | 845,024 | 8463 | 76 | 5.5 |
| *Clostridium perfringens* | 965,958 | 438 | 26 | 0.4 |
| *Clostridium botulinum* | 55 | 42 | 9 | 0.2 |
| Shigatoxigenic *Esc. coli* (STEC), O157 | 63,153 | 2138 | 20 | 2.9 |
| STEC, non O157 | 112,752 | 271 | 20 | |
| Enterotoxigenic *Esc. coli* (ETEC) | 17,894 | 12 | 0 | 0 |
| *Listeria monocytogenes* | 1591 | 1455 | 255 | 27.6 |
| *Salmonella* spp. (nontyphoidal) | 1,027,561 | 19,336 | 378 | 30.6 |
| *Shigella* spp. | 131,254 | 1456 | 10 | 0.8 |
| *Staphylococcus aureus* | 241,148 | 1064 | 6 | 0.1 |
| *Streptococcus* Group A. | 11,217 | 1 | 0 | 0 |
| *Vibrio parahaemolyticus* | 34,664 | 100 | 4 | |
| Other *Vibrio* | 17,564 | 83 | 8 | 1.7 |

*(continued)*

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**Table 24.3 Estimated Yearly Cases of Foodborne Diseases and Related Deaths in the United States (Continued)**

| *Pathogens* | *Cases* | *Hospitalizations* | *Deaths* | *% Deaths* |
|---|---|---|---|---|
| *Vibrio cholerae* | 84 | 2 | 0 | 0 |
| *Vibrio vulnificus* | 96 | 93 | 36 | 1.0 |
| *Yersinia enterocolitica* | 97,656 | 533 | 29 | 0.1 |
| **Parasites** | | | | |
| *Giardia intestinali* | 78,840 | 225 | 2 | 0.1 |
| *Toxoplasma gondii* | 86,686 | 4428 | 327 | 20.7 |
| *Cryptosporidium parvum* | 57,616 | 210 | 4 | 0.4 |
| *Cyclospora cayetanensis* | 11,407 | 11 | 0 | 0 |
| *Trichinella spiralis* | 156 | 6 | 0 | 0 |
| **Viruses** | | | | |
| Norovirus | 5461 | 14,663 | 149 | 6.9 |
| Rota virus | 15,443 | 348 | 0 | 0 |
| Astrovirus | 15,443 | 87 | 0 | 0 |
| Hepatitis A | 1566 | 99 | 7 | 0.2 |
| Sapovirus | 15,443 | 87 | 0 | 0 |
| Total known | 9,388,074 | 55,962 | 1350 | |
| Total unknown | 38,392,704 | 127,839 | 1686 | |
| Grand total | 47,780,778 | 183,801 | 3036 | |

*Source:* Scallan, E. et al., *Emerg. Infect. Dis.*, *17*, 7–15, 2011.

parasites are *Toxoplasma gondii*, *Giardia intestinalis*, and *Cryptosporidium* (Table 24.3). Several factors may be involved in the high incidence caused by pathogenic bacteria: Many pathogenic bacteria are found in the raw food materials of animal and plant origin, many are present in the food environments, many grow very effectively in different foods, and many are not killed by the conditions used for processing of different foods.

## *Types of Microbial Foodborne Diseases*

Foodborne diseases in humans result from the consumption of either food and water contaminated with viable pathogenic bacterial cells (or spores in the case of infant botulism) or food containing toxins produced by toxigenic bacteria and molds. On the basis of mode of illness, these can be arbitrarily divided into three groups: intoxication or poisoning, infection, and toxico-infection (Table 24.4).

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**Table 24.4 Microbial Foodborne Diseases and Causative Pathogens**

| *Types of Disease* | *Causative Microorganism* | *Microbial Group* | *Major Symptom Type* |
|---|---|---|---|
| **Intoxication** | | | |
| Staph poisoning | *Staphylococcus aureus* | Bacteria, Gm+[a] | Vomiting, diarrhea |
| Botulism | *Clostridium botulinum* | Bacteria, Gm+ | Neurologic |
| Mycotoxin poisoning | Mycotoxin producing strains (e.g., *Aspergillus flavus*) | Molds | Carcinogenic, Hepatotoxic |
| **Infection** | | | |
| Salmonellosis | Over 2000 *Salmonella enterica* serovars (except *Sal*. Typhi and Paratyphi) | Bacteria, Gm–[a] | Diarrhea |
| *Campylobacter* enteritis | *Campylobacter jejuni* and *Cam. coli* strains | Bacteria, Gm– | Diarrhea |
| Yersiniosis | Pathogenic strains of *Yersinia enterocolitica* | Bacteria, Gm– | Diarrhea |
| Enterohemorrhagic *Escherichia coli* (EHEC) | *Esc. coli* O157:H7, *Esc. coli* O26:H11 | Bacteria, Gm– | Hemorrhagic diarrhea, Hemolytic uremic syndrome (HUS) |
| Enteropathogenic *Esc. coli* (EPEC) | *Esc. coli* O111:H12 | Bacteria, Gm– | Hemorrhagic diarrhea |
| Shigellosis | Four *Shigella* species (e.g., *Shi. dysenterae*) | Bacteria, Gm– | Bloody mucoid diarrhea |
| *Vibrio parahaemolyticus* gastroenteritis | Pathogenic strains of *Vib. parahemolyticus* | Bacteria, Gm– | Diarrhea, hepatitis |
| *Vibrio vulnificus* infection | *Vib. vulnificus* | Bacteria, Gm– | Diarrhea, hepatitis |
| Brucellosis | *Brucella abortus* | Bacteria, Gm– | Gastric and nongastric |
| Listeriosis | *Listeria monocytogenes* | Bacteria, Gm+ | Fever, meningitis, abortion, diarrhea (rare) |
| Viral infections | Pathogenic enteric viruses (e.g., Hepatitis A virus) | Virus | Fever, diarrhea, hepatitis |

*(continued)*

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**Table 24.4 Microbial Foodborne Diseases and Causative Pathogens (Continued)**

| *Types of Disease* | *Causative Microorganism* | *Microbial Group* | *Major Symptom Type* |
|---|---|---|---|
| Mad Cow disease | Bovine spongiform encephalopathy (BSE) (e.g., prion) | Protein | Neurologic |
| **Toxico-Infection** | | | |
| *Clostridium perfringens* gastroenteritis | *Clo. perfringens* | Bacteria, Gm+ | Diarrhea, vomiting |
| *Bacillus cereus* gastroenteritis | *Bacillus cereus* | Bacteria, Gm+ | Vomiting, diarrhea |
| *Escherichia coli* gastroenteritis | *Enterotoxigenic Esc. coli* (ETEC) serotype O15:H11 | Bacteria, Gm– | Travellers' diarrhea |
| Cholera | *Vibrio cholerae* | Bacteria, Gm– | Diarrhea |
| **Gastroenteritis by Opportunistic Pathogens** | | | |
| *Aeromonas hydrophila* Gastroenteritis | *Aeromonas hydrophila* | Bacteria, Gm– | Diarrhea |
| *Plesiomonas shigelloides* gastroenteritis | *Plesiomonas shigelloides* | Bacteria, Gm– | Diarrhea |

[a] Gm+, Gm–: Gram-positive and -negative, respectively.

### *Intoxication*

Illness occurs as a consequence of ingestion of a preformed bacterial or mold toxin (mycotoxin) from its growth in a food. A toxin has to be present in the contaminated food in an active form. Once the microorganisms have grown and produced a toxin in a food, there is no need of viable cells during the consumption of the food for illness to occur. An example is Staphylococcal food poisoning.

### *Infection*

Illness occurs as a result of the consumption of food and water contaminated with enteropathogenic bacteria or viruses. It is necessary that the cells of enteropathogenic bacteria and viruses remain alive in the food or water during consumption. The viable cells, even if present in small numbers, have the potential to establish and multiply in the digestive tract to cause the illness (e.g., Salmonellosis, hepatitis A virus infection).

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### Toxico-Infection

Illness occurs from the ingestion of a large number of viable cells of some pathogenic bacteria through contaminated food and water. Generally, the bacterial cells sporulate, colonize, or die and release toxin(s) to produce the symptoms (e.g., *Clo. perfringens* gastroenteritis).

In addition to the pathogenic microorganisms associated with foodborne illnesses, there are some bacterial species and strains, normally considered nonpathogenic, that are capable of causing gastroenteritis, especially in susceptible individuals. They are designated as opportunistic pathogens. They are normally required to be alive and present in large numbers when consumed through a contaminated food.

## Predominant Food Types Associated with Foodborne Diseases of Bacterial and Viral Origin

Certain food types or foods prepared under specific conditions and environments have been implicated more frequently with foodborne disease than others. Some of the factors could be the presence of a pathogen in the raw materials in higher frequency with a greater chance of contamination of the finished products, the ability of a pathogen to grow advantageously in a particular type of food, a greater chance of failure in quality control in a specific environment, and a higher possibility of contaminating the finished products by food handlers. Some of these aspects can be explained from the foodborne disease outbreak data presented in Table 24.5. Foods of animal origin (meat, fish, eggs, and dairy products), fresh fruits and vegetables, nuts, and spices are major source for outbreaks in recent years. However, the general trend for outbreaks in recent years is associated more with fresh produce involving lettuce, sprouts, cantaloupes, etc. The primary pathogens involved in many of these outbreaks are *Salmonella*, *Esc. coli*, *Listeria*, and norovirus.[6–8] *Salmonella* serovars were involved in a large proportion of the outbreaks because many food animals harbor *Salmonella* as carriers in the digestive tract and thus can contaminate meat, eggs, and dairy products and the environment. Peanuts and peanut butter and spices were also involved with several *Salmonella* outbreaks, possibly resulting from contamination from birds or environmental sources. Processed fish, especially those that are smoked, are found to contain large numbers of *Lis. monocytogenes*. In recent years, fish consumption has increased, and foodborne disease outbreaks from fishery products have also increased. Similarly, fruits and vegetables can be contaminated with *Esc. coli*, *Lis. Monocytogenes*, and *Salmonella* from untreated manure/fertilizer of animal origin, irrigation water, and the soil. In contrast, salads, which are handled extensively, can be contaminated with several pathogens of human origin. The high incidence of *Clo. perfringens* in Mexican foods and *Bac. cereus* in Chinese fried rice or pasta is likely a result of the methods used in the preparation of certain ethnic foods. Ready-to-eat processed lunchmeats (bologna, hotdogs) and soft cheeses made with unpasteurized milk (e.g., Mexican-style soft cheese) have high incidences of *Lis. monocytogenes*. However, one can speculate from the high incidence of *Salmonella* in both multiple foods and unknown food categories that many were probably foods of animal origin.

## Predominant Places of Food Consumption Associated with Confirmed Foodborne Disease Outbreaks of Bacterial and Viral Origin

An analysis of the relationship between the places of food consumption and the number of foodborne disease outbreaks revealed that, at least in the United States, the highest number of

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**Table 24.5 Foods and Pathogens Involved in Outbreaks from 2000–2012**

| *Year* | *Foods Involved* | *Place* | *Pathogen* | *No. Illnesses* | *No. Deaths* |
|---|---|---|---|---|---|
| 2012 | Tuna fish | USA | *Salmonella* Bareilly | 258 | – |
| 2011 | Lettuce | USA | *Esc. coli* O157:H7 | 60 | – |
| 2011 | Strawberries | USA | *Esc. coli* O157:H7 | 15 | 1 |
| 2011 | Cantaloupe | USA | *Lis. monocytogenes* | 146 | 31 |
| 2011 | Sprouts | Europe (Germany) | *Esc. coli* O104:H4 | 3911 | 48 |
| 2011 | Papaya | USA | *Salmonella* | 106 | – |
| 2011 | Alfalfa sprouts | USA | *Salmonella* | 140 | – |
| 2010 | Celery | USA | *Lis. monocytogenes* | 10 | 5 |
| 2010 | Lettuce | USA | *Esc. coli* O145 | 26 | |
| 2010 | Eggs | USA | *Salmonella* Enteritidis | 1939 | – |
| 2009–2010 | Black and red pepper | USA | *Salmonella* Montevideo | 272 | |
| 2009 | Alfalfa sprouts | USA | *Salmonella* | 235 | |
| 2009 | Frozen raspberries | Finland | Norovirus | 200 | |
| 2009 | Peanut butter | USA | *Salmonella* Typhimurium | 714 | 9 |
| 2008 | Mexican-grown peppers | USA | *Salmonella* Saintpaul | 1017 | 2 |
| 2007 | Chicken and turkey pot pies | USA | *Salmonella* | 401 | – |
| 2007 | Peanut Butter | USA | *Salmonella* | 425 | – |
| 2007 | Frozen ground beef patties | USA | *Esc. coli* O157:H7 | 40 | – |
| 2008 | Ground beef | USA | *Esc. coli* O157:H7 | 45 | – |
| 2007 | Milk or milk-related products | USA | *Lis. monocytogenes* | 5 | 3 |
| 2006 | Green onions | USA | *Esc. coli* O157:H7 | 67 | – |
| 2006 | Spinach | USA | *Esc. coli* O157:H7 | 199 | 3 |
| 2002 | Hamburger | USA | *Esc. coli* O157:H7 | 18 | – |

*(continued)*

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**Table 24.5 Foods and Pathogens Involved in Outbreaks from 2000–2012 (Continued)**

| *Year* | *Foods Involved* | *Place* | *Pathogen* | *No. Illnesses* | *No. Deaths* |
|---|---|---|---|---|---|
| 2002 | Processed chicken | USA | *Lis. monocytogenes* | 46 | 7 |
| 2000 | Bean sprouts | USA | *Salmonella* | 23 | – |
| 2000 | Dairy farms | USA | *Esc. coli* O157:H7 | 56 | – |
| 2000 | Delicatessen turkey | USA | *Lis. monocytogenes* | 30 | 4 deaths, 3 miscarriages |
| 2000 | Homemade Mexican-style cheese | USA | *Lis. monocytogenes* | 12 | 5 miscarriages |

incidences occurred with foods served at food establishments (Table 24.6). These include fast-food services, restaurants, cafeterias, and schools. Several factors could be associated with this.[9] The total number of meals served is very high. Also, high proportions of food are served within a very short period of time. In addition, the number of people involved in handling the food is very high, many of which may not have training in safe food handling.

This can result in a greater chance of contamination and failure to observe proper sanitation. Improper cooling of foods, cross-contamination, and contamination of food from pets could be the reasons for the foodborne diseases with foods served in homes. Mishandling of foods, temperature abuse, long intervals between preparation and consumption, and improper sanitation could be associated with the outbreaks at picnics. The exact sources could not be identified in 30% of the outbreaks; however, from the available information, it is most likely that many of them were probably with foods served at food establishments. Although not listed in the table, the relative

**Table 24.6 Predominant Establishments of Food Consumption Associated with Confirmed Foodborne Diseases Due to Pathogenic Bacteria and Viruses Between 1983 and 1987 in the United States**

| *Establishment* | *No. Outbreaks* | *%* |
|---|---|---|
| Homes | 149 | 24.8 |
| Food services[a] | 225 | 37.5 |
| Picnic[b] | 45 | 7.5 |
| Processing plants[c] | N/A | N/A |
| Unknown[d] | 181 | 30.2 |
| Total | 600 | 100 |

[a] Includes fastfood establishments, restaurants, cafeterias, school lunch programs.
[b] Includes family picnics, church picnics, camp.
[c] Data not available.
[d] Exact place(s) could not be identified.

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frequency of foodborne disease outbreaks with foods from large commercial processors is low. This is generally because of their use of good quality control, sanitation, and testing of the products for the pathogens. However, when a contamination of food by a pathogen occurs in a big processing establishment, a large number of people over a wide area are affected.[9]

## Predominant Contributing Factors Associated with Confirmed Foodborne Disease Outbreaks from Pathogenic Bacteria and Viruses

Before consumption, foods are exposed to many different environments and conditions. These dictate whether a pathogen present initially will survive or be killed, whether recontamination can occur, or whether a pathogen can multiply to reach a high population to cause disease. The foodborne disease outbreak data between 1983 and 1987 in the United States revealed that the most predominant cause of the outbreaks was improper holding (refrigerated) temperature of the food, which was responsible for 34.6% outbreaks. The temperature abuse resulted in the growth of pathogens (bacteria) reaching a level that causes illness following consumption. The other factors, in order of importance, were contamination of foods resulting from poor personal hygiene (20.5% outbreak), survival of pathogens after cooking at lower temperatures than specified (16.7%), cross-contamination of foods from equipment (previously contaminated with pathogens) (15.3%), and food from unsafe sources, such as raw foods (5.5%). These five factors, in combination, were responsible for 92.6% of the outbreaks caused by pathogenic bacteria and viruses.[9]

## Influence of Month (of the Year) to Number of Foodborne Disease Outbreaks Caused by Pathogenic Bacteria and Viruses

In general, foodborne outbreaks of pathogenic bacterial and viral origin are more prevalent during the summer months. The compiled data between 1983 and 1987 show that in the United States about 66% of the outbreaks occurred during May to September with the highest incidence in August.[9] Between November and April, the incidence was reduced to about 34%. During commercial processing, the food products are exposed to indoor temperatures, which may not vary greatly in the summer or the winter. However, both raw products and processed products could be exposed to outside temperatures for a long time during transportation, displays in retail stores, at food establishments, and at home. Also, during the summer, picnics and outdoor eating occur more frequently. A high temperature during the summer can stimulate rapid growth of the contaminating pathogens, reaching high levels, even from a low initial level, within a relatively shorter period. During the winter months, the growth rate can be greatly reduced; fewer outbreaks are reported to occur during February.

## Influence of Location on Foodborne Diseases of Pathogenic Bacterial and Viral Origin

It has been mentioned previously that, in general, the incidence of foodborne disease outbreaks is lower in the developed countries than in the developing countries. Several socioeconomic reasons and climatic conditions have major roles in this difference. However, the frequency of outbreaks can vary greatly among regions, both in the developed and developing countries. In the United States, foodborne disease outbreaks have been recorded more frequently in some states than in others. States with high populations, large numbers of industry, and warm climates have more

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incidences. Some of the states in this group are New York, Washington, California, Hawaii, and Pennsylvania. Some other factors, such as a large ethnic population, greater frequency of migration and traveling of people, as well as better surveillance systems, could also be the reasons for the high incidence rate. In comparison, states with lower populations had very few incidents. Imported foods from countries around the globe were also responsible for foodborne outbreaks in the United States. In 2013, the CDC, based on the data collected from 2005 to 2010 reported that imported foods from 15 countries were responsible for 39 outbreaks involving 2348 illnesses in the United States (see Chapter 29).[10]

## Human Factors in Foodborne Disease Symptoms

When a group of people consumes food contaminated with live cells of pathogens or their toxins, it does not make everybody develop disease symptoms.[9] Also, among those who develop the symptoms, all may not show either the same symptoms or the same severity of any symptom. This is probably a result of the difference in resistance among individuals. One of the factors involved in developing symptoms from the consumption of a contaminated food is susceptibility of an individual to the contaminants. In general, infants and old, sick, pregnant, and immunodeficient people (e.g., AIDS patients) are more susceptible than normal adults and healthy individuals. The chance of developing disease symptoms is directly related to the amount of a contaminated food consumed. This is related to the number of viable cells of a pathogen or the amount of a toxin consumed by an individual. The virulence of a pathogen or a toxin consumed through a food also determines the onset of a disease and severity of symptoms. For some highly virulent pathogens, such as *Esc. coli* serovar O157:H7, O104:H4 consumption of as low as 10 viable cells can cause disease in an infant. In contrast, for some pathogens, such as *Yersinia enterocolitica*, consumption of as high as 1 million or more viable cells is necessary for the symptoms to develop.

## Acceptance Quality of Food Resulting from Growth of Pathogens

Pathogenic viruses and protozoan parasites need viable host cells for growth; thus, in prepared foods, they cannot grow, and they do not affect the food quality. Pathogenic bacteria can grow in many foods. When the environment is suitable, only a few viable cells present initially can reach a high level, maybe several millions per gram or milliliter. However, growth of some pathogens, even to a high level, may not alter the color, texture, and odor of a food (such as *Sta. aureus*). People can consume this food without suspicion and develop symptoms of a foodborne illness.

## Sequence of Events in a Foodborne Disease

For a foodborne disease to occur, several events have to happen in sequence (Figure 24.2).[9] An understanding of these sequences is helpful in investigating the cause (the source and means of transmission) of a foodborne disease. It also helps in recognizing how the sequence can be broken in order to stop a foodborne disease. Initially, there has to be a source of a pathogen. Next, the pathogen has to contaminate a food. Consumption of the food contaminated with a pathogenic virus or parasites may lead to viral or parasitic infection. For bacterial pathogens (and toxicogenic molds), the contaminated food has to support growth and be exposed for a certain period of time at a suitable temperature to enable the pathogens to grow. However, for some potent pathogens (such as *Esc. coli* O157:H7) growth may not be necessary to cause a foodborne infection. For intoxication, the growth should reach a sufficient level to produce enough toxins so that when

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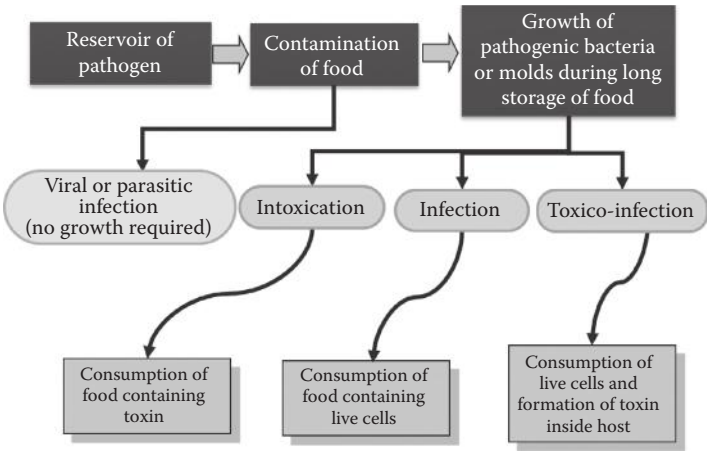



**Figure 24.2 Sequence of events that lead to foodborne disease by bacteria and viruses.**

the food is consumed, the individual develops the symptoms. For bacterial infection, viable cells of a pathogen need to be consumed in sufficient numbers, which vary greatly with pathogens, to survive stomach acidity, establish in the digestive tract, and cause illness. In the case of toxico-infection, viable cells should be consumed either in very high numbers (for those that cannot multiply in the digestive tract, such as *Clo. perfringens*) or in reasonable numbers (for those that can multiply in the digestive tract, such as *Vibrio cholerae*), so that toxins released by them in the digestive tract can produce the symptoms.

## Current Trends

In 1997, a summary review on foodborne illnesses in the United States between 1988 and 1992 was published. At present, through FoodNet, a foodborne disease surveillance network of the regulatory agencies has been set up, which makes it possible to obtain available foodborne outbreak reports relatively quickly. In addition, a recent article from the CDC (2011), based on a scientific approach, listed the estimated foodborne illnesses for each of the currently known foodborne pathogens (Table 24.3).[11]

### *FoodNet*

The CDC, along with the Food Safety Inspection Service (FSIS) of the USDA, the federal Food and Drug Administration (FDA), and the eight Emerging Infections Program (EIP) sites, developed the Foodborne Diseases Active Surveillance Network (FoodNet) Program in an effort to reduce foodborne diseases in the United States. The major objectives of FoodNet are to determine (1) the frequency and severity of foodborne diseases; (2) the proportion of common foodborne diseases that result from eating specific foods; and (3) the epidemiology of new bacterial, parasitic, and viral foodborne pathogens. To obtain the information, FoodNet conducts active surveillance and related epidemiological studies. FoodNet can determine the effectiveness of the programs initiated by the interagency National Food Safety Initiative to reduce foodborne diseases. One example is to study the effectiveness of the USDA Pathogen Reduction and Hazard Analysis

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Critical Control Point (HACCP) Rule in reducing the number of cases of major foodborne diseases in the United States each year. FoodNet is a sentinel network that can respond rapidly to new and emerging foodborne pathogens and evaluate new prevention and control strategies in order to safeguard the food and health of the consumers.[12]

FoodNet was established in 1996 by directly linking CDC, USDA-FSIS, and the FDA with the state and local health departments in the states of California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee. As of 2009, FoodNet conducted population-based active surveillance for confirmed cases of 10 pathogens, namely, *Campylobacter*, *Salmonella*, *Shigella*, Shigatoxigenic *Esc. coli* (STEC) O157:H7, STEC non-O157 (started from 2005), *Lis. monocytogenes*, *Yer. enterocolitica*, *Vibrio* spp., *Cryptosporidium*, and *Cyclospora* (the last two have been included since 1997), covering about 44–47 million persons each year.

The findings between 2000 and 2009 are summarized in Table 24.7. Over the 10-year period, the incidence of several pathogens declined while many showed an upward trend. Incidence of *Yersinia* decreased 53%, *Shigella* 55%, *Listeria* 26%, *Campylobacter* 30%, STEC O157:H7 41%, and *Salmonella* 10%. Incidence of *Sal*. Typhimurium decreased by 51%, Agona 48%, Heidelberg 33%, and Thompson 33% while the incidence of other serovars increased: Newport 64%, Oranienburg 39%, Enteritidis 32%, and Javiana by 120%. Incidence of *Vibrio* species increased 85%, whereas the incidence of *Cryptosporadium* remained unchanged.[12]

**Table 24.7 Rate of Incidence of Nine Foodborne Diseases in the United States During 2000–2009[12]**

| *Pathogens* | *Rate per 100,000 Population* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *2000* | *2001* | *2002* | *2003* | *2004* | *2005* | *2006* | *2007* | *2008* | *2009* |
| **Bacteria** | | | | | | | | | | |
| *Campylobacter* spp. | 15.7 | 13.8 | 13.37 | 12.6 | 12.9 | 12.72 | 12.71 | 12.79 | 12.68 | 13.02 |
| *Lis. monocytogenes* | 0.3 | 0.3 | 0.27 | 3.3 | 2.7 | 0.3 | 0.31 | 0.27 | 0.29 | 0.34 |
| Salmonella | 14.4 | 15.1 | 16.10 | 14.5 | 14.7 | 14.55 | 14.81 | 14.92 | 16.20 | 15.19 |
| *Shigella* spp. | 7.9 | 6.4 | 10.34 | 7.3 | 5.1 | 4.67 | 6.09 | 6.26 | 6.59 | 3.99 |
| STEC O157 | 2.1 | 1.6 | 1.73 | 1.1 | 0.9 | 1.06 | 1.31 | 1.20 | 1.12 | 0.99 |
| STEC non-O157 | NR | NR | NR | NR | NR | 0.33 | 0.46 | 0.57 | 0.45 | 0.57 |
| *Vibrio* spp. | 0.2 | 0.2 | 0.27 | 3.0 | 2.8 | 0.27 | 0.34 | 0.24 | 0.29 | 0.35 |
| *Yer. enterocolitica* | 0.4 | 0.4 | 0.44 | 4.0 | 3.9 | 0.36 | 0.35 | 0.36 | 0.96 | 0.32 |
| **Parasites** | | | | | | | | | | |
| *Cryptosporidium* | 1.5 | 1.5 | 1.42 | 10.9 | 13.2 | 2.95 | 1.91 | 2.67 | 2.25 | 2.86 |
| *Cyclospora* | 0.1 | 0.1 | 0.11 | 0.3 | 0.3 | 0.15 | 0.09 | 0.03 | 0.04 | 0.07 |

STEC, Shigatoxigenic *Esc. coli*; NR, not recorded.

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### Estimated Foodborne Illnesses

For a long time, there was a difference in opinion regarding the actual number of foodborne illness cases versus the number of cases reported annually in the United States. The average number of about 20,000 cases reported annually was thought to be too low. A realistic figure was estimated to be anywhere in the range of 5–25 million and there were individuals who thought it to be as high as 80 million.

Recently, a group of researchers at CDC published a report on the estimated number of cases of foodborne illnesses from both known as well as unknown causes.[1] They compiled and analyzed the information gathered by several surveillance systems (e.g., FoodNet) and other relevant sources. According to this report, the total number of foodborne illnesses from known and unknown causes was estimated to be 48 million annually with 128,000 hospitalizations and 3000 deaths in the United States. Among these, the known pathogens account for an estimated 9.4 million illnesses, 56,000 hospitalizations, and 1351 deaths (Table 24.3). Of these, nontyphoidal *Salmonella* was responsible for 28% of these deaths, followed by *Toxoplasma gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%).

They indicated that via different surveillance programs, an accurate number of illnesses associated with outbreaks caused by some pathogens were available. However, sporadic illnesses, especially for pathogens like *Bac. cereus*, *Clo. perfringens*, and *Sta. aureus* are not accurately available through such programs. For those pathogens, the outbreak-related cases were multiplied by 10 to obtain the total number of illnesses through sporadic as well as outbreak incidences. The basis of using this multiplier was from data where both sporadic and outbreak illnesses were available, such as for *Salmonella* and *Shigella*.

Some other important aspects mentioned in this report are that even with the current surveillance programs, the foodborne illnesses are underreported (Figure 24.1); many pathogens that are transmitted through food can also be transmitted through water and person to person, thus obscuring the role of foodborne transmission. Also, some of the current foodborne illnesses are caused by pathogens that have not yet been identified or caused by emerging pathogens (see Chapter 29).[1]

## Conclusion

The material presented in this chapter shows that foodborne diseases, either sporadic or outbreaks, can be caused by different pathogenic microorganisms, some of which are more predominant than others. The different parameters associated with the predominant factors (pathogenic bacteria and viruses) were also described. Finally, the sequence of events necessary for a foodborne disease to occur was discussed. This information will be helpful in understanding the material presented in the next chapters.

**QUESTIONS**

1. List the factors associated with a foodborne disease outbreak.
2. Define or explain an outbreak, a reported case, and a confirmed outbreak.
3. Survey 25 of your friends and relatives about the number of individuals had, at least once, a digestive disorder in the last 2 years. From this, calculate approximately how many people can be affected with foodborne disease in the United States per year. Is it close to 20,000? 5 million? 25 million? or 80 million?

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4. From the above survey estimate the most common food type (animal, plant) associated with foodborne diseases.
5. From the survey in question 4 use one incidence and discuss the symptoms, time of occurrence of symptoms after eating, the likely pathogen, probable source of the pathogen, and the predisposing factors that caused the disease.
6. Define foodborne intoxication, infection, and toxicoinfection, and give two examples for each.
7. Discuss why pathogenic bacteria are the predominant agents in foodborne diseases.
8. "Foods of animal origin are more frequently associated with foodborne disease outbreaks." Justify or explain the statement.
9. List the possible reasons of higher incidence of foodborne diseases with food served at the food service establishments.
10. List in order of importance, the five most important contributing factors in the occurrence of foodborne disease of pathogenic bacterial and viral origin.
11. List the sequence of events in case of a salmonellosis outbreak.
12. Briefly discuss what you have learned from the material in this section.

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# Chapter 25

# Foodborne Intoxications

## Introduction

Foodborne intoxication or food poisoning of microbial origin occurs from the ingestion of a food containing a preformed toxin from bacteria, such as *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum*, and mycotoxin from mold. Molds and mycotoxins are discussed in Chapter 28. Although *Bacillus cereus* can also form a heat-stable toxin and produce intoxication, this aspect is discussed with toxico-infection. The discussions include relative importance of a disease, characteristics of the microorganism(s) involved, predominant types of food, nature of the toxin(s), disease and the symptoms, preventative measures, and, for some, analysis of actual outbreak. Some general characteristics of food poisoning include the following:

1. The toxin is produced by a pathogen while growing in a food.
2. A toxin can be heat labile or heat stable.
3. Ingestion of a food containing an active toxin, not viable microbial cells, is necessary for poisoning (except for infant botulism or hidden botulism, in which viable spores need to be ingested).
4. Symptoms generally occur quickly, as early as 30 minutes after ingestion.
5. Symptoms differ with the type of toxin ingested; enterotoxins produce gastrointestinal symptoms, and neurotoxins produce neurological symptoms.
6. The febrile symptom is not present.

## Staphylococcal Intoxication

### *Importance*

Staphylococcal food poisoning (also known as staphylococcal gastroenteritis, staph food poisoning), caused by toxins of *Staphylococcus aureus*, is considered to be one of the most frequently occurring foodborne diseases worldwide.[1] In the United States, at least before the 1980s, it was implicated in many outbreaks. However, in recent years, the number of staphylococcal food



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poisoning outbreaks has declined. CDC reports indicate that during 1972 to 1976, it was associated with 21.4% of the foodborne disease outbreaks affecting 29.7% of total cases; in contrast, between 1983 and 1987, there were 5.2% staphylococcal foodborne outbreaks affecting 5.8% of the cases with no deaths. This decline is probably a reflection of the better use of refrigerated temperatures to store food and improved sanitary practices that can control contamination and growth of *Sta. aureus*. Even then, the number of outbreaks and number of cases of staphylococcal gastroenteritis is much higher than several other microbial foodborne disease outbreaks. In 2011, the CDC reported that *Sta. aureus* is responsible for about 240 cases with six deaths annually in the United States.[2] The European Food Safety Authority (EFSA) reported that in 2009 there were 293 outbreaks caused by *Staphylococcus* spp. and bacterial toxins (produced by *Bacillus*, *Clostridium*, and *Staphylococcus*), which were the fourth most common causative agent in foodborne outbreaks.

## *Characteristics of* Staphylococcus aureus[1,3]

### Organisms

*Sta. aureus* are gram-positive cocci; occur singly or in grape-like clusters (Figure 25.1); and are nonmotile, noncapsular, and nonsporulating. It produces a carotenoid pigment resulting in a golden-colored colony, hence the name aureus (which means golden). *Sta. aureus* is routinely isolated from foods by culturing of the most common selective medium, Baird-Parker (BP), which can be complemented with rabbit plasma fibrinogen (BP-RPF).

### Growth

Most strains ferment mannitol and produce coagulase, thermonuclease, and hemolysin but differ in their sensitivity to bacteriophages. The cells are killed at 66°C in 12 minutes and at 72°C in 15 seconds. *Sta. aureus* are facultative anaerobes but grow rapidly under aerobic conditions. They can ferment carbohydrates and also cause proteolysis by the extracellular proteolytic enzymes. They are mesophiles with a growth temperature range of 7°C–48°C with fairly rapid growth between 20°C and 37°C. Other important growth characteristics are their ability to grow at relatively low $A_W$ (0.86), low pH (4.8), and high salt and sugar concentrations of 15% and in the presence

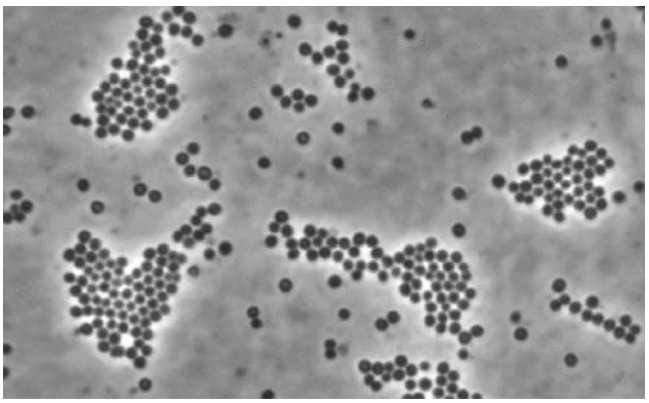


**Figure 25.1** ***Staphylococcus aureus*** **cells. Phase contrast microscopy (1000× magnification).**

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of $NO_2$. However, their growth can be reduced by combining two or more parameters. Because of their ability to grow under several adverse conditions, *Sta. aureus* can grow in many foods. Normally, they are poor competitors in the presence of many other microorganisms found in foods. But their ability to grow under adverse environments gives them an edge in growth in many foods where others do not grow favorably.

### Habitat

Enterotoxin-producing *Sta. aureus* strains have generally been associated with staphylococcal food intoxication. Although strains of several other *Staphylococcus* species are known to be enterotoxin producers, their involvement in food poisoning is not fully known. *Sta. aureus*, along with many other staphylococci, are naturally present in the nose, throat, skin, and hair (feathers) of healthy humans, animals, and birds. *Sta. aureus* can be present in infections, such as cuts in skin and abscesses (boils, carbuncles) in humans, animals, and birds, and cuts in hands and facial erupted acne in humans. Food contamination generally occurs from these sources.

## Toxins and Toxin Production

Enterotoxigenic strains of *Sta. aureus* produce 21 different enterotoxins: A, B, C1, C2, C3, D, and E through V (also designated as SEA, SEB, etc.). They are serologically distinct, heat-stable proteins of molecular weight 26–30 kDa and differ in toxicity. Toxins are water soluble and may be resistant to proteolytic enzymes. The genes encoding enterotoxin production are present in prophage, plasmids, pathogenicity islands, enterotoxin gene cluster (*egc*) and staphylococcal cassette chromosome (SCC). Toxins are produced when the food, primarily protein-rich food, is left at room temperature for long periods. The toxins vary in heat stability. Generally, they can withstand a temperature of 60°C for 16 hours. SEB is more stable than SEA, and it has been considered as a potential weapon in bioterrorism.[4] Normal temperature and time used in processing or cooking foods will not destroy the potency of the toxins. Outbreaks from SEA are more frequent; one reason could be because of its high potency.

The rate of toxin production by a strain is directly related to its rate of growth and cell concentrations. Optimum growth occurs around 37°C–40°C. Under optimum conditions of growth, toxins can be detected when a population has reached over a few million per gram or milliliter of food and generally in about four hours. Some of the lowest environmental parameters of toxin production are 10°C, pH 5.0, or $A_W$ 0.86. However, by combining two or more parameters, the lowest ranges can be adversely affected.

## Disease and Symptoms

Staphylococcal toxins are enteric toxins and cause gastroenteritis. A healthy adult has to consume about 30 g or mL of a food containing 100–200 ng toxins produced by $10^6$–$10^7$ cells per gram or milliliter; infants and old and sick individuals need less. Upon consumption, the toxin stimulates the vagus nerve in the stomach and induces severe vomiting (Figure 25.2). The symptoms occur within two to four hours with a range of 30 minutes to eight hours and are directly related to the potency and amounts of toxin ingested and an individual's resistance. The disease lasts for about one to two days and is rarely fatal and only six deaths are reported to occur annually in the United States.

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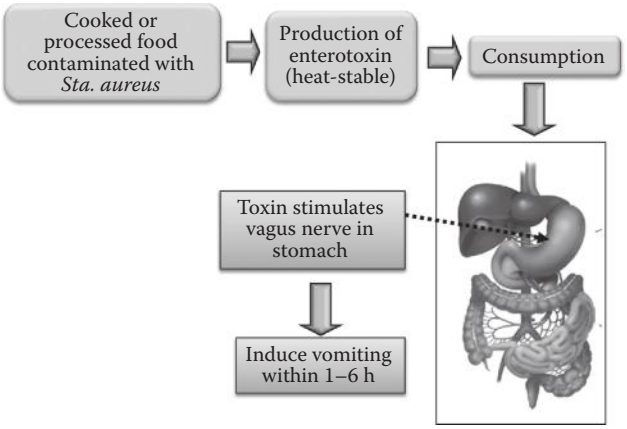



**Figure 25.2 Pathogenic mechanism of intoxication with enterotoxin from *Staphylococcus aureus*.**

The primary symptoms from the stimulation of the autonomic nervous system by the toxins are salivation, nausea and vomiting, abdominal cramps, and diarrhea. Some secondary symptoms are sweating, chills, headache, and dehydration. However, the symptoms and their severity vary among individuals in an outbreak.

## *Food Association*

Many foods have been implicated with staphylococcal foodborne outbreaks. In general, the bacterium grows in the food and produces toxins without adversely affecting the acceptance quality. Many protein-rich foods, foods that are handled extensively, foods in which associated bacteria grow poorly, and foods that have been temperature abused are associated with staphylococcal gastroenteritis. Some of the foods that have been more frequently implicated include ham, corned beef, salami, bacon, barbecued meat, salads, baking products containing cream, custard (puddings), salad dressings, sauces, and cheeses. The relative frequencies of different foods involved in staphylococcal food poisoning in the United States between 1973 and 1987 are presented in Table 25.1. Pork, baking products, beef, turkey, chicken, and eggs are associated with the high percentages of outbreaks. Different types of salads, because of extensive handling and a high chance of temperature abuse, have been implicated in relatively high numbers in staphylococcal food poisoning. From 1983 to 1987, salads were associated with 13% of total staphylococcal food poisoning outbreaks in the United States. They are included in the table in the "other" category. Three major contributing factors in these outbreaks from 1983 to 1987 were improper holding temperature (51.6%), poor personal hygiene (23.4%), and contaminated equipment (17.2%). Major sources of outbreaks (where foods were prepared or served) were food services (24.7%), homes (14.9%), and picnics (8.5%). A high percentage of outbreaks occurred between May and October (63.8%) with the highest frequency in August (21.3%). In the case of imported foods, a raw or processed food exported from a country can have *Sta. aureus* toxins but can cause food poisoning in a different country. This is exemplified by at least six outbreaks of staphylococcal gastroenteritis in 1989 in the United States from the consumption of dishes prepared using enterotoxin-containing canned mushrooms processed in a plant in the People's Republic of China. *Sta. aureus* probably grew prior to canning, and the enterotoxin, being heat stable, remained potent after canning.

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**Table 25.1 Food Types Involved in Confirmed Staphylococcal Food Poisoning Outbreaks in the United States from 1973 to 1987**

| *Food Type* | *% Outbreaks*[a] |
|---|---|
| Pork | 16.2 |
| Bakery products | 7.1 |
| Beef | 6.0 |
| Turkey | 5.5 |
| Chicken | 3.8 |
| Eggs | 2.5 |
| Fish | 1.3 |
| Dairy products | 1.7 |
| Fruits and vegetables | 1.1 |
| Ethnic foods | 1.1 |
| Other | 37.2[b] |
| Unknown | 6.5 |

[a] Out of a total of 367 outbreaks.
[b] The foods included in the "other" category are not known. However, salads were implicated in many outbreaks. Between 1983 and 1987, salads were involved in about 13% of the total staphylococcal foodborne disease outbreaks.

## *Prevention (Reduction) of the Disease*

The normal occurrence of *Sta. aureus* in raw food materials, among food handlers, and in many food environments makes it impossible to produce nonsterile foods that are free of this bacterium. Thus, a zero tolerance is not economically possible to achieve. One needs to recognize that many foods can contain *Sta. aureus*, and consumption of a food containing 100 or 500 cells per gram (or milliliter) is, in all probability, not going to make a person sick (unless the food has large amounts of preformed toxin). To reduce the incidence of staphylococcal food poisoning, the aim will be to reduce the initial load of *Sta. aureus* in a food by proper selection of the quality of the raw materials and ingredients, sanitation of the food environments, and proper personal hygiene among the food handlers.[3–5] People with respiratory diseases, acute types of facial acne, skin rashes, boils, and cuts on their hands should not handle the food. Where possible, the products should be heat-treated to ensure killing of the live cells. Following heating, recontamination of the products should be avoided. The most important aim should be that the processed products and ready-to-eat foods should be chilled to ≤5°C quickly. Suitable preservatives can also be used to kill or arrest growth. Care should be taken so that the inside of the food, not just the surface, reaches the chilled temperature, preferably within one hour. Finally, the food should not be subjected to temperature abuse and stored for a long period of time at growth temperature prior to eating. One should recognize that once the heat-stable toxins are formed, heating prior to eating will not ensure safety.

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## Identification Methods

To associate a food implicated in staphylococcal food poisoning, the food, or foods, or vomit samples are analyzed for the presence of high levels of enterotoxigenic *Sta. aureus* cells and enterotoxin(s) (Chapter 42). An enumeration technique in one or more selective differential agar media to determine the load of viable cells of *Sta. aureus* followed by several biochemical tests, such as hemolysis, coagulase, thermonuclease reactions, or ability of a pure culture to produce enterotoxin, are performed to link the potential causes of the food poisoning outbreaks.

The enterotoxin(s) from the food or vomit samples are extracted and tested, either by biological means or by serological means, to associate them with the outbreak. In the biological method, animals (such as cats, monkeys, or dogs) are given the enterotoxin preparation orally or injected intraperitoneally or intravenously. Vomiting symptoms by the test animals is a positive indication of the presence of staphylococcal enterotoxin.

In serological methods, the enterotoxins are purified and examined by one of the several recommended immunological methods. Not only are these tests very sensitive, but they also allow the identification of the types of enterotoxins involved in a food poisoning case.

## Analysis of an Outbreak

A foodborne disease outbreak affecting 52 of 101 people who attended a dinner (foods prepared at home) was reported on December 6, 1986, in Riverton, Wyoming. Of these, 49 people needed immediate medical attention. The symptoms developed between less than one to seven hours after the meal and included nausea (100%), vomiting (98%), diarrhea (90%), abdominal cramps (83%), prostration (62%), chills (52%), sweating (35%), and blood pressure–temperature depression (21%). When the regulatory people came to investigate after 36 hours, samples of foods or vomit of the patients from the doctors were not available. However, one vomit sample frozen by a patient was available and, when analyzed, was found to have $12–19 \times 10^6$ per gram coagulase-positive *Sta. aureus*. Meat obtained from a leftover turkey carcass had $1 \times 10^6$ per gram coagulase-positive *Sta. aureus*. An investigation revealed that a person who deboned and handled the cooked turkey had erupting facial rash (acneiform). The turkeys (three total) were improperly cooled following cooking and held on an improperly heated steam table for about four hours prior to serving.

This is a classical case of a staphylococcal food poisoning outbreak in which more than half of the people who ate foods involving several preparations developed many of the classical symptoms, some within 30 minutes. Although the food samples served in the dinner or vomit samples from the attending physicians or medical facilities were not available, analysis of a vomit sample saved by a patient revealed the presence of very high numbers of coagulase-positive *Sta. aureus* and meat available from a leftover turkey carcass also had fairly large numbers of *Sta. aureus*. So, from the symptoms and indirect evidence, the outbreak was concluded to be an incidence of staphylococcal food poisoning.

The sequences of events were most likely as follows: The erupting facial rash of the food handler was most probably the source of the pathogen. The pathogen was transmitted by the individual through the hands during the deboning of the cooked turkey. *Sta. aureus* is capable of growing in meat under a suitable environment. The turkey, following cooking and prior to serving, was temperature abused for a long period of time, thus enabling the contaminants to grow and reach very high populations. However, the acceptance quality of the meat was not adversely affected. The stage was set and many consumed enough toxin with turkey (and could be with other servings also) to develop the symptoms.

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## Botulism by *Clostridium botulinum*[5,6]

### *Importance*

Foodborne botulism results following consumption of food containing the potent botulinum toxin of *Clostridium botulinum*. It is a neurotoxin and produces neurological symptoms along with some gastric symptoms. Unless prompt treatment is administered, it is quite fatal. Infant botulism occurs from the ingestion by the infant of *Clo. botulinum* spores that germinate, grow, and produce toxins in the GI tract and cause specific symptoms.

In the United States, the average number of outbreaks per year is about 15–16. From 1973 to 1983, there were a total of 231 outbreaks. This represents about 8% (231 out of 2841) of the total reported foodborne outbreaks involving less than 4% (4984 out of 124,994) of the total cases. However, out of a total of 247 deaths (2 per 1000 cases) from all foodborne diseases, 47 died of botulism during this period (9.5 per 1000 cases) even with the medical treatment available in this country. It is now estimated botulism causes about 55 cases with nine deaths annually in the United States.[2] A majority of the outbreaks occur with foods prepared at home. Ethnic food preparations have been implicated in many cases.

### *Characteristics*

#### *Organisms*

Cells of *Clo. botulinum* strains are Gram-positive rods and occur as single cells or in small chains; many are motile, obligate anaerobes and form single terminal spores. Cells are sensitive to low pH (<4.6), low $A_W$ (0.93), and moderately high salt (5.5%). Spores do not germinate in the presence of nitrite (250 ppm). Spores are highly heat resistant (killed at 115°C) but the cells are killed at moderate heat (pasteurization). Toxins are produced during growth. Strains can either be proteolytic or nonproteolytic.

#### *Growth*

*Clo. botulinum* strains, on the basis of the type of toxin produced, have been divided into seven types: A, B, C, D, E, F, and G. Of these, A, B, E, and F have been associated with human foodborne intoxications while involvement of serotype G in human infection is not established yet. Type A strains are proteolytic, type E strains are nonproteolytic, but types B and F strains can be either proteolytic or nonproteolytic. The proteolytic strains can grow between 10°C and 48°C with the optimum at 35°C. The nonproteolytic strains grow optimally at 30°C with a range between 3.3°C and 45°C. Optimum growth facilitates optimum toxin production. An anaerobic condition is necessary for growth. Spore germination and outgrowth prior to cell multiplication is favored in the environment of cell growth. Similarly, conditions that prevent cell growth also adversely affect spore germination. As indicated before, either pH 4.6, $A_w$ 0.93, or 5.5% NaCl can prevent cell growth; but by using two or more parameters along with lower temperatures, the lower growth limits of any of the above parameters can be greatly reduced.[4]

#### *Habitat*

Spores of *Clo. botulinum* are widely distributed in soil, sewage, mud, sediments of marshes, lakes and coastal waters, plants, and the intestinal contents of animals and fish. Fruits and vegetables

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can be contaminated with spores from soil; fish from water and sediments, and various other foods can be contaminated from many of the above sources. While types A and B spores are more prevalent in soil, sewage, and fecal matters of animals, type E spores are generally found in marine environments. Type A spores are predominant in the western United States, and type B spores are found predominantly in the eastern United States and different parts of the world.

## *Toxins and Toxin Production*

The botulinum neurontoxin (BoNT) is a 150 kDa protein toxin produced by *Clo. botulinum*. It is an A-B type toxin consisting of two subunits: A subunit is 50 kDa, and B is 100 kDa. The toxin is produced as a large derivative toxin, which is inactive. Bacterial or stomach protease converts the inactive toxin to its active form. BoNT produced by nonproteolytic strains are activated by host protease (trypsin) in the digestive tract, whereas BoNT from proteolytic strain is activated by bacterial protease. In general, toxins associated with food intoxication in humans (types A, B, E, and F) are extremely potent, and only a small amount of toxin is required to produce the symptoms and death. Following ingestion, toxin molecules are absorbed from the upper part of the intestine through the intestinal wall and spread via the blood to the peripheral nerves. The B subunit of toxin binds to the receptor (sialic acid containing glycoprotein) in nerve cells and facilitates internalization of subunit A inside the nerve cells. A subunit has endopeptidase (metalloprotease) activity that cleaves synaptobrevin, a protein that controls the release of neurotransmitter acetylcholine in the neuromuscular junction, thereby interfering with the flow of nerve impulses (Figure 25.3). This results in irreversible flaccid paralysis of all involuntary muscles. The toxin moves slowly through the body. The toxin is

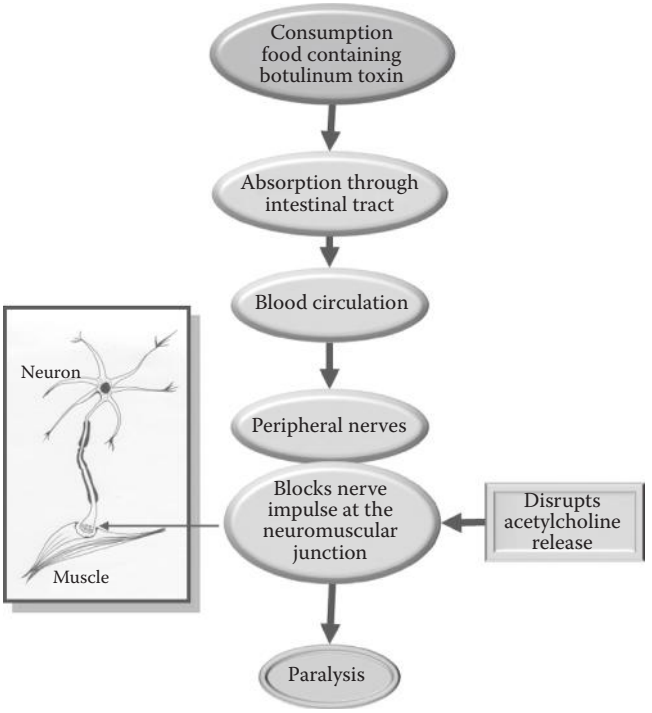



**Figure 25.3 Sequence of events in foodborne botulism (*Clostridium botulinum*).**

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heat labile and can be destroyed in a contaminated food by high and uniform heat, such as 90°C for 15 minutes or boiling for five minutes. Radiation at 5–7 mrad can also destroy it.

Cell growth is necessary for toxin production. At optimum growth temperatures, toxins are produced in large amounts. However, at both extreme growth ranges, enough toxins can be produced by a strain in a food to cause disease and death following ingestion.

## Disease and Symptoms

There are five types of botulism: foodborne botulism, infant botulism, hidden botulism, wound botulism, and inadvertent botulism.

### Foodborne Botulism

Foodborne botulism is caused by the ingestion of the BoNT formed in a food (Figure 25.3). The toxins are absorbed from the intestine and spread to the peripheral nerves, and they block the transmission of impulse. However, at the initial stage (generally 12–36 hours, but it can be two hours), some gastrointestinal disorders (e.g., nausea, vomiting, diarrhea, and constipation) may be evident. Neurological symptoms develop within a short time, especially if the amount of botulinum consumed is high. As they are highly potent toxins, only a very small amount (1 ng/kg body weight) is necessary for severe symptoms and even death. In general, neurological symptoms include blurred or double vision; difficulty in swallowing, breathing, and speaking; dryness of the mouth; and paralysis of different involuntary muscles that spreads to the diaphragm, lungs, and heart. Death usually results from respiratory failure.

### Infant Botulism

*Clo. botulinum* spores, ingested by human infants through food, such as honey, and the environment, can germinate in the intestine and produce toxins to cause infant botulism. The spores fail to produce the same disease, generally, in individuals above one year of age. In these individuals, the well-established normal population of gastrointestinal microflora prevent spore germination and cell multiplication by *Clo. botulinum*. Both types A and B have been identified in infant botulism cases. The symptoms consist of general weakness, inability to suck and control the head, loss of reflexes, and constipation. Foods, such as honey and corn syrup, and dirt have been linked as sources of *Clo. botulinum* spores in infant botulism cases.

### Hidden Botulism

Hidden botulism, also known as the adult variant of infant botulism, is seen in adults suffering from chronic gastrointestinal disorders, such as infectious bowel disease (IBD) or other gastrointestinal abnormalities. This disease is called "hidden" because the source of toxin is not so obvious, and no known food is linked to this disease. *Clo. botulinum* is almost always found in the feces, which aids in diagnosis of this disease.[5] Besides *Clo. botulinum*, *Clo. baratii* has been implicated to cause this disease.

### Wound Botulism

In wound botulism, spore-laden dust lodged in wounds as a result of a gunshot on the battlefield or an accidental cut will germinate and produce toxins.

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### Inadvertent Botulism

In inadvertent botulism, therapeutic (to cure dystonia, a chronic muscle tension disorder) or cosmetic (to remove wrinkles) use of BoNT can cause this type of botulism.

The toxins are antigenic; thus, antitoxins are available. Soon after the onset of the symptoms and if the amount of a toxin consumed is fairly low, trivalent antitoxins (against BoNT A, B, and E) can be administered to treat the disease. Antitoxin will neutralize the unbound toxins. Recovering patients can suffer from permanent neurological disorder because of the damage to nerve cells. But in some advanced cases, especially if the diagnosis is delayed, antitoxin administration may not be successful. In the United States, even with available facilities, botulism accounts for about 19% of the total foodborne fatal cases.

## Food Association[7,8]

The events in foodborne botulism involve contamination of a food with *Clo. botulinum* spores, survival of the spores during processing, and the ability of the spores to germinate and outgrow and the cells to multiply when the product is abused (temperature and time). Results presented in Table 25.2 show that the largest number of outbreaks are associated with fruits and vegetables. These were mainly low-acid vegetables (e.g., green beans, corn, spinach, asparagus, peppers, carrots, and mushrooms) and fruits (e.g., figs and peaches). The next highest incidence was with fin fish, and they include fermented, improperly cooked, and smoked fish and fish eggs. Type E was

**Table 25.2 Food Types Involved in Confirmed Botulism Outbreaks from 1973 to 1987 in the United States**

| *Food Types* | *No.* | *%* |
|---|---|---|
| Beef stew | 2 | 0.9 |
| Chicken | 1 | 0.4 |
| Dairy products | 1 | 0.4 |
| Finfish | 35 | 15.2 |
| Fruits and vegetables | 99 | 42.9 |
| Mexican food | 3 | 1.3 |
| Mushrooms | 5 | 2.2 |
| Beverages (nondairy) | 5 | 2.2 |
| Pork | 1 | 0.4 |
| Turkey | 1 | 0.4 |
| Other[a] | 40 | 17.3 |
| Unknown | 38 | 16.4 |

[a] Not identified but probably include several types of food, many of which were of "unlikely" categories, and no single type was involved in a large number of outbreaks.

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associated predominantly with fish while types A and B were associated with vegetables. The major cause of outbreaks was improper home canning of the contaminated products. Between 1983 and 1987, out of 231 botulism outbreaks in the United States, 56 occurred at home. Between 1990 and 2000, there were 263 cases from 160 foodborne botulism events, of which 103 (39%) cases and 58 events occurred in Alaska. Toxin type A caused 51% of all cases while Toxin E was involved in 90% of Alaska cases. Home-canned vegetables were involved in 44% cases.[9] The occurrence of botulism from meat, poultry, and dairy products is low. This is probably because they are mostly heated and eaten quickly. Several outbreaks from unlikely foods (sautéed onions, baked potatoes, home-canned bamboo shoots) have been recorded, but in many instances, the foods were subjected to temperature abuse (held for a long time at warm temperature). Some condiments, such as chili peppers, relish, and sauce, have also been associated with outbreaks.

Growth of proteolytic strains in meats and low-acid, high-protein vegetables generally produce obnoxious odors and gas. In low-protein vegetables, these characteristics may not be obvious. The growth of nonproteolytic strains, even when growing in meat, fish, and other high-protein foods, do not produce spoilage characteristics. As the toxins are heat labile, high uniform heating (90°C for 15 minutes or boiling for five minutes) of a suspected food will destroy the toxins and make the food theoretically safe. However, as only a small amount of toxin is enough to cause disease, it is better to avoid a suspected food.

## Prevention of Botulism[6]

The single most important control method is to use proper temperature and time in home canning of low-acid products. Commercial processors use the 13D concept (Chapter 33). Directions for pressure canning of foods at home are available, and they should be strictly followed. Proper and uniform cooking of some foods (e.g., fish) at high temperatures should be followed as outlined.[6] (1) Foods cooked at temperatures in which spores survive should be stored at low temperatures (at 3°C or below); (2) at refrigerated temperatures (4°C–5°C), storage should not be prolonged (the 10-day rule) unless some additional precautions are used, such as $NO_2$, low pH, low $A_W$, NaCl, and others; (3) a heat treatment of 90°C for 10 minutes or equivalent lethality (e.g., 80°C for 129 minutes, 85°C for 36 minutes) combined with storage at chill temperatures (designed to give a 6D process for nonproteolytic *Clo. botulinum*); (4) a pH ≤ 5.0 throughout the food, combined with storage at chilled temperatures; (5) a NaCl concentration of ≥3.5% throughout the food, combined with storage at chilled temperatures; (6) an $A_W \leq 0.97$ throughout the food, combined with storage at chilled temperatures; and (7) combinations of heat treatment and other preservative factors, which can be shown consistently to prevent growth and toxin production by *Clo. botulinum*, combined with storage at chilled temperatures. Suspected foods should be properly heated prior to consumption, but it is better not to eat them. Even tasting a small amount of a suspected food without giving high and uniform heat treatment can be dangerous.

## Identification Methods

In a suspected food, the presence of *Clo. botulinum* can be determined by enumeration techniques using selective agar media and anaerobic incubation, followed by biochemical and toxicological testing. The presence of toxins in the food is more often tested. This involves injection of a food extract intraperitoneally to mice. Development of characteristic neurological symptoms, followed by death in 92 hours suggests the presence of toxins. The people engaged in testing for the organism or the toxins need to immunize themselves prior to handling the materials.

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### *Analysis of a Foodborne Botulism Case*

On May 4, 1992, in New Jersey, a man of Egyptian origin developed dizziness, facial drooping, dry mouth, weakness, and respiratory difficulties. Within two days, two more family members developed similar symptoms. The cases were diagnosed as botulism and treated with trivalent (A, B, E) botulinal antitoxins. The source of the toxin was traced to an ethnic fish preparation made with uneviscerated, salt-cured fish. On May 3, the family obtained the product from outside and consumed it without cooking.

The sequence of events was most probably as follows: The fish had *Clo. botulinum* spores in the gut (probably type E) from the water and water sediment. The uneviscerated fish were cured in salt, the concentration of which was not high enough to prevent germination, cell growth, and toxin production. The product was consumed, probably in different amounts, without cooking by the family members, who developed symptoms at different times. The outbreak could have been avoided by removing the viscera, curing the fish in recommended salt concentrations, and heating prior to eating.

## Conclusion

Foodborne pathogenic microorganisms producing toxins while growing in foods and food ingredients are involved in human foodborne intoxication. The toxins can be proteins or small organic molecules and heat labile or heat stable. A toxin, depending upon the pathogen, can produce only enteric symptoms or neurological symptoms or cancer. The incidence of foodborne intoxication can be reduced by using proper sanitation and preservation of foods during processing and subsequent handling.

**QUESTIONS**

1. List five characteristics of foodborne intoxication.
2. Describe some growth characteristics of *Staphylococcus aureus* strains that give them an advantage for growth and toxin production in a food. Name two known food types that have the highest incidence of staphylococcal poisoning and state the predisposing causes.
3. List the characteristics of the enterotoxins of *Sta. aureus* and discuss the mechanisms by which they produce enteric symptoms.
4. List the symptoms of staphylococcal food poisoning and discuss how the disease can be differentiated from other food poisoning diseases of microbial origin.
5. From a case of a staphylococcal food poisoning outbreak (get it from the instructor or the Internet), determine the sequence of events in the outbreak. What precautions could have been taken to avoid the incidence?
6. Describe how foodborne botulism differs from infant botulism.
7. List the types of *Clostridium botulinum* associated with food intoxication and describe the changes that can occur from their growth in a food high in protein and a food low in protein.
8. List the toxins associated with foodborne botulism in humans and describe the mechanisms by which the toxins produce neurological symptoms.
9. Discuss how a combination of several environmental parameters can be used to control growth of *Clostridium botulinum* in a food.
10. List two food groups frequently associated with botulism in the United States. Suggest four methods that could reduce the incidence.

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# Chapter 26

# Foodborne Bacterial Infections

## Introduction

Foodborne infection occurs from the consumption of food (and water) contaminated with pathogenic enteric bacteria and viruses. Many pathogens are included in this group. Viruses are discussed in Chapter 28. However, many are involved more frequently than others, and they are discussed in this chapter. The discussions include their relative importance, characteristics, food association, toxins, disease symptoms, and prevention. For some, detection methods and case histories are also included. The following are some characteristics of foodborne infections:

1. Live cells of the enteric pathogens (bacteria and viruses) have to be consumed through food.
2. The surviving cells (from gastric environment) penetrate through the mucus membrane and establish in the epithelial cells of the intestines, multiply, and produce toxins and other virulence factors.
3. Dose levels that cause infection vary greatly. Theoretically, one live cell has the potential to produce the disease. Experts estimate that consumption of approximately 10 cells (for an extremely virulent species and strain, such as *Escherichia coli* O157:H7) to approximately $10^5$ cells or more (for a less virulent species and strain, such as *Yersinia enterocolitica*) might be required for the disease.
4. Symptoms generally occur after 24 hours, which, depending on the pathogen, can be both enteric and nonenteric in nature.
5. Enteric symptoms are local and result from enteric infection and the effect of toxins. Symptoms include abdominal pain, diarrhea (sometimes accompanied with blood), nausea, vomiting, and fever. Examples of pathogens include *Salmonella*, *Shigella*, enteropathogenic *Esc. coli* (EPEC), *Vibrio parahaemolyticus*, *Campylobacter jejuni*, and *Yer. enterocolitica*.
6. Nonenteric symptoms (along with enteric symptoms) result when the pathogens or their toxins pass through the intestine and invade or affect other internal organs and tissues. Symptoms depend on the types of organs and tissues affected but are accompanied by fever. Examples of pathogens include *Listeria monocytogenes*, enterohemorrhagic *Esc. coli* (EHEC), *Vib. vulnificus*, and Hepatitis A virus.



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## *Salmonella enterica*

### *Importance*

Prior to the 1940s, *Salmonella enterica* serovar Typhi and Paratyphi were considered the major causes of worldwide foodborne and waterborne diseases in humans caused by *Salmonella*. However, with the pasteurization of milk and chlorination of water supplies, the spread of typhoid and paratyphoid fever through food and water was greatly reduced, at least in developed countries. As efficient techniques for the isolation and identification of other *Salmonella* serovars from foods and environmental samples were developed, it became apparent that the worldwide incidence of foodborne salmonellosis caused by other *Salmonella* is quite high. Since the 1950s, it has been recognized that foodborne salmonellosis has been the major cause of all foodborne diseases by pathogenic bacteria and viruses, both in number of incidents (sporadic and outbreaks) and number of cases. Although, at present, scientific information about their habitats, mode of transmission in foods, growth characteristics, and survival parameters are available, and methods to control their contamination of foods have been developed, foodborne salmonellosis is still the leading cause of foodborne bacterial diseases and is the second leading cause of all foodborne diseases (norovirus is the first) in the United States and other developed countries. *Salmonella* is estimated to cause 1.2 million illnesses each year in the United States, with 23,000 hospitalizations and 450 deaths. This is quite puzzling. The control measures seem to be working for several other foodborne pathogens, at least in the United States, such as *Clostridium perfringens*, *Bacillus cereus*, *Yer. enterocolitica*, *Vib. parahaemolyticus*, and probably *Staphylococcus aureus*. Even incidences from *Sta. aureus* and *Lis. monocytogenes* seem to have declined since the 1990s. It is somewhat astonishing why it is not working against *Salmonella*. Between 1969 and 1976, the average number of reported foodborne salmonellosis outbreaks was about 37 per year in the United States. In contrast, between 1983 and 1987, the average number of outbreaks per year was over 68. In 2009, *Salmonella* was responsible for 113 outbreaks while in 2010, 121 outbreaks. Not only is the incidence not decreasing, it continues to increase at a high rate. It is difficult to point out the exact cause(s) of this increase. Maybe it is related to the large number of serotypes present in high frequency in the carrier state in food animals, birds, pets, insects, and humans, and their ability to grow in foods, or it may be the way the food animals and birds are raised, processed, and marketed, or it may be a result of better surveillance systems by the regulatory agencies, or maybe our lifestyles and food habits give these pathogens an edge.

Tauxe[1] indicated that the present increase in salmonellosis (including foodborne salmonellosis) in the United States could be related to four factors: the increase in number of antimicrobial-resistant *Salmonella* isolates, the increase in individuals with immunodeficiency who are extremely susceptible to *Salmonella*, the increase in egg-associated *Salmonella enterica* serovar Enteritidis contamination resulting from the increase in laying hens with infected ovaries, and food production in centralized facilities that can lead to, if contamination occurs, extremely large and widespread outbreaks. It is necessary to understand the importance of these factors in regard to the increase of salmonellosis and to develop corrective measures to control the incidence.

There are about 2600 serovars (based on somatic, flagellar, and capsular antigen types) of *Salmonella* and some that are capable of causing salmonellosis in humans. Along with fecal-oral direct transmission, contaminated food and water can cause salmonellosis. Recently, a better system of nomenclature has been developed to group these serovars in a few species instead of considering each as a separate species.[2]

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## Current Nomenclature System

The serovars of *Salmonella* were found to have a high degree of relatedness in the DNA. It was proposed on this basis that the genus *Salmonella* should have only one species, *Salmonella enterica*, and six subspecies, two of which are from the *Arizona* group (Table 26.1). The specific epithets that are used before (e.g., *typhimurium* for *Salmonella typhimurium*) should be considered as a serotypic name, and the strains of a serotype can be distinguished by numerals, letters, and a combination as of now. It is recommended that in formal publications, the complete name of the species and subspecies should be given (e.g., *Salmonella enterica* subsp. *enterica*). Then serotype names could be used (e.g., ser. Typhimurium; not italicized). The currently acceptable format is *Salmonella enterica* ser. Typhimurium.[2]

In this text, the previous method has been replaced for most serovars (e.g., *Sal. typhimurium*). The two serotypes that have been associated with frequent incidence of foodborne illness of *Salmonella*, *Sal. enterica* ser. Typhimurium and *Sal. enterica* ser. Enteritidis, are in subspecies 1. *Salmonella* is again grouped based on its sensitivity to specific phage types (PT) and designated PT4, PT8, PT13, PT13a, PT23, DT104 (definitive type), and so forth.

### Predominant Serotypes in Salmonellosis

Among the six subspecies, *Salmonella enterica* subsp. *enterica* includes most serotypes that are frequently associated with foodborne salmonellosis. However, two of the more than 2000 serotypes, serotype Typhimurium and serotype Enteritidis, are involved in higher frequencies worldwide. While many animals, including food animals and pets, harbor serotype Typhimurium in a carrier state, serotype Enteritidis can infect the ovaries of poultry, especially chicken, and can be transmitted through eggs.[3] Increasing numbers of serotype Enteritidis have also been isolated from chicken carcass rinse in samples collected between 2000 and 2005, and the predominant phage types are PT 13 and PT 8. The CDC in the United States has identified the top 20 serovars of *Salmonella enterica* serotypes that were involved in recent outbreaks (Table 26.2).

Because of the increase in antibiotic use in the feed of food animals and birds, there is an increase in multidrug resistant (MDR) strains among *Sal. enterica* serotypes. One that has created

**Table 26.1 Six Subspecies of *Genus Salmonella***

| |
|---|
| 1. *Salmonella enterica* subsp.[a] *enterica* |
| 2. *Salmonella enterica* subsp. *salamae* |
| 3a. *Salmonella enterica* subsp. *arizonae* |
| 3b. *Salmonella enterica* subsp. *diarizonae* |
| 4. *Salmonella enterica* subsp. *houtenae* |
| 5. *Salmonella enterica* subsp. *bongori* |

[a] The relationship between this subspecies scheme and Kauffman's subgenus scheme are as follows: subspecies 1 corresponds to subgenus I, subspecies 2 corresponds to subgenus II, subspecies 3a and 3b correspond to subgenus III, and *Arizona hinshawii*, subspecies 4 corresponds to subgenus IV, and subspecies 5 constitutes six atypical serotypes from subgenus II and IV.

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**Table 26.2 Top twenty *Salmonella enterica* Serotypes That Are Responsible for Majority of Outbreaks in Recent Years[a], 2009**

| *Rank* | *Serotype* | *Percent* |
|---|---|---|
| 1 | Enteritidis | 17.5 |
| 2 | Typhimurium | 15.0 |
| 3 | Newport | 9.3 |
| 4 | Javiana | 4.9 |
| 5 | Heidelberg | 3.5 |
| 6 | Montevideo | 3.1 |
| 7 | I 4,[5],12:i:- | 2.4 |
| 8 | Oranienburg | 2.2 |
| 9 | Saintpaul | 2.1 |
| 10 | Muenchen | 2.0 |
| 11 | Braenderup | 1.8 |
| 12 | Infantis | 1.6 |
| 13 | Thompson | 1.2 |
| 14 | Mississippi | 1.1 |
| 15 | Paratyphi B | 1.1 |
| 16 | Typhi | 1.1 |
| 17 | Agona | 1.0 |
| 18 | Schwarzengrund | 0.9 |
| 19 | Bareilly | 0.7 |
| 20 | Hadar | 0.7 |
| | Others | 27.2 |
| | Total | 100 |

[a] An overview of National *Salmonella* Surveillance is available online at http://www.cdc.gov/nationalsurveillance/PDFs/NationalSalmSurveillOverview_508.pdf.

concern is the MDR *Sal. enterica* ser. Typhimurium definitive phage type (DT) 104. The strains are resistant to several antibiotics, including ampicillin, chloramphenicol, streptomycin, spectinomycin, sulfonamides, florfenicol, and tetracycline. DT104 has also been reported to be resistant to nalidixic acid and ciprofloxacin. In addition, an emerging strain, DT204, resistant to eight or nine antibiotics, is also a concern. It is suspected that antibiotic resistance may be transferred among strains or serovars by different methods of genetic recombinations, especially under antibiotic

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selective pressure. Multidrug resistance in DT104 is a result of chromosomal integration of a 43 kb Salmonella genomic island 1 (SGI1) carrying antibiotic resistant genes.

During the last 10 years, foodborne salmonellosis from *Sal. enterica* ser. Enteritidis has increased greatly, and at present, the frequency of incidences from it is as high as that caused by *Sal. enterica* ser. Typhimurium. Among the strains, serotype Enteritidis PT4 and several other types are involved in salmonellosis in high frequency. The reasons for this could be the way the poultry is raised and also increased consumption of poultry.

## Characteristics

The *Salmonella* cells are Gram-negative, nonsporulating, facultative anaerobic motile rods. They form gas while growing in media containing glucose. Generally, they ferment dulcitol, but not lactose; utilize citrate as carbon source; produce hydrogen sulfide, decarboxylate lysine, and ornithine; do not produce indole; and are negative for urease. They are mesophilic with an optimum growth temperature between 35°C and 37°C but generally have a growth range of 5°C–46°C. They are killed by pasteurization temperatures and sensitive to low pH (4.5 or below) and do not multiply at $A_W$ 0.94, especially in combination with a pH at 5.5 and below. The cells survive under frozen and dried states for a long time. They are capable of multiplying in many foods without affecting the acceptance qualities. In recent years, *Salmonella* outbreaks associated with low-moisture foods, including peanuts, almonds, peanut butter, spices, and wheat flours indicate that they can survive at $A_W$ of as low as 0.2 for an extended period of time (over 1000 days).[4]

## Habitat

Salmonellae are natural inhabitants of the gastrointestinal tracts of domesticated and wild animals, birds, pets (including turtles and frogs), and insects. In animals and birds, they can cause salmonellosis and then persist in a carrier state. Humans can also be carriers following an infection and shed the pathogens through feces for a long time. They have also been isolated from soil, water, and sewage contaminated with fecal matters.

## Disease and Symptoms[5,6]

Human salmonellosis is different from typhoid and paratyphoid fever caused by *Sal. enterica* ser. Typhi and *Sal. enterica* ser. Paratyphi, respectively. Although there are some *Salmonella* serovars specific against different animals and birds, all are considered to be potential human pathogens capable of causing salmonellosis. Foodborne salmonellosis is characterized by gastrointestinal disorders manifested predominantly by diarrhea and abdominal cramp. A dose of $>10^{2-3}$ cells is needed to be consumed to initiate infection; however, there are some virulent strains of which ingestion of fewer cells can cause the disease. Strains that are sensitive to gastric acidity generally need more cells to establish in the intestine and cause the disease; conversely, acid-resistant strains may require fewer cells to cause the disease. The infectious dose decreases if the pathogen is consumed with food that neutralizes the stomach acidity, such as milk, cheese, and so forth. Progression of disease also depends on the physiological state of the host. Elderly persons with high gastric pH are vulnerable to salmonellosis.

Following ingestion, the pathogen colonizes in the small and large intestines, and most of the pathological lesions are reported to be found in the large intestine rather than in the small intestine. The organism adheres to the mucosal cells using fimbriae or other adhesion factors and

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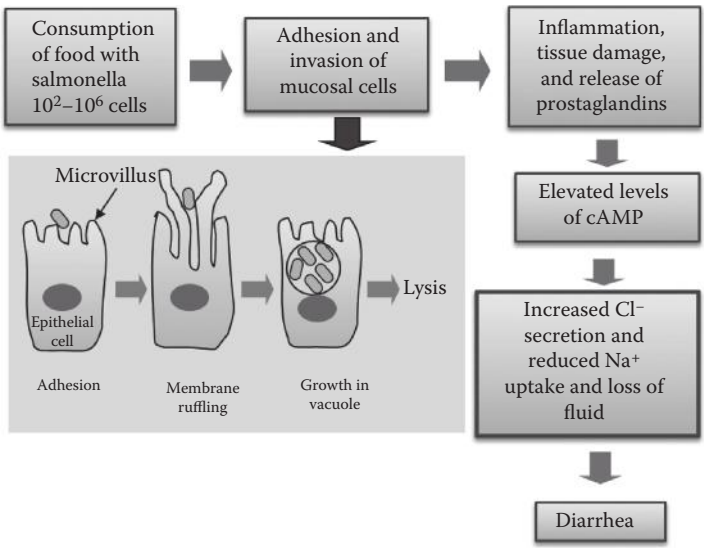



**Figure 26.1 Schematic drawing showing steps involved in *Salmonella* pathogenesis.**

then actively invades mucosal cells (Figure 26.1). Bacteria can also enter through M cells in Peyer's Patch, a localized lymphoid tissue in the small intestine. *Salmonella* multiplies inside epithelial cells and macrophages, neutrophils, and eventually lyses the cells. As a result, inflammation and severe edema occur in the site of infection and lead to mucosal damage. The inflammatory cells, such as neutrophils, and activated macrophages release prostaglandins that increase the cyclic adenosine monophosphate (cAMP) levels in mucosal cells, which inhibits the uptake of $Na^+$ and release of $Cl^-$ ions. An electrolyte imbalance facilitates fluid loss resulting in diarrhea. A thermostable cytotoxic factor produced by *Salmonella* is also thought to be responsible for inflammation and tissue damage, resulting in the loss of fluid and electrolytes. Production of the enterotoxin is directly related to the growth rate of the pathogens.

The symptoms appear within eight to 42 hours, generally in 24–36 hours. The symptoms last for about two to three days but, in certain individuals, can linger for a long time. An individual remains in a carrier state for several months following recovery.

Not all individuals ingesting the same contaminated foods will develop symptoms nor will those who develop symptoms have all the symptoms in the same intensity. It varies with the state of health and natural resistance of an individual. The general symptoms are abdominal cramps, diarrhea, nausea, vomiting, chills, fever, and prostration. It can be fatal, especially to the sick, infants, and the elderly.

## *Food Association*

Foods of animal origin have been associated with large numbers of outbreaks. These include beef, chicken, turkey, pork, eggs, milk, and products made from them. In addition, many different types of foods have been implicated in both sporadic cases and outbreaks (Table 26.2). These foods were contaminated directly or indirectly with fecal matters from carriers (animals, birds, and humans) and eaten either raw or improperly cooked or contaminated following adequate heat treatment. Cross-contaminations at home and at food services are the major sites of contamination of heated foods with *Salmonella*. Salmonellae have also been isolated from many foods of plant origin (resulting from

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use of sewage as fertilizer or washing products with polluted water), such as cantaloupe, tomatoes, nuts, and seafood, such as finfish (harvested from polluted water). Recent surveys show that soil and irrigation water are major sources for *Salmonella* contamination in fresh produce.[7]

Although there are about 2600 serotypes of *Salmonella*, only a small number of them have been frequently associated with foodborne illnesses. This could be a result of geographical distribution of the serotypes as well as the pathogenicity of a serovar or a strain. *Sal. enterica* ser. Typhimurium has been associated in the United States as the major causative agent of foodborne salmonellosis (over 20% of the total cases). However, since the 1980s, foodborne salmonellosis from *Sal. enterica* ser. Enteritidis has increased, mainly from contaminated shell eggs; in recent years, it has been involved in the same number of cases as *Sal. enterica* ser. Typhimurium. The exact cause of the predominance of *Sal. enterica* ser. Enteritidis is not yet clearly understood. The methods used in raising food animals and birds and in the processing of foods of animal origin are suspected to have an important role.

## Prevention and Control

Raw foods of animal origin that are heat-treated before consumption can have *Salmonella*. However, in the United States (and other developed countries), as per regulatory requirements, heat-treated and ready-to-eat foods that contain *Salmonella* in portions (samples) tested are considered to be adulterated and should not be sold. Many food-processing industries have in-house *Salmonella* (as well as several other pathogens) surveillance programs to control the presence of *Salmonella* in their products. The regulatory agencies also have programs to educate consumers at home and food handlers in food service places to control *Salmonella* contamination in foods. These include proper cooking of foods (minimum to pasteurization temperature and time, such as 71.7°C for 15 seconds or equivalent) and prompt cooling (to 3°C–4°C or freezing, if not used in two hours); prevention of cross-contamination of a ready-to-eat food with a raw food through cutting boards, equipment, utensils, and hands; use of proper sanitation and personal hygiene; not handling a food while sick; and properly reheating a food refrigerated for a long time.

## Detection Method

The methods involve preenrichment of a sample of food in a nutrient broth, followed by selective enrichment, streaking on a selective-differential agar medium, and biochemical and serological confirmation (see Chapter 42). Several rapid methods, based on specific immunological characteristics and nucleotide base sequence in the nucleic acids, have been developed.

## A Case Study

In July of 1989, 21 out of 24 people who attended a baby shower at a home in New York had gastroenteritis with severe diarrhea, vomiting, fever, and cramps within 6–57 hours after the party.[8] Twenty people needed medical help, of which 18 were hospitalized with one at 38 weeks pregnancy; the infant was delivered while she was ill and developed septicemia. *Sal. enterica* ser. Enteritidis was isolated from all 21 people (rectal swab) and the infant. All 21 ill attendees, but not the three who remained well, ate a homemade baked ziti pasta dish consisting of one raw egg, ricotta cheese, cooked tomato, and meat sauce, mixed together in a pan and refrigerated overnight. Before serving, the preparation was baked for 30 minutes at 350°F (176.7°C). Several attendees commented that the center of the ziti was cold when served. *Sal. enterica* ser. Enteritidis was

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isolated from the leftover baked ziti and from the unused eggs from the carton. *Sal. enterica* ser. Enteritidis was also isolated in laying hens at the poultry farm that supplied the eggs.

The poultry farm should have tested the birds and culled those carrying the pathogen as a regulatory requirement. However, at home, proper methods should have been taken in the preparation of the dish to avoid the incidence. It is likely, as only one egg was used for a serving of 24+ people, that *Sal. enterica* ser. Enteritidis was present initially in the food in small numbers, and multiplied before or during (slow) refrigeration. Also, if the dish was taken out of the refrigerator a long time prior to baking, *Salmonella* cells could have multiplied. During the 30 minutes of baking, the content was not thoroughly and evenly heated (the center was cold), so some *Sal. enterica* ser. Enteritidis cells survived and, when ingested, caused salmonellosis. The dish could have been prepared thin and baked immediately after mixing for a longer time for the food to attain a high and uniform temperature and then refrigerated quickly. It could have been reheated to a high and uniform temperature prior to serving to prevent this salmonellosis outbreak.

## *Listeria monocytogenes*

### *Importance*

Human listeriosis has been recognized for a long time. However, the presence of *Lis. monocytogenes* in many foods of animal and plant origin and illnesses resulting from the consumption of contaminated foods were recognized rather recently in the 1980s. Human listeriosis is considered by some to be an opportunistic rare disease. Individuals with normal health may not develop the symptoms or show a very mild enteric form of the disease. However, it is highly fatal (30%–40%) to fetuses, newborns, infants, the elderly, pregnant women, and immunocompromised people, such as those with cancer (receiving chemotherapy), renal diseases, heart diseases, and AIDS. Individuals receiving organ transplants and treated with immunosuppressive drugs are also susceptible to listeriosis. In addition, its ability to grow in many foods at refrigerated temperatures helps the organism to reach from a low initial level to an infective-dose level during storage of refrigerated foods, which include those that originally harbored the pathogen and those that were post-heat contaminated. The increase in consumption of many types of ready-to-eat foods that are stored for fairly long periods of time, and the fact that many of these foods are consumed without properly reheating or by microwave heating, has given an edge for this pathogen to cause the disease. Many technological developments used for the production of these ready-to-eat foods may have steps that can contaminate food with the pathogens in low levels, which then can reach a higher level during subsequent refrigerated storage prior to consumption. Any temperature abuse, even for a short time, can accelerate the growth rate.

It is quite clear that many of the above conditions have given an advantage to *Lis. monocytogenes* to become a newly emerging foodborne pathogen in many countries. However, an understanding of the type of foods that are mostly involved in listeriosis, the food processing steps that can contaminate ready-to-eat foods with this pathogen, and the special groups of people that are most susceptible to the disease helped the regulatory agencies develop procedures to reduce the number of foodborne listeriosis cases. These have been achieved through changes in the processing steps and testing of the foods so that contaminated ready-to-eat foods do not reach the consumers and by educating the susceptible consumer groups about food choices, eating habits, and sanitary practices in food preparation. As a result, in developed countries, the number of listeriosis cases has dropped; in the United States since 1991, the number of human listeriosis cases has fallen

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from 2000 cases per year in 1986 to about 1000 cases per year in 1991. Now, an estimated 1600 cases with 255 deaths are reported per year with a fatality rate of 16%. The infectious dose for this pathogen varies between individuals, with the immunosuppressed being most susceptible. Because this pathogen is highly infectious, there is no study done with volunteer feeding trials to establish an infectious dose. On the basis of the epidemiological data, it is estimated to be in the range of 100–1000 cells in an immunocompromised host, and because of its high fatality rate, the U.S. government has established a zero-tolerance policy for this pathogen in ready-to-eat products. In Canada and some European countries, the acceptance level is 100 cells per 25 g ready-to-eat product. In healthy individuals, the disease is less severe and is manifested as febrile gastroenteritis, and a dose of $10^8$–$10^{10}$ cells is required to cause such disease.

## Classification

The genus *Listeria* contains six species, including *Lis. monocytogenes*, *Lis. ivanovii*, *Lis. seeligeri*, *Lis. innocua*, *Lis. welshimeri*, and *Lis. grayi*. Three new species have been added to the genus *Listeria* since 2010: *Lis. marthii*, *Lis. rocourtiae* and *Lis. weihenstephanensis*. *Lis. monocytogenes* is considered to be pathogenic to humans and animals, and *Lis. ivanovii* is pathogenic only to animals, primarily the ruminants (cattle and sheep); however, it caused disease in humans in recent outbreaks. The remainder species in the genus are considered nonpathogenic. *Lis. monocytogenes* is comprised of 13 serogroups: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab, and 7. While 1/2a and 1/2b were the predominant serogroups isolated in foodborne human listeriosis in Europe, 4b was predominant in Canada and the United States. *Lis. monocytogenes* again is grouped into three distinct lineages (I, II, III) based on their genetic fingerprint patterns and pathogenic potential. Lineage I contains primarily epidemic clones and is responsible for most outbreaks; lineage II contains clones that are responsible for sporadic human disease, and lineage III contains clones that rarely cause human disease but may be responsible for animal listeriosis. Lineage III is again subdivided into IIIA and IIIB; IIIA is rhamnose-positive and IIIB is rhamnose-negative. Thirteen serogroups mentioned above fall within one of the three lineages, which is summarized in Table 26.3.

## Characteristics

*Lis. monocytogenes* is a gram-positive, psychrotrophic, facultative anaerobic, nonsporulating, motile, small rod. It displays a characteristic tumbling motility that is facilitated by the presence of peritrichous flagella. Motility is temperature dependent, showing high motility at 20°C–30°C when flagellar expression is maximum. Flagella are highly antigenic and thus are used for antibody production for use in immunoassay for detection of *Listeria* species. In a fresh culture, the cells

**Table 26.3 Classification of *Listeria monocytogenes* Based on Genomic Fingerprinting and Pathogenic Potential (Epidemic Data)**

| *Groups* | *Outbreaks* | *Pathogenic Potential* | *Predominant Serotype* |
|---|---|---|---|
| Lineage I | Epidemic clones and responsible for most outbreaks | High | 1/2b, 3b, 4b, 4d, 4e |
| Lineage II | Sporadic listeriosis cases | Medium | 1/2a, 3a, 1/2c, 3c |
| Lineage III | Rarely cause human disease | Low | 4a, 4c |

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may form short chains, but under stress conditions, such as in the presence of high salt (>10%) or temperature (>45°C), the cells may appear elongated or be present in long chains. It is hemolytic and produces β-hemolysis on sheep or horse blood agar plates. In the CAMP (Cristie, Atkinson, Munch, Peterson) test, in the presence of β-lysin positive *Sta. aureus*, it produces a characteristic large zone of hemolysis at the junction of two cultures on the blood agar plate, and this property allows differential identification of *Lis. monocytogenes* from hemolytic *Lis. ivanovii*, which does not produce such a hemolytic zone with *Sta. aureus*. In contrast, *Lis. ivanovii* exhibits a positive CAMP test (zone of hemolysis) at the junction when streaked with *Rhodococcus equii*.

*Lis. monocytogenes* is a psychrotroph and grows between 1°C and 44°C with optimum growth at 30°C–37°C. At 7°C–10°C, it multiplies relatively rapidly. It ferments rhamnose but not xylose and ferments glucose without producing gas. It can grow in many foods and environments. The cells are also relatively resistant to freezing, drying, high salt (>10%) and pH 5.0 and above. *Lis. monocytogenes* is also acid tolerant, which is essential for survival in stomach acid. The acid tolerance is a result of the presence of a glutamate decarboxylase (GAD) enzyme system. It is sensitive to pasteurization temperature (71.7°C for 15 seconds or 62.8°C for 30 minutes), but when inside the white blood cells, a temperature of 76.4°C–77.8°C for 15 seconds is required to kill the cells.

## Habitat

*Lis. monocytogenes* is primarily a saprophyte[9] and is isolated from many environmental samples, such as soil, sewage, water, and dead vegetation. It is isolated from the intestinal contents of domesticated animals and birds. Humans can also carry the organisms in the intestine without any symptoms. As carriers, humans may harbor the organism in the gall bladder. A large proportion of uncooked meat, milk, egg, seafoods, and fish, as well as leafy vegetables and tubers (potatoes and radishes, in particular), are found to contain *Lis. monocytogenes*. Many heat-processed foods, such as pasteurized milk and dairy products, and ready-to-eat meat preparations have also been found to contain the organism. *Lis. monocytogenes* is isolated in high frequency in different places of food processing and storage areas and are responsible for post-processing contamination of RTE products.[10,11]

## Virulence Factors

*Lis. monocytogenes* possesses a large number of virulence factors (Table 26.4) that are essential for pathogenesis.[12,13] The virulence genes are located primarily in a 9.0 kb region in the chromosome called the pathogenicity island (PAI). A majority of these genes are regulated by two regulatory factors; protein regulatory factor (PrfA) and sigma B. Carbon sources, temperature, salt, bile salts, and acidic and anaerobic environments differentially regulate the expression of virulence genes and thus affect the severity of infection in a host.

## Disease and Symptoms

*Listeria monocytogenes* causes two forms of diseases: (1) febrile gastroenteritis and (2) invasive systemic diseases.[12,13]

### Febrile Gastroenteritis

The exact mechanism of gastroenteritis is not known; however, epidemiological study suggests that this form is mostly associated with healthy individuals, and the infectious dose is in the range

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**Table 26.4 Major Virulence Proteins in *Listeria monocytogenes***

| *Virulence Factors* | *Size* | *Function* |
|---|---|---|
| Protein regulatory factor (PrfA) | 27 kDa | Regulation of virulence protein expression |
| Internalin (InlA) | 80 kDa | Responsible for invasion into intestinal epithelial cells and placenta during pregnancy |
| Internalin B (InlB) | 70 kDa | Entry into hepatocytes and hepatic phase of infection |
| Virulence invasion protein (Vip) | 96 kDa | Invasion of epithelial cells |
| Listeria adhesion protein (LAP) | 104 kDa | Adhesion to intestinal epithelial cells |
| Listeria adhesion protein (LapB) | 170 kDa | Adhesion and invasion of epithelial cells |
| Autolysin amidase | 102 kDa | Adhesion to host cells |
| Listeriolysin (LLO) | 58–60 kDa | A hemolysin responsible for lysis of red blood cells. It also aids in bacterial escape from vacuole inside the cell |
| Actin polymerization protein (ActA) | 90 kDa | Nucleation of actin tail for bacterial movement inside the cytoplasm |
| Bile salt hydrolase (BSH) | 36 kDa | Survival in gut |
| Phospholipase C (PLC) | 29–33 kDa | Lyses of vacuole membrane |
| Metalloprotease | 29 kDa | Helps synthesis of PLC |

of $10^8$–$10^{10}$ cells. Most often, the symptoms appear within one to seven days following ingestion and include mild flu-like symptoms with slight fever, abdominal cramps, and diarrhea. The symptoms subside in a few days, but the individual sheds *Lis. monocytogenes* in the feces for some time.

### *Invasive Systemic Disease*[13–15]

This form of disease is associated with immunologically challenged populations. These groups include pregnant women, unborn fetuses, infants, elderly people with reduced immunity from diseases, and people taking special medications, such as steroids and chemotherapeutic agents to treat cancer. The infective dose in these people is considered to be about 100–1000 cells. Following ingestion, bacteria pass through the stomach unharmed and then reach the small intestine. GAD (glutamate decarboxylase) helps bacterial survival in the stomach acid while bile salt hydrolase protects *Lis. monocytogenes* from antimicrobial action of bile salts. Bacteria rapidly pass through the intestine via the Peyer's patch, a localized lymphatic tissue, where M cells phagocytose and allow bacterial translocation to the lamina propria and then to blood circulation. *Lis. monocytogenes* can also actively pass through the epithelial barrier (Figure 26.2). First, bacteria bind to epithelial cells using adhesion proteins like Listeria adhesion protein (LAP), fibronectin binding protein (Fbp), and amidase and then invade epithelial cells using Internalin A, Internalin B, LapB,

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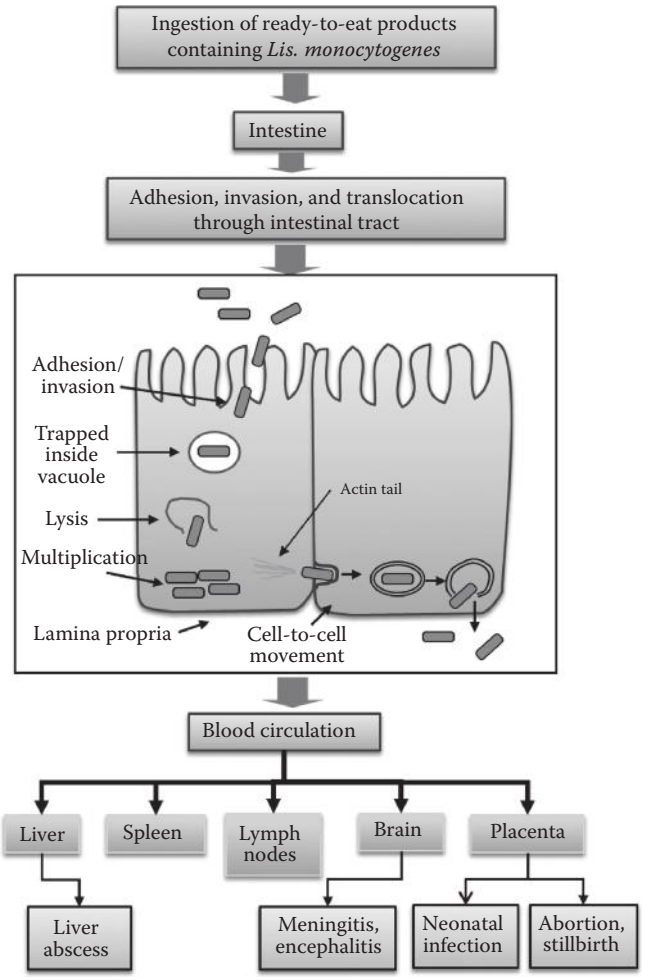



**Figure 26.2 Mechanism of *Listeria monocytogenes* pathogenesis and tissue distribution.**

or Virulence invasion protein (Vip). Once inside, the bacterium is trapped inside the vacuole, and it immediately escapes by forming pores with the help of listeriolysin O and phospholipase. *Lis. monocytogenes* multiplies inside the cytoplasm and then moves from cell-to-cell by forming a comet-like actin tail with the aid of actin polymerization protein (ActA) and the phospholipase enzyme.

*Lis. monocytogenes* also survives inside macrophage in the bloodstream and is transported to the liver, spleen, and lymph nodes within 12–24 hours of ingestion. Persistent infection allows the bacterium to infect the central nervous system. It crosses the blood brain barrier, causing inflammation of the meninges and brain stem. In pregnant women, it can pass through the placental barrier, infecting the fetus. Abortion and stillbirth follows. The incubation period for invasive disease is about two to three weeks before the symptoms are visible. Symptoms include bacteremia (septicemia) resulting in fever and headache, meningitis, encephalitis, endocarditis, liver abscess,

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and others. The fatality rate among fetuses, infected newborn infants, and immunocompromised individuals is very high.

## Food Association

Foodborne listeriosis in humans is mainly sporadic; however, outbreaks were reported from the consumption of contaminated coleslaw, pasteurized milk, raw milk and dairy products, soft cheeses (Mexican style, Brie, and Liederkranz), meat pâté, turkey franks, cold cut meats, improperly cooked chicken, and smoked mussels. Fruits and vegetables, including celery and cantaloupes, are involved in recent fatal outbreaks in the United States. Sporadic listeriosis was also documented from the consumption of these foods either at home or at delicatessen counters. Heat-treated foods were either not properly heated or were contaminated following heating. As many raw foods of both animal and plant origin harbor *Lis. monocytogenes*, consumption of raw foods or recontaminated heat-processed foods have been the cause of listeriosis. Growth during long refrigerated storage and temperature abuse prior to eating has been implicated in many cases. Individuals that suffered most from listeriosis and fatality from the disease were the sensitive groups.

## Prevention and Control

Because of the ubiquitous presence of *Lis. monocytogenes*, it is impossible to have foods free of this pathogen. However, a strong *Listeria* control program at the commercial production facilities has been imposed by the regulatory agencies and industries in many countries. This has helped to greatly reduce the number of listeriosis cases since 1991. However, in recent years, there has been an increase in incidents of sporadic and outbreaks of listeriosis as well as in product recalls. In 1999, there were a total of 24 recalls. In the United States, this includes the absence of *Lis. monocytogenes* in 25-g portions of cooked, ready-to-eat meat and poultry products from each lot; a positive lot is considered adulterated and discarded. A plant producing contaminated products is then thoroughly tested and subjected to proper sanitation (hazard analysis critical control point; see Appendix C) until products free of *Lis. monocytogenes* are produced. Because of the effectiveness of the current regulatory procedures, there is a possibility that, in the future, the current stringent zero tolerance may be slightly relaxed.

In addition to the control measures in the processing facilities, regulatory agencies have implicated consumer education to reduce foodborne listeriosis. This includes thoroughly cooking raw foods of animal origin; thoroughly washing raw vegetables and fruits before eating; keeping uncooked meats separate from vegetables, cooked foods, and ready-to-eat foods; not consuming raw milk or foods made with raw milk; and washing hands, knives, and cutting boards after handling uncooked foods.

The Food and Drug Administration (FDA) conducted a risk assessment study in 2003 for *Lis. monocytogenes* with several foods to provide a better guideline and warning to the high risk populations. They analyzed large numbers of ready-to-eat foods and categorized them into five major groups based on their potential associated risks: very high, high, moderate risk, low, and very low risk foods (Table 26.5).

In addition, special recommendations have been provided to high-risk individuals[16]: avoid soft cheeses (Mexican style, feta, Brie, Camembert, blue-veined, cream, or cottage cheeses); reheat (until steaming) all refrigerated leftover foods and ready-to-eat foods before eating; and pregnant women, elderly people, and immunocompromised people should avoid foods from the delicatessen.

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**Table 26.5 Food Groups with Associated Risk for *Listeria***

| *Risk Category* | *Foods* |
|---|---|
| Very high | Deli meats and frankfurters (not reheated) |
| High | High fat–containing dairy products, pasteurized fluid milk, pâté and meat spreads, soft unripened cheese, smoked seafood, unpasteurized fluid milk |
| Moderate | Cooked RTE crustaceans, deli salads, dry/semidry fermented sausages, fresh soft cheese, fruit, semisoft cheese, soft ripened cheese, vegetables, frankfurters (reheated) |
| Low | Preserved fish, raw seafood |
| Very low | Cultured milk products, hard cheese, ice cream and frozen dairy products, processed cheese |

## *Detection Method*

The most commonly used method involves preenrichment and enrichment steps in recommended broths and streaking on specific selective-differential agar media plates. The suspected colonies are then tested for biochemical and serological profiles. Several rapid methods have also been developed based on immunological characteristics and nucleic acid base sequences (see Chapter 42).

## *A Case Study*

In December of 1988 in Oklahoma, a cancer patient developed septic listeriosis after consumption of turkey franks following heating in a microwave oven.[17] *Lis. monocytogenes* serotype 1/2a was isolated from the patient, from the remaining franks in the opened package (in high numbers) in the refrigerator, and several other foods in the same refrigerator. Isolation of the same serotype from unopened packages (in low numbers) of the same brand of turkey franks facilitated the regulatory agency's determination that the contaminated turkey franks were the cause of this foodborne listeriosis. Subsequent investigations, by the regulatory agencies after four months, revealed that the plant processing this brand of franks had the same serotype of *Lis. monocytogenes* (as well as other serotypes) in the processing environment, and the heated franks were recontaminated from the conveyor belt following peeling of casings and before repackaging (see Chapter 29).

This incidence reveals several important aspects of foodborne human listeriosis: (1) the person affected with listeriosis is immunocompromised; (2) ready-to-eat franks were the source of the pathogenic serotype; (3) microwave heating, the way used by the individual, did not kill the pathogen; (4) the pathogen grew during refrigerated storage in the franks; (5) several other foods in the same refrigerator were also cross-contaminated; (6) the pathogenic strain was present in the processing environment (along with other serotypes) even after four months; and (7) recontamination of franks following heating occurred from contaminated equipment prior to packaging.

To control this incidence of listeriosis, proper sanitation of the processing plant would be the primary requirement. At the consumer level, the incident indicates the importance of proper heating of the product before eating (and prevention of cross-contamination of refrigerated ready-to-eat food, especially by the high risk individual).

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## Pathogenic *Escherichia coli*

### *Importance*

Since its discovery in 1885 by Theodor Escheric, *Escherichia coli* was considered as a harmless, gram-negative, motile, nonsporulating, rod-shaped, facultative anaerobic bacterium, a normal inhabitant of the intestinal tract of humans and warm-blooded animals and birds. Because it is normally present at a very high level (in millions per gram of the content of the large intestine), for a long time it has been used as an index organism of possible fecal contamination and the presence of enteric pathogens in food and water (see Chapter 30). Since the mid-1940s, evidence has accumulated that certain *Esc. coli* strains cause diarrhea, particularly in infants, and they were designated as EPEC. However, current evidence indicates that pathogenic strains of *Esc. coli* are more than one type. They are subdivided into six groups based on their ability to produce toxins and to adhere to and to invade epithelial cells. They are enterotoxigenic *Esc. coli* (ETEC), enteropathogenic *Esc. coli* (EPEC), enteroinvasive *Esc. coli* (EIEC), enterohemorrhagic *Esc. coli* (EHEC), enteroaggregative *Esc. coli* (EAEC), and diffuse-adhering *Esc. coli* (DAEC). The mechanism of infection and the symptoms produced by these are somewhat distinct but may show some overlapping characteristics (Figure 26.3).

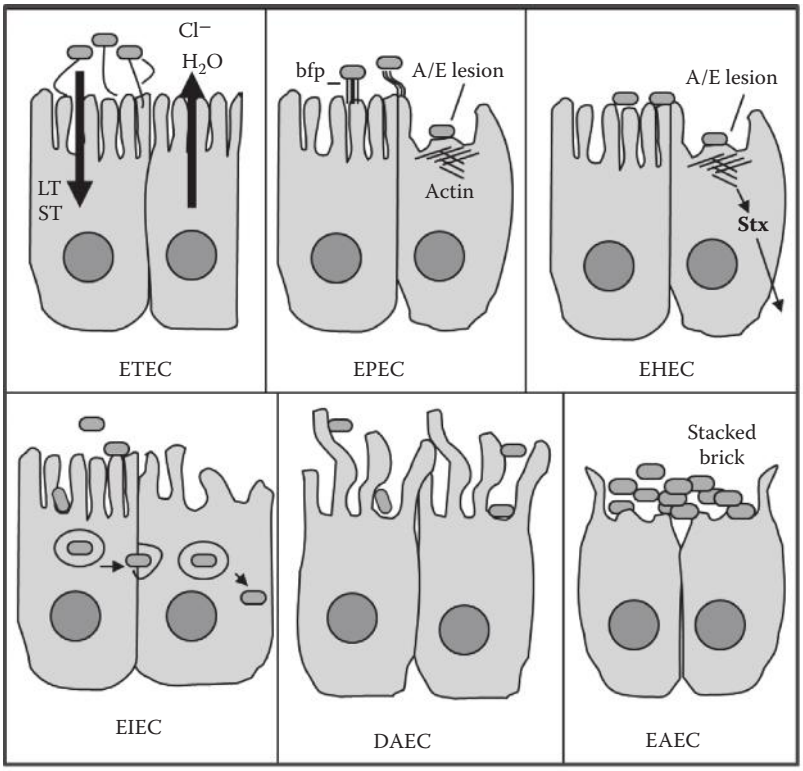



**Figure 26.3 Mechanism of *Escherichia coli*–induced cell damage in intestinal villous epithelial cells by enterotoxigenic *Esc. coli* (ETEC), enteropathogenic *Esc. coli* (EPEC), enterohemorrhagic *Esc. coli* (EHEC), enteroinvasive *Esc. coli* (EIEC), diffusely adhering *Esc. coli* (DAEC), and enteroaggregative *Esc. coli* (EAEC). LT, heat-labile toxin; HT, heat-stable toxin; bfp, bundle-forming pili; A/E lesion, attachment/effacement lesion showing actin accumulation; Stx, Shiga-like toxin.**

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### *Enterotoxigenic* Escherichia coli *(ETEC)*[18,19]

These strains are the major cause of diarrhea among travelers (also known as Traveler's Diarrhea, Montezuma's Revenge, or Delhi belly) as well as in infants in many developing countries with poor sanitation. It is estimated the 300,000–500,000 deaths are associated with ETEC in children under the age of five. The presence of disease is a result of the ability of the pathogens to colonize by adhering to the intestinal epithelial cells using pili or colonization factor antigen (CFA) and then producing either heat labile (LT) or heat stable (ST) or both LT and ST toxins.[18] LT toxin binds to a GM1 ganglioside receptor in enterocytes, and it does not cause any histological changes in the mucosal layers. Little or no inflammation is observed in the intestine. The toxin(s) induces intracellular cAMP levels, which, in turn, activate cystic fibrosis transmembrane receptor (CFTR) chloride channel resulting in increased membrane permeability, electrolyte imbalance ($Na^+$, $K^+$), and severe fluid loss (watery diarrhea) (Figure 26.3).

The symptom is gastroenteritis, like a mild form of cholera. It is fatal in children because of excessive fluid loss and dehydration. In addition, other signs and symptoms include headache, fever, nausea, and vomiting. The pathogen is spread directly or indirectly by human carriers. Both food and water have been implicated in outbreaks and sporadic cases in humans. In 1983, imported Brie cheese contaminated with O27:H7 serotype caused outbreaks in several countries, including the United States. Ingestion of large numbers of cells ($10^8$–$10^9$) is necessary for an individual to develop the symptoms.

### *Enteropathogenic* Escherichia coli *(EPEC)*[20,21]

These strains are important in infant diarrhea worldwide, especially in places with poor sanitation. Atypical EPEC (aEPEC) is more prevalent than the typical EPEC (tEPEC) in both developed and developing countries. They are transmitted directly or indirectly through human carriers. Several serotypes (O111:H12; O55:H6) are implicated in waterborne and foodborne disease outbreaks in different countries. These pathogens do not produce any toxins, but they intimately attach to the epithelial cells with the help of bundle-forming pili (bfp) and a virulence factor called the attachment-effacement factor or Intimin. The *Esc. coli* attaching and effacing (*eaeA*) gene encodes Intimin, which is responsible for intimate contact, resulting in severe lesions on the epithelial layer called attachment-effacement lesions that destroys the absorptive villi, resulting in malabsorption and diarrhea (Figure 26.3). The *eaeA* gene is located in the LEE (locus of enterocyte effacement) pathogenicity island. One needs to ingest high numbers of cells ($10^6$–$10^9$) to develop the symptoms, which could appear within three hours. The predominant symptoms are gastroenteritis, profuse watery diarrhea, vomiting, and low-grade fever.

Although there is no clear differentiation for these four subgroups, both EIEC and EHEC strains seem to fit with the enteric pathogens associated with foodborne infection. Thus these two groups are discussed in this chapter. Both EPEC and ETEC groups are included in the chapter discussing toxicoinfection (Chapter 27).

### *Enteroinvasive* Escherichia coli *(EIEC)*[22]

These strains are known to cause dysentery, like shigellosis. They first bind to epithelial cells and invade and move from cell to cell spreading infection in the intestines (Figure 26.3). Cell damage results in bloody mucoid diarrhea similar to bacillary dysentery caused by *Shigella*. Human carriers, directly or indirectly, spread the disease. Ingestion of as many as $10^6$ cells may be necessary for an individual to develop the symptoms. An outbreak in the United States as early as 1971 was

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recognized from the consumption of imported Camembert cheese contaminated with serotype O124:H17. The pathogens produce virulence factors that are responsible for invasion to epithelial cells and enable the pathogen to move from cell to cell and set up infection in the colon. The genes for invasion are encoded in a 140-MDa plasmid.[23]

### *Disease and Symptoms*

The disease and symptoms are like shigellosis. Following ingestion of the pathogen (about $10^6$ cells) and an incubation period, symptoms appear as abdominal cramps, profuse diarrhea, headache, chills, and fever. A large number of pathogens are excreted in the feces. The symptoms can last for 7–12 days, but a person can remain a carrier and shed the pathogens in feces for a long time.[23]

### *Food Association*

Only humans are known to be the host of the pathogen, and a food can get contaminated directly or indirectly though fecal contamination. Outbreaks from the ingestion of foods contaminated with this pathogen have been recorded. The 1971 outbreak in the United States from the ingestion of an imported cheese was traced to contamination of the processing plant equipment from a malfunctioning water filtration system. In 1983, another outbreak on a cruise ship was related to potato salad contaminated by a carrier food handler.

### *Prevention*

The pathogen is sensitive to pasteurization temperatures. Thus, proper heat treatment, elimination of post-heat contamination for a ready-to-eat food, and refrigeration of a food soon after preparation are necessary to control the disease. In addition, proper sanitation at all stages of food processing and handling will be an important factor. Finally, individuals suspected of being carriers should not handle food, especially ready-to-eat food.

## *Enteroaggregative* Escherichia coli

EAEC causes diarrhea in children and adults, lasting more than 14 days and is prevalent in developing countries. The diarrhea in children is similar to ETEC and causes mild but significant mucosal damage. During infection, it expresses aggregative adherence fimbriae that help bacteria to form characteristic stacked brick–like aggregates on the intestinal cells and causes mucosal damage. In addition, it produces two types of toxins: EAST (enteroaggregative heat stable toxin) and a hemolysin, which form pores. Generally, EAEC do not produce Shiga toxin, but a new EAEC serovar (O104:H4) has emerged in 2011 that expresses Stx and caused disease similar to EHEC (see below) and was responsible for the largest outbreak in Germany from consumption of sprouts affecting 3842 people with 53 deaths (see Chapter 29).

## *Enterohemorrhagic* Escherichia coli *(EHEC),* *Shiga-Toxigenic* Escherichia coli *(STEC)*[24]

The strains in this group (a principal serogroup is O157:H7) have been recognized relatively recently (1982) as the cause of severe bloody diarrhea (hemorrhagic colitis) and hemorrhagic uremic syndrome (HUS) in humans. Other serogroups in EHEC are O157:NM and less common

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serotypes, such as those that belong to O55, O26, O113, and O117. One of the main characteristics of these pathotypes is the production of Shiga-toxin (Stx), which is an important virulence factor, responsible for HUS. Because all EHEC produce Stx, they are also known as Shiga-toxigenic *Esc. coli* (STEC) or verotoxigenic *Esc. coli* (VTEC) resulting from cytotoxic effects on Vero cells (the African Green Monkey kidney cell line). Because Stx production has been attributed to be the major virulence factor, recent emphasis is on classifying organisms based on their ability to produce Stx; hence they are called STEC. In 2011, the U.S. Department of Agriculture identified seven major STEC serovars, including O26, O45, O103, O111, O121, O145, and O157, as adulterants, and meats contaminated with any of these serovars cannot be sold for human consumption. Animals, particularly dairy and beef cattle, are thought to be the carriers. Ingestion of as few as 10–100 cells can produce the disease, especially in sensitive individuals.

## *Gastroenteritis Resulting from EHEC/STEC*[25]

### *Characteristics*

The principal serotype associated with enterohemorrhagic colitis is *Esc. coli* O157:H7. In contrast to other *Esc. coli*, it generally does not ferment sorbitol or have glucuronidase activity. Like other *Esc. coli*, it grows rapidly at 30°C–42°C, grows poorly at 44°C–45°C, and does not grow at 10°C or below. Strains resistant to pH 4.5 or below have been identified. The organism is destroyed by pasteurization temperatures and time and killed at 64.3°C in 9.6 seconds. The cells survive well in food at –20°C.

### *Toxins*

*Esc. coli* O157:H7 produces Verotoxins (VT) or Shiga-like toxins (Stx). There are two distinct Stx: Stx1 and Stx2. A single STEC can produce Stx1 only, Stx2 only, or both. Stx2 is more potent than Stx1 and is thought to be responsible for pathogenesis. Stx binds to a specific glycolipid receptor, globotriaosylceramide (Gb3) located in intestinal and kidney cells and leukocytes. The toxin is also called verotoxin (VT) because of its cytotoxic effect on Vero cells. The toxin then blocks protein synthesis, resulting in cell death. EHEC also produces an intimate adhesion factor called Intimin encoded by the *eaeA* gene, which is responsible for attachment/effacement (A/E) lesion in the intestine similar to EPEC. EHEC also produces enterohemolysin, which is encoded in a 60-MDa plasmid and translocated intimin receptor (TIR), which serves as a host cell receptor for Intimin.

### *Disease and Symptoms*

Upon consumption of contaminated food or water, the bacteria first colonize in the intestine by adhering to the epithelial cells. The protein Intimin facilitates intimate adhesion forming a pedestal-like structure on the microvilli, producing the characteristic A/E lesion. This destroys the absorptive vili resulting in malabsorption, and the lesion resembles EPEC-mediated destruction (Figure 26.3). Bacteria then produce toxins (Stx) that damage colon epithelial cells, resulting in hemorrhagic colitis. Toxins (Stx1 or Stx2) are also absorbed into the bloodstream and damage the small blood vessels in the intestine, kidneys, and brain and also damage the kidney tubules. This results in hemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Symptoms occur three to nine days after ingestion and generally last for 4–10 days. The colitis symptoms include a sudden onset of abdominal cramps, watery diarrhea (which in 35%–75% of

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cases turns to bloody diarrhea), and vomiting. Fever may or may not be an associated symptom. Damage to the lining of the large intestine causes bleeding. Toxins also cause breakdown of red blood cells and clotting in the small blood vessels of the kidney, causing kidney damage and occasional kidney failure, causing HUS. It can be fatal, particularly in children. TTP results from a blood clot in the brain with seizures, coma, and often death.

### *Food Association*

The pathogen is generally present in the intestine of animals, particularly in cattle, without producing symptoms.[26,27] Stx-producing *Esc. coli* (STEC) has been also isolated from the feces of chicken, goats, sheep, pigs, dogs, cats, and seagulls. Food of animal origin, especially ground beef, has been implicated in many outbreaks in the United States, Europe, and Canada. Irrigation water and soils are the major risk factors for STEC transmission. Beef cattle operation can contaminate irrigation water, or untreated manures can directly contaminate produce. In 2006, a major outbreak involving multiple states was associated with spinach, and the contamination source was attributed to the nearby beef cattle operation. The affected people were found to have consumed spinach and lettuce in salad or improperly cooked, contaminated hamburgers. Since then, numerous fresh produce–related outbreaks were associated with STEC.[28] In a 1993 outbreak, affecting over 500 people and causing four deaths, consumption of hamburgers served by a fast-food chain in Washington, Nevada, Oregon, and California was implicated. The hamburgers, contaminated with *Esc. coli* O157:H7, were cooked at a temperature that failed to kill the pathogen. In addition to ground beef, other foods, such as raw milk, mayonnaise, apple cider, some fruits, uncooked sausages, fermented hard salami, sprouts, and salad have been implicated. Investigations revealed the presence of *Esc. coli* O157:H7 in many different types of foods of animal origin, such as ground beef, pork, poultry, lamb, and raw milk, in low percentages. The organism was isolated in low frequencies from dairy cows as well as calves and chickens.

### *Prevention*

Proper sanitation, cooking or heating at appropriate temperatures, proper refrigeration, and prevention of cross-contamination should be practiced in order to control the presence of *Esc. coli* O157:H7 in a ready-to-eat food. The Food Safety Inspection Service (FSIS) in the United States has provided the following guidelines to control foodborne illness from this pathogen: Use only pasteurized milk; quickly refrigerate or freeze perishable foods; never thaw a food at room temperature or keep a refrigerated food at room temperature over two hours; wash hands, utensils, and work areas with hot soapy water after contact with raw meat and meat patties; cook meat or patties until the center is gray or brown; and prevent fecal-oral contamination through proper personal hygiene. Because fresh produce is becoming the major source of many foodborne pathogens, including STEC, efforts are made to decontaminate produce surfaces using liquid (hypochlorite solution, hydrogen peroxide) or gaseous (ozone, chlorine dioxide gas) sanitizers.

## *Shigella* Species[22]

### *Importance*

The genus *Shigella* contains four species: *Shigella dysenteriae*, *Shi. flexneri*, *Shi. boydii*, and *Shi. sonnei*, and each species has several serovars. *Shi. dysenteriae* is responsible for a brisk but deadly

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epidemic outbreak, *Shi. flexneri* and *Shi. sonnei* cause endemic disease, and *Shi. boydii* causes rare disease. Only humans and some primates serve as their hosts. The organisms are either transmitted directly through fecal-oral routes or indirectly through fecal-contaminated food and water. While in most developed countries transmission through drinking water has been reduced, in developing countries contaminated drinking water is a major cause of shigellosis or bacillary dysentery. The disease is prevalent in some geographic locations, particularly in Asia, Mexico, and South America. It occurs more frequently in places with poor sanitation. In the United States, shigellosis occurs more among migrant workers, on Indian reservations, in poor urban institutions, and in daycare centers. Children below five years of age are more affected. In developing countries, there is a high fatality rate among children suffering from shigellosis. In the United States, before the 1950s, the predominant species was *Shi. dysenteriae*. At present, the predominant species is *Shi. sonnei*. In the United States, between 1983 and 1987, there were 44 outbreaks, affecting 9971 people with two deaths. In one outbreak in 1987, several thousand people were affected by eating commercial meals prepared under poor hygienic conditions that resulted in contamination of food with *Shi. sonnei*. The CDC estimates *Shigella* spp. cause about 130,000 cases with 10 deaths annually in the United States.[29] In general, food service establishments have been implicated in more outbreaks, and poor personal hygiene has been the major cause. The disease is more predominant during late spring to early fall.

### Characteristics

The cells of the species are Gram-negative, nonmotile, facultative anaerobic rods. They are generally catalase positive and oxidase and lactose negative. They ferment sugars, usually without forming gas. On the basis of DNA homology, both *Shigella* and *Escherichia* can be included in one genus, and because of many biochemical similarities, the separation between the two genera is not clear. There are suggestions that *Shigella* species could be pathogenic variants of *Esc. coli*. They are more like Shiga-toxin-producing *Esc. coli* with *Shigella* O antigen.

The strains grow between 7°C and 46°C with an optimum at 37°C. The cells are not as fragile as once thought. They survive for days under different physical and chemical stresses, such as refrigeration, freezing, 5% NaCl, and pH 4.5. They are killed by pasteurization. The strains can multiply in many types of food when stored in the growth temperature range.

### Habitat

The intestine of humans and some primates are the only habitats known. Humans can carry the organism in the intestine and shed it in the feces without showing any symptoms. Following recovery from shigellosis, an individual can remain a carrier for months.

### Toxins

The strains are believed to carry plasmid-encoded invasive traits that enable the shigellae cells to invade epithelial mucosa of the small and large intestines. Once engulfed by the epithelial cells, they produce an exotoxin that has an enterotoxigenic property. The toxin is designated as Shiga toxin (Stx). The invasive trait is expressed at 37°C but not at 30°C. Shigellae cells growing at 30°C need a few hours of conditioning at 37°C before they can invade intestinal epithelial cells. The engulfed shigellae cells kill the epithelial cells and then attack fresh cells, causing ulcers and lesions.

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### *Disease and Symptoms*

The infective dose is very low, approximately 10 cells in adults. Bacteria, after ingestion with contaminated food or beverages, pass through the stomach and small intestine and reach the large intestine. The disease is restricted to the intestinal mucosa and invades the epithelial cells of the colon. Shigellae are also taken up by M cells in the Peyer's patch, which also facilitate invasion. Bacteria produce large numbers of virulence proteins, such as IpaA, IpaB, IpaC, IpaD, IcsA, and so forth, that allow bacteria to invade host epithelial cells, to allow cell-to-cell movement by initiating actin polymerization, and to induce programmed cell death (Figure 26.4). LPS produced by *Shigella* also activates macrophages to produce cytokine (IL-1) that induces inflammation. The symptoms occur in 12 hours to seven days but generally in one to three days. In case of mild infection, symptoms last for five to six days, but in severe cases, symptoms can linger for two to three weeks. Certain individuals might not develop symptoms. An infected person sheds the pathogens long after the symptoms have stopped. The symptoms are the consequence of both the invasiveness of epithelial mucosa and the enterotoxin and include abdominal pain, diarrhea often mixed with blood, mucus and pus, fever, chills, and headache. Generally, children are more susceptible to the disease than adults.

### *Food Association*

Shigella cells are present in food only through fecal contamination, directly or indirectly, from a person either suffering from the disease, a carrier, or a person who has not developed symptoms yet but is shedding the pathogens in feces. Direct contamination occurs from poor personal hygiene. Indirect contamination occurs from the use of fecal-contaminated water to wash foods that are not subsequently heat processed. Also, cross-contamination of ready-to-eat foods can be involved in an outbreak. Foods often implicated in shigellosis are those that are handled too much and are ready-to-eat.

In many developed countries, the most frequently involved foods are different types of salads (potato, tuna, shrimp, and chicken) with potato salads ranked at the top. Foods that are chopped,

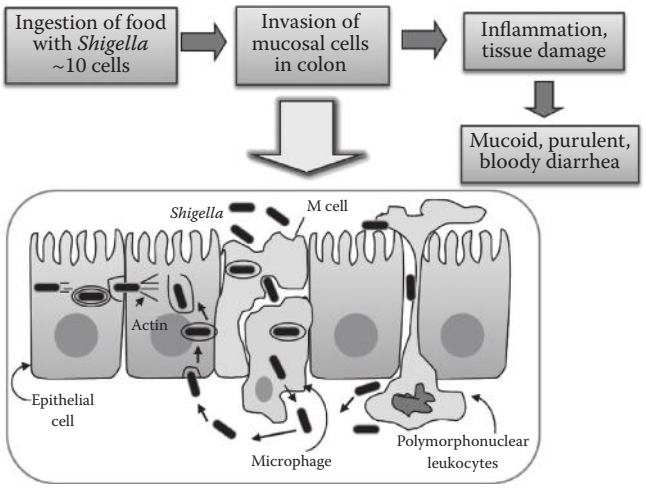



**Figure 26.4** ***Shigella*** **pathogenesis showing bacterial invasion and movement inside intestinal epithelial cells.**

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diced, or cut prior to eating, such as vegetables used in salads, have also been involved in outbreaks. Shellfish harvested from sewage-polluted water and eaten raw have been associated with shigellosis. Many foods support their growth. As the infective dose is very low, growth in food is probably not an important factor for the disease.

### Prevention

Foodborne shigellosis, at least in the developed countries, is caused by contamination of foods by food handlers shedding the pathogen in the feces and having poor personal hygiene. To prevent contamination of ready-to-eat food by such individuals, it is necessary to forbid them to handle such foods. Quite often, this is impossible, especially if the individual is a carrier. Proper education of the food handlers about the importance of good personal hygiene and the need to not handle food if one suspects having a digestive disorder is important. Use of rigid sanitary standards to prevent cross-contamination of ready-to-eat food, use of properly chlorinated water to wash vegetables to be used for salads, and refrigeration of foods are necessary to reduce foodborne shigellosis. Vaccination of children in developing countries to control Shigellosis have been proposed.[30]

## *Campylobacter* Species[31]

### Importance

Genus *Campylobacter* contains 16 species and six subspecies, and many of them cause human gastroenteritis; however, *Cam. jejuni* and *Cam. coli* are considered the most common causative agents of human diarrheal disease in many countries worldwide. In many countries, the number of cases of campylobacteriosis probably far exceeds the combined number of salmonellosis and shigellosis cases. Epidemiological data have confirmed this in Canada, the U.K., and Scotland. Isolation of *Campylobacter* spp. from a suspected sample requires specific methods. After developing the method and incorporating it to isolate suspected foodborne pathogens, *Cam. jejuni* has been confirmed as a causative agent in many foodborne illnesses. Since it was first recognized as the cause of an outbreak in 1979, *Cam. jejuni* has been implicated in 53 foodborne outbreaks in the United States between 1979 and 1987, affecting 1547 individuals and resulting in two deaths. In 2009 and 2010, *Campylobacter* spp. was responsible for 13 and 21 outbreaks, respectively, in the United States, resulting in 600 illnesses.[32] It is now estimated that *Campylobacter* causes about 845,000 cases with 76 deaths annually in the United States.[29] The foods implicated most often in campylobacteriosis were raw milk and improperly cooked chicken. Although several *Campylobacter* spp. have been associated with foodborne campylobacteriosis, *Cam. jejuni* has been isolated in most incidents; the discussion here is on *Cam. jejuni*.

### Characteristics

*Cam. jejuni* is a gram-negative, motile, nonsporulating, rod-shaped bacterium. The cells are small, fragile, and spirally curved. The strains are microaerophilic and catalase and oxidase positive. The strains require a microaerophilic environment of approximately 5% oxygen, 8% $CO_2$, and 87% $N_2$ for growth. Growth temperature ranges between 32°C and 45°C with optimum approximately 42°C. They grow better in amino acids than in carbohydrates. They generally grow slowly and are not good competitors while growing with other bacteria. They generally do not grow well in many

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foods. They are sensitive to many environmental parameters, including oxygen (in air), NaCl (above 2.5%), low pH (below pH 5.0), temperature (below 30°C), heat (pasteurization), and drying. However, they survive well under refrigeration and for months in the frozen state.

## Habitat

*Cam. jejuni* is an enteric organism. It has been isolated in high frequency from the feces of animals and birds. Human carriers were also found to shed the organisms in feces. Fecal materials from poultry were found to contain $\geq 10^6$ cells/g in some instances. Water, sewage, vegetables, and foods of animal origin are easily contaminated with *Cam. jejuni* excreted through feces.

## Pathogenic Factors and Toxins[33]

Pathogenesis of *Cam. jejuni* is dependent on its ability to adhere to and invade epithelial cells (Figure 26.5). Flagella, fimbriae, and other adhesion proteins (PEB1 and CadF) serve as adhesion factors. *Cam. jejuni* produce cytolethal distending toxins (CDT), hemolysin and phospholipase, which are responsible for enteric disease symptoms. The toxin cross-reacts with cholera toxin, and the toxin production trait is plasmid linked. In addition, the strains produce an invasive factor that enables the cells to invade and establish in epithelial cells in both the small and large intestines in humans.

## Disease and Symptoms[33]

The infective dose for campylobacteriosis is considerably low, only approximately 500 cells. Following ingestion, motile bacteria reach the mucus layer. Chemotaxis and a requirement for iron drive the bacteria to reach to the epithelial surface where they colonize. Toxin production causes cell damage (death), inflammation, and fluid loss resulting in diarrhea that appears in two to five days. Symptoms generally last for two to three days but can linger for two weeks or more

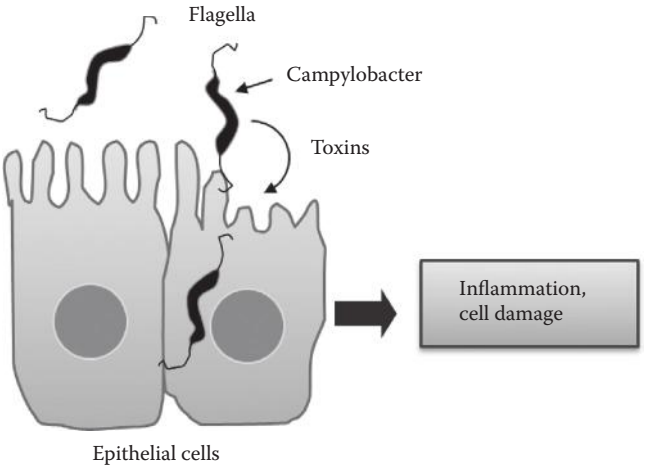



**Figure 26.5** ***Campylobacter jejuni*** **pathogenesis: Campylobacter cells can invade and produce toxins to cause cellular damage.**

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(Figure 26.5). Persons with no visible symptoms can shed the cells in feces for a long time. The main symptoms are enteric and include abdominal cramps, profuse diarrhea, nausea, and vomiting. Other symptoms include fever, headache, and chills. In some cases, bloody diarrhea has been reported. An individual can have a relapse of symptoms after a short interval.

Consequence of campylobacteriosis in humans result in a chronic disease called Guillain-Barre syndrome, a debilitating generalized paralysis. Chronic infection promotes antibody production against *Campylobacter* antigen, which reacts with nerve cells causing impaired nerve function. Another consequence of campylobacteriosis is arthritis, also known as Reiter's syndrome.

### Food Association

Because the organism is present in high frequency in animals, birds, and the environment, many foods, both from plant and animal sources, can be contaminated with *Cam. jejuni*. The foods can be contaminated directly with fecal material from animals and infected humans or indirectly from sewage and contaminated water. *Cam. jejuni* has been isolated at a very high frequency from raw meats (beef, lamb, pork, chicken, and turkey), milk, eggs, vegetables, mushrooms, and clams. In heat-processed food, their presence has been related to cross-contamination following heat treatment or to improper heating. The use of animal feces as fertilizers was found to contaminate vegetables. Outbreaks of campylobacteriosis result from the consumption of raw milk, improperly cooked chicken, dairy products, bakery products, turkey products, Chinese food, eggs, and others. Consumption of raw milk and chicken were implicated in many outbreaks. Although the organism is a poor competitor against other microorganisms present in a food and generally does not grow well in foods, enough cells can survive in a contaminated food to provide the dose required for the disease.

### Prevention

It is rather difficult to control the access of *Cam. jejuni* to raw foods, particularly foods of animal origin. However, proper sanitation can be used to reduce its load in raw foods during production, processing, and future handling. Preventing consumption of raw foods of animal origin, heat-treatment of a food when possible, and preventing post-heat contamination are important to control campylobacteriosis in foods of animal origin. Contamination of vegetables can be controlled by not using animal feces as fertilizer and not using contaminated water to wash vegetables (especially ready-to-eat types). Contamination from humans can be reduced by establishing good personal hygiene and not allowing sick individuals to handle foods, especially ready-to-eat foods.

## *Yersinia enterocolitica*[34]

### Importance

The genus *Yersinia* now includes 17 species, and only three species are pathogenic to humans: *Yersinia enterocolitica*, *Yer. pseudotuberculosis*, and *Yer. pestis*. The most important aspects about the organism and the disease are that *Yer. enterocolitica* is a psychrotroph and can grow at 0°C, and its symptoms include a sharp abdominal pain with fever, mimicking appendicitis. *Yer. pseudotuberculosis* is quite common in animals (pigs, cats, dogs, beavers) and birds and has been isolated in foods, and its involvement in foodborne illnesses is confirmed. *Yer. pestis* is also a pathogenic

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member of the genus, but it causes plague in humans transmitted by rodents and their fleas. Foodborne outbreaks with *Yer. pestis* is rare, but it has been associated with consumption of camel meat and can cause severe gastroenteritis[35] Only *Yer. enterocolitica* and its association in foodborne illnesses are discussed here.

Foodborne yersiniosis was first confirmed in the United States in 1976 following an outbreak among a large number of school children from the consumption of chocolate milk contaminated with *Yer. enterocolitica*. In many other countries, foodborne yersiniosis was recorded earlier. In Denmark, yersiniosis is one of the most common forms of gastroenteritis. Following the first outbreak in the United States, *Yer. enterocolitica* was designated as an emerging foodborne pathogen. Since the first outbreak in the 1970s, several others were recorded until the early 1980s. In 1982, three outbreaks were recorded, one from the consumption of contaminated tofu packed with contaminated spring water and the other two from the consumption of contaminated pasteurized milk. But yersiniosis is not a frequent cause of foodborne infection in the United States. No incidents were reported between 1982 and 1987, but one was reported in 1988. However, recent data suggest that an estimated 98,000 cases of human diseases occur as a result of *Yer. enterocolitica* infection annually in the United States, and 90% of those are foodborne.

## Characteristics

*Yer. enterocolitica* cells are gram-negative short rods (coccobacilli) that are non-spore-forming, motile below 37°C, and facultative anaerobic. The strains grow between 0°C and 44°C with optimum growth at 25°C–29°C. Growth occurs in milk and raw meat at 1°C but at a slower rate. Cells can grow in 5% NaCl and at a pH above 4.6. Cells are sensitive to pasteurization. There are six major biotypes designated 1A, 1B, 2, 3, 4, and 5, and except biotype 1A, all biotypes are pathogenic and carry a virulence plasmid, pVY. Certain strains of biovar 1A has been reported to cause gastroenteritis and several outbreaks[36] The major pathogenic serotypes are O:1,2,3; O:2,3; O:3; O:8; O:9; O:4,32; O:5,27; O:13a,13b; O:18; O:20; and O:21.

## Habitat

*Yer. enterocolitica* is a normal inhabitant of intestines of food animals (pigs, cattle, sheep, and goats) and birds, pets (dogs and cats), wild animals (rodents, hares, raccoons, foxes), and humans. Pigs serve as a major reservoir, and pork products are thought to be the primary source for human infection. Human carriers do not show any disease symptoms. Different types of food can be contaminated from these sources.

## Virulence Factors

Not all strains can cause yersiniosis. Most strains isolated from the environment are nonpathogenic. Pathogenic strains are predominant in pigs. Both the pathogenic and nonpathogenic strains produce a heat stable enterotoxin (Yst); thus, toxin production is not directly related to the ability of a strain to cause yersiniosis. However, toxin production is thought to be temperature dependent, producing toxins when grown at 25°C but not at 4°C. Pathogenic strains carry several virulence factors encoded in chromosomes (Invasin, AiL, Yst) and in a 70-kb virulence plasmid (pVY), which encodes virulence genes for YadA (Yersinia adhesion protein), and Yersinia outer membrane proteins, such as YopH, YopB, YopD, YopE, etc., required for adhesion, invasion, anti-phagocytic action, and colonization in intestinal epithelial cells and lymph nodes. Only after colonization

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does the Yst enterotoxin cause fluid secretion in the intestine. The pathogenic strains vary in serological characteristics. In the United States, the most common serovar implicated in yersiniosis is 0:8.

### Disease and Symptoms[34,37]

Foods that are incriminated for yersiniosis are generally cycled through refrigeration. Thus, the bacterium has to adapt in the human host (37°C) prior to initiating infection. Generally, a high dose (>$10^4$ cells) is required for the disease. Once ingested, the bacterium adheres and colonizes in the small intestine using adhesion factor YadA and is taken up by M cells in the Peyer's patch. Engulfed bacteria are then released from the M cells in the basal layer and invade epithelial cells using the Invasin protein and YadA. Bacteria survive inside the macrophages and spread to the regional lymph nodes, liver, and spleen. In the intestine, the enterotoxin Yst promotes fluid secretion, resulting in diarrhea. Young children are more susceptible to foodborne yersiniosis. Symptoms are severe abdominal pain at the lower quadrant of the abdomen mimicking appendicitis, diarrhea, nausea, vomiting, and fever. *Yersinia* multiplies in mesenteric lymph nodes leading to lymphadenitis, which can lead to appendicitis. Symptoms generally appear 24–30 hours following consumption of a contaminated food and last two to three days. The disease can be fatal in rare cases. Severity of infection is pronounced in immunocompromised hosts as septicemia, pneumonia, meningitis, endocarditis, and so forth. Long-term sequelae of yersiniosis is manifested as reactive arthritis and erythema nodosum.

### Food Association[38,39]

*Yer. enterocolitica* strains are found in the environment; thus many foods can harbor the organism. However, pork products are the most common source for this organism. It has been isolated from raw milk, processed dairy products, raw and improperly cooked meats, chitterling, fresh vegetables, and improperly chlorinated water. Foods implicated in yersiniosis include raw and pasteurized milk, ice cream, and improperly cooked meats. Because the cells are heat sensitive, a properly pasteurized or heated food can have this pathogen from recontamination following heat treatment. A food can also be contaminated from a human carrier or a pet. As the cells can grow at refrigerated temperatures, even a low initial load can reach a high level during extended storage of refrigerated foods.

### Prevention

Because the strains are psychrotrophs, refrigeration cannot be used to control their growth. Good sanitation at all phases of handling and processing and proper heat treatment are important to control the occurrence of yersiniosis. Consumption of raw milk or meat cooked at low temperatures should be avoided.

## *Vibrio* Species

The genus *Vibrio* is comprised of 30 species, and four species have been implicated in foodborne illnesses: *Vibrio cholerae* (O1 and non-O1 serogroups), *Vib. mimicus*, *Vib. parahaemolyticus*, and *Vib. vulnificus*. *Vib. parahaemolyticus* and *Vib. vulnificus* are discussed in this section, and *Vib. cholera* and *Vib. mimicus* are included in the toxico-infection section (Chapter 27). Although there

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is no clear-cut basis for this differentiation, the absence of fever among individuals affected by both *Vib. cholerae* and *Vib. mimicus* is used to separate the two species from *Vib. parahaemolyticus* and *Vib. vulnificus* infection.

## Vibrio parahaemolyticus[40,41]

### Importance

Gastroenteritis caused by *Vib. parahaemolyticus* is quite common in Japan and accounts for 40%–70% of the total bacterial foodborne diseases. The high incidence is directly related to the consumption of raw seafoods, such as oysters. In the United States, its involvement in foodborne infection was first recognized in 1971 from a large outbreak associated with the consumption of steamed crabs contaminated with the pathogen following heat treatment. Several other large-scale outbreaks were confirmed in the 1970s. However, in the 1980s, the number of outbreaks and number of cases per outbreak reduced greatly. Between 1980 and 1987, there were only 12 outbreaks involving a total of 75 people and no fatalities. Between 2009 and 2010 there were four outbreaks associated with 33 illnesses.[32] The CDC estimates annually about 35,000 people acquire *Vib. Parahaemolyticus*, and 86% of them are foodborne in the United States.[29] *Vib. parahaemolyticus* serovars O3:K6 is associated with outbreaks worldwide, and other serotypes, O4:K68 and O1:KUT, are increasingly associated with foodborne diseases.

### Characteristics

The cells are gram-negative, nonsporulating, and motile with straight or curved rods. They are generally catalase and oxidase positive. *Vib. parahaemolyticus* cells are either swimmer-aided by a single polar flagellum or swarmer resulting from the presence of lateral flagella. The strains grow in a medium containing glucose without producing gas but are unable to ferment lactose and sucrose. They can grow over a temperature range of 5°C–42°C with the optimum at approximately 30°C–37°C. The cells are halophiles and multiply rapidly in the presence of 3%–5% NaCl but are sensitive to 10% salt. Under optimum growth conditions, cells can multiply in approximately 15 minutes. Growth is restricted at pH 5.0 or below. The cells are extremely sensitive to drying, heating (pasteurization), and refrigeration and freezing.

### Habitat

*Vib. parahaemolyticus* strains are halophilic bacteria distributed in coastal waters worldwide. They are found in estuarine environments and show a seasonal variation, being present in the highest numbers during the summer months. During the winter months, they remain in the estuarine bottom on chitinous materials of plankton.

### Virulence Factors and Toxin Production

Not all strains of *Vib. parahaemolyticus* are pathogenic. The foodborne pathogenic strains produce many virulence factors: thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH), siderophores (called vibrioferrin), and Type 3 secretion system (T3SS). In addition, capsule (K antigens) and biofilm productions help in pathogenesis. TDH (23-kDa hemolysin) is considered to be the most important toxin. TDH producers are designated as Kanagawa phenomenon-positive,

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KP+ because of their ability to cause hemolysis when grown on Wagatsuma agar. KP+ strains are pathogenic while KP− strains are generally nonpathogenic. Most strains isolated from natural sources (estuarine water, plankton, shellfish, and finfish) are Kanagawa-negative. However, some Kanagawa-negative strains have also been associated with foodborne outbreaks. All pathogenic strains were found to adhere to human fetal intestinal cells in cell cultures. The toxin production rate and its level are directly related to cell growth, cell concentrations, and pH of the environment. Heating does not destroy the toxin that is formed (in a food).

### *Disease and Symptoms*[42]

The cells are sensitive to low stomach pH. Generally, an individual has to consume $10^{5-7}$ cells of a Kanagawa-positive strain for symptoms to develop. However, an increase in pH from consumption of bicarbonates and foods can reduce the infective dose. Once *Vib. parahaemolyticus* reach the intestine, it adheres/colonizes epithelial cells, using a multivalent adhesion molecule (MAM). Iron acquisition using siderophore (vibrioferrin) is also important for bacterial survival and growth. Bacteria secrete hemolysins (TDH and TRH) and form pores in epithelial cells. The relatively large pore size allows both water and ions to flow through a membrane. Symptoms appear 10–24 hours following ingestion of live cells and last for two to three days. Symptoms include nausea, vomiting, abdominal cramps, watery diarrhea, headache, fever, and chills. Sometimes, it may cause bloody diarrhea. The disease is not normally fatal. But systemic spread of the organism in blood may lead to septicemia resulting in organ failure, shock, and death.

### *Food Association*

*Vib. parahaemolyticus* strains have been isolated in high numbers from various types of seafoods harvested from estuarine environments, especially during the summer months. The outbreaks, as well as sporadic cases of gastroenteritis, were linked to the consumption of raw, improperly cooked, or post-heat-contaminated seafoods, including fish (mackerels, sardines, codfish), oysters, crabs, shrimp, clams, octopus, and lobsters. In unrefrigerated raw and cooked seafoods, *Vib. parahaemolyticus* can grow rapidly, especially at 20°C–30°C. In temperature-abused seafoods, cells can reach an infective dose level very rapidly even from a low initial population. Many outbreaks in the United States were identified as being a result of inadequate cooking and cross-contamination of cooked seafoods, followed by improper holding temperatures.

### *Prevention*

Several factors need to be considered in controlling gastroenteritis from *Vib. parahaemolyticus*. The seafoods harvested from an estuary should be assumed to contain *Vib. parahaemolyticus*, some strains of which can be pathogenic. In unrefrigerated raw, improperly heated, or post-heat-contaminated seafood, the cells can multiply rapidly. Once the pathogenic strains grow to infective numbers, even heat treatment cannot destroy the toxin. With this understanding, the control methods should include the following: no consumption of raw seafoods, proper heat treatment of seafoods, proper sanitation to avoid cross-contamination of heated foods, proper refrigeration of raw and heated products, and consumption of the food within a reasonable period of time. Temperature abuse, even for a short duration, of a seafood should be avoided. Persons with chronic liver disease should avoid raw oysters to prevent *Vib. parahaemolyticus* infection.

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## Vibrio vulnificus[43]

*Vib. vulnificus* is often responsible for serious septicemia and would cause infection. It is a gram-negative, lactose-positive, salicin-positive, halophilic bacteria found in the estuarine environment in coastal waters globally. It is considered a highly lethal pathogen because of its ability to invade the bloodstream. *Vib. vulnificus* has been associated with fulminating septicemia from the consumption of contaminated seafood as well as progressive cellulitis and tissue necrosis, resulting from wound infection. Following consumption of contaminated seafood, in many cases raw oysters or clams, the cells penetrate the intestinal wall and produce primary septicemia in 20–40 hours. *Vib. vulnificus* produces several virulence factors, including the ability to survive stomach acid, capsular polysaccharide, lipopolysaccharide, cytotoxins, pili, and flagellum, which allow the bacterium to enter into the bloodstream to cause septicemia. Symptoms are chills, fever, and prostration with occasional vomiting and diarrhea. The fulminating septicemia sets up rapidly, which can be fatal in many cases. The infection and fatality rate is very high (40%–60%) among people with liver and gastric diseases and immunodeficiencies. This organism can cause severe wound infection in individuals with preexisting cuts, skin lesions, and punctures in the skin during swimming or during handling of seafoods (especially the fisherman). In severe cases, tissue necrosis and secondary sepsis may develop; thus, surgical debridement or amputation may be required. The fatality rate for wound infection is 20%–30% and is related to underlying conditions. Control measures should include methods discussed for *Vib. parahaemolyticus*. Susceptible individuals should avoid consumption of raw seafoods, such as oysters.

## *Brucellosis*[44]

Human brucellosis is a zoonotic infection caused by *Brucella* spp., namely, *Brucella abortus*, *Bru. suis*, and *Bru. melitensis*. They are Gram-negative, nonmotile, nonsporeforming, aerobic small rods pathogenic to animals and humans. In infected animals, the organisms are located in the uterus of pregnant animals and in the mammary glands of lactating females. Thus, the pathogens can be excreted in milk. People working with animals can become infected with *Brucella* spp. Also, people working with meat can be infected. Consumption of raw milk and some products made from raw milk (such as some imported cheese) have been implicated in foodborne brucellosis. The cells survive for a long time in milk and milk products. Pasteurization of milk and milk products kills *Brucella* cells. The infectious dose for human infection is 10–100 cells. Symptoms of brucellosis in humans include undulant fever with irregular rise and fall of temperature, profuse sweats, body aches, aching joints, chills, and weakness. Symptoms appear in 3–21 days following consumption of a contaminated food. Between 1983 and 1987, there were two outbreaks in the United States from the consumption of imported cheese affecting 38 people with one death. The CDC estimates about 839 cases of brucellosis in the United States annually and 50% of which are foodborne.[29] Control measures for foodborne brucellosis include pasteurization of milk, manufacturing of dairy products from pasteurized milk, and proper sanitation to prevent recontamination of pasteurized products.

## *Streptococcal Infection*

*Streptococcus pyogenes*, in Group A, is a pathogen and has been isolated from lactating animals with mastitis. It is a gram-positive coccus and has been associated with human pharyngitis with symptoms of sore throat, fever, chills, and weakness. In some cases, nausea, vomiting, and diarrhea can

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be present. Some strains can cause scarlet fever.[45] Foodborne infections have been recorded from the consumption of contaminated raw milk and milk products made with raw milk and different types of salads (contaminated by infected food handlers). In the United States, between 1983 and 1987, seven outbreaks affected 1019 individuals. In one outbreak in 1983, 553 people were affected from eating a contaminated potato salad. According to 2011 report, Group A streptococci is responsible for about 11,200 illnesses each year in the United States.[29] In addition to Group A streptococci, some other streptococci have been implicated in foodborne infections. Control measures include pasteurization of dairy products and avoiding the consumption of raw milk and products made with raw milk. People who are suffering from streptococcal infections or are carriers should not handle ready-to-eat foods such as salads. Proper sanitation in all phases of processing and proper refrigeration will help reduce the incidence.

### *Q Fever*

Q fever in humans is caused by a unique bacterium (rickettsia), *Coxiella burnetii*. Animals carry this organism without any symptom. People handling animals, raw milk, and meat can be infected by the rickettsia and develop symptoms of Q fever. Symptoms include fever, malaise, anorexia, muscular pain, and headache. Symptoms appear two to four weeks after infection and last for approximately two weeks. Foodborne infection occurs from the consumption of raw or improperly pasteurized milk and milk products. *Cox. burnetii* is more resistant to heat than many pathogenic bacteria. Because of this, the current temperature and time of pasteurization of milk have been set to either 145°F (62.8°C) for 30 minutes or 160°F (71.1°C) for 15 seconds so that *Cox. burnetii* is killed. Recently (in 2011), consumption of raw milk in Michigan (United States) resulted in a *Cox. burnetii* outbreak that infected five people.[46]

## Conclusion

Foodborne infection is caused by several enteric pathogenic bacteria. The viable cells survive the GI environment and set up infection in the intestine to produce enteric symptoms and fever. Several can also infect the organs inside the body and produce specific diseases. Several pathogens of this group can also produce a high rate of mortality. All of them are sensitive to low heat and killed by pasteurization. Proper sanitation, heat treatment, and refrigeration can be adopted to reduce their incidence. However, a few can multiply even at 1°C; to overcome problems from them, foods should not be refrigerated for a long time and a refrigerated food should be reheated uniformly before consumption.

**QUESTIONS**

1. List some characteristics of foodborne microbial infections. Define enteric and nonenteric infections and give examples of three pathogens in each group.
2. How do symptoms of foodborne infection, intoxication, and toxico-infection differ?
3. Explain the possible reasons for the current increase in foodborne salmonellosis in a developed country.
4. Briefly discuss the current scheme used in the taxonomy of *Salmonella* serovars.
5. Discuss the regulatory requirements and suggestions directed to control foodborne salmonellosis in the United States.

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6. From the epidemiology of a recent foodborne salmonellosis outbreak (instructor may provide or obtain from the Internet), analyze the causes of the incidence and suggest methods that could be implemented to prevent this.
7. How does foodborne listeriosis differ from foodborne salmonellosis?
8. How can a pasteurized ready-to-eat food be contaminated with *Lis. monocytogenes*? What are the implications of this contamination in a refrigerated ready-to-eat food?
9. List the individuals most susceptible to foodborne listeriosis.
10. "Since 1991, foodborne listeriosis cases have declined." What could have helped in this reduction in the United States?
11. Define nonpathogenic and pathogenic *Esc. coli*. How are the pathogenic strains grouped? What are the characteristics of their toxins?
12. Describe the pathogenicity of foodborne EHEC infection in humans.
13. In a 1993 multistate foodborne outbreak, EHEC was isolated from the victims, improperly cooked hamburger patties, and raw hamburger. A large amount of ground beef (several thousand pounds) was suspected of being contaminated with this pathogen. Some food microbiologists believe that when a large volume of a product is contaminated with a pathogen, there is a greater likelihood that a step in the processing operation is directly contaminating the product (which, in turn, could have been previously contaminated from a small amount of contaminated raw material or environment). With this idea in mind, develop a scenario that might have been involved in the EHEC contamination in large amounts of hamburger patties in the incidence.
14. List two similarities and two differences between foodborne salmonellosis and shigellosis. Define Shiga toxin.
15. Describe food association for a shigellosis outbreak and preventative measures to control the disease. Why can food animals not be the source of *Shigella*?
16. "Campylobacteriosis is emerging as a major foodborne illness in many countries." Justify this statement.
17. Discuss the food association of and preventative measures against the causative pathogen associated with campylobacteriosis.
18. What is the common sequele of campylobacteriosis?
19. Describe two important characteristics of the causative pathogen associated with foodborne yersiniosis.
20. List the symptoms of yersiniosis and indicate how it is often misdiagnosed.
21. Discuss the control measures to reduce the incidence of foodborne yersiniosis.
22. List the *Vibrio* species considered to be human pathogens and what type of disease is associated with *Vib. vulnificus*?
23. Discuss how foods can be the vehicle of gastroenteritis caused by *Vib. parahaemolyticus*.
24. Describe the pathogenicity of *Vib. vulnificus* in humans.
25. Briefly describe foodborne infection caused by *Bru. abortus*, pathogenic *Streptococcus*, and *Cox. burnetii*.

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# Chapter 27

# Foodborne Toxico-Infections

## Introduction

The pathogenesis and disease symptoms of several pathogens associated with foodborne and waterborne gastroenteritis are somewhat different from classical food poisoning or foodborne infection caused by the pathogens described in Chapters 25 and 26. Although the differences are not always very clear, in this chapter, the gastroenteritis caused by *Clostridium perfringens*, *Bacillus cereus*, *Vibrio cholerae*, and enterotoxigenic *Escherichia coli* is described as a toxico-infection. The first two are Gram-positive spore-formers, and the last two are gram-negative short rods. For each pathogen, importance, characteristics, nature of toxins, food association, and control measures are discussed. For some, detection methods and analysis of outbreaks are also included. The following are some characteristics of foodborne toxico-infection:

1. For spore-formers, ingestion of large numbers of live vegetative cells is usually necessary.
2. Vegetative cells of spore-formers do not multiply in the digestive tract but sporulate and release toxins.
3. For gram-negative bacteria, live cells can be ingested in moderate numbers.
4. Gram-negative cells rapidly multiply in the digestive tract.
5. Many cells also die, releasing toxins.
6. Toxins of both groups produce the gastroenteritis symptoms.

## *Clostridium perfringens*[1]

### *Importance*

*Clostridium perfringens* causes various disease in humans, animals, and birds, including necrotic enteritis, gangrene in animals and humans, and food poisoning or gastroenteritis in humans. Gastroenteritis caused by *Clo. perfringens* has several specific characteristics. In most outbreaks, large numbers of cases are involved. In the United States, in the 1960s and 1970s, it was involved



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in more than 7% of the total foodborne outbreaks and more than 10% of the total number of cases. In the 1980s, the incidence dropped to approximately 3% of the total outbreaks, affecting approximately 5% of the total cases. The outbreaks generally occurred with some foods that were prepared in advance by heating and then kept warm for several hours before serving. In the majority of the instances, these situations were associated with feeding many people within a short period of time in cafeterias, restaurants, schools, and banquets (banquet disease). Between 1983 and 1987, out of 24 outbreaks, 12 were associated with such institutions and only one outbreak occurred with food prepared at home. Between 1993 and 1997, there were 57 outbreaks with 2772 cases. In 2009 and 2010, there were a total of 57 outbreaks associated with *Clo. perfringens* in the United States, affecting over 3000 people.[2] It is now estimated that this pathogen causes approximately 960,000 cases with 26 deaths annually in the United States.[3] As the disease produces mild symptoms, many incidents are probably not reported. Thus, the reported cases could be only a fraction of the actual numbers and the annual estimates are 248,520 cases.

## Characteristics

The cells are gram-positive, motile rods, and are spore-formers. Cells vary in size and can form small chains. *Clo. perfringens* is anaerobic but can tolerate some air (oxygen). The vegetative cells are sensitive to low-heat treatment (pasteurization), but the spores are extremely heat resistant, and some can survive even boiling for several hours. The cells are resistant to D-cycloserine. In the presence of suitable substrates, $H_2S$ is formed during growth. The cells need several amino acids for growth. Thus, they can grow very effectively in many protein foods. The temperatures of growth of vegetative cells and germination of spores and outgrowth range between 10°C and 52°C. The optimum growth occurs at approximately 45°C. At optimum conditions, cell multiplication can be very rapid, in approximately nine minutes. The cells fail to grow well at pH < 5.0, in NaCl concentrations > 5%, $A_W$ < 0.93, and in 500 ppm nitrite.

## Habitat

Spores and vegetative cells are found in soil; dust; the intestinal contents of animals, birds, and humans; and sewage. Many foods, particularly raw foods, get contaminated from these sources.

## Toxins and Toxin Production

There are five types of *Clo. perfringens* (A, B, C, D, and E) based on the production of four types of extracellular toxins (alpha, beta, epsilon, and iota). Type A strains are predominantly involved in foodborne toxico-infection. *Clo. perfringens* enterotoxin (CPE), a 34-kDa protein that is associated with the foodborne disease, is a heat-labile protein. Enterotoxin production is encoded by the *cpe* gene located in a plasmid or in the chromosome. CPE is an intracellular protein produced by the cells during sporulation in the intestine and released. Unlike toxins of food-poisoning microorganisms, the enterotoxin is produced in the digestive tract during sporulation. The environmental parameters for the production of enterotoxin are directly related to the sporulation environment. Figure 27.1 shows sequential events that lead to enterotoxin (CPE) production and clostridial food poisoning. There are some reports that, in addition to the intestine, sporulation and enterotoxin production to certain levels can also occur in some foods.

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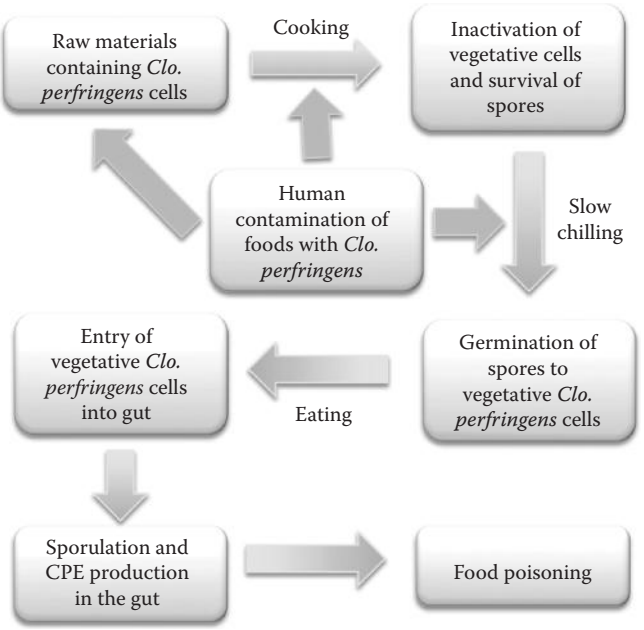



**Figure 27.1 Sequential events leading to *Clostridium perfringens* contamination, enterotoxin production (CPE), and food poisoning. (Adapted and modified from Lindström, M. et al., *Food Microbiol.*, 28, 192–198, 2011.)**

## *Disease and Symptoms*[4,5]

Once the enterotoxin (CPE) is produced in the intestinal lumen, it binds to claudin protein (receptor) in the tight junction on intestinal epithelial cells, inserts into the membrane, and the toxin is then nicked, and this changes membrane permeability, resulting in loss of water, $Na^+$, and $Cl^-$. The enterotoxin causes only gastroenteritis. CPE also causes epithelial cell death and leads to damage in microvilli, epithelial sloughing, and necrosis, further triggering fluid and electrolyte loss. The symptoms appear 8–24 hours following ingestion of a large number of viable cells ($\geq 5 \times 10^5$/g) through a food. The main symptoms are diarrhea and abdominal pain. Nausea, vomiting, and fever also can occur but are less common. Fatality, although rare, can occur among the very young, elderly, and sick. Symptoms generally disappear within 24 hours. It is considered a mild disease and is seldom reported.

## *Food Association*

Raw meat from animals and birds is most commonly contaminated with the spores and cells from the digestive tract content, whereas vegetables and spices commonly get them from soil and dust. As the bacterium has some amino acid requirements for growth, meat and meat-containing foods provide good environments for cell growth. The foods commonly incriminated with the outbreaks include meat stews (beef and poultry), roasts, meat pies, casseroles, gravies, sauces, bean dishes, and some Mexican foods (tacos and enchiladas). Table 27.1 lists the food types involved in 190 *Clo. perfringens* gastroenteritis outbreaks in the United States from 1973 to 1987. Among the

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**Table 27.1 Food Types Involved in *Clo. perfringens* Outbreaks from 1973 to 1987 in the United States**

| *Food Types* | *No. of Outbreaks*[a] | *%* |
|---|---|---|
| Beef | 51 | 26.3 |
| Mexican foods | 23 | 11.9 |
| Turkey | 19 | 9.8 |
| Chicken | 9 | 4.6 |
| Pork | 8 | 4.1 |
| Finfish | 3 | 1.6 |
| Shellfish | 2 | 1.0 |
| Vegetables | 1 | 0.5 |
| Others | 46 | 23.8 |
| Unknown | 28 | 14.4 |

[a] Total cases were 12,234.

foods listed, protein-rich foods were involved in high percentages. The three most important contributing factors in these outbreaks, in order of importance, were improper holding temperature, inadequate cooking, and contaminated equipment. The most important predisposing cause was cooking food in large volumes in advance, allowing it to cool slowly, then holding for a long period and often serving without reheating. Cooking kills the vegetative cells, but the spores survive. Large volumes of the food provide an anaerobic environment, and slow cooling not only provides the temperature for the spores to germinate and outgrow but also allows cells to multiply rapidly to reach high population levels before eating. The food may not show any loss of acceptance quality.

## *Prevention*[6]

The presence of *Clo. perfringens* spores and cells in foods is not uncommon. Unless viable cells are present in high numbers before eating, they cannot cause gastroenteritis; thus, the aim is to keep the cell numbers low. This can be achieved by using proper sanitation in all phases of food preparation and handling. Food should be cooked to the highest temperature recommended to kill the cells and as many spores as possible. The food should be cooled quickly and uniformly (preferably within one hour) to refrigerated temperature. A shallow container, in contrast to a deep container, facilitates rapid cooling (below 10°C or 50°F). If a food is stored for a long time, it is important to reheat it quickly and uniformly (to kill vegetative cells) and keep it hot (above 60°C or 140°F) while being served.

## *Detection Method*

The detection method involves enumeration of the incriminated food and fecal samples for *Clo. perfringens* in a selective agar medium and incubation of plates under anaerobic conditions.

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### Analysis of an Outbreak

In March 1989, 300 of 420 employees became ill after attending a corporate luncheon the previous day. Among the 113 people interviewed, diarrhea, abdominal cramps, and nausea were reported. The symptoms started between 2 and 18 hours (mean average 9.5 hours) following the luncheon, and the disease lasted approximately 12 hours. Stool specimens contained high numbers of *Clo. perfringens*. The menu consisted of chicken, roast beef and gravy, lasagna, and mixed vegetables. Interviews revealed that 98% of those who ate the roast beef became ill; thus, it was implicated as the likely source. Examination of the catering facilities revealed poor sanitation and refrigeration facilities. The roasts, each approximately 19 lb (8.6 kg), were cooked three days before the luncheon date and kept in the refrigerator. Examination of the remaining roast beef showed the presence of *Clo. perfringens* at more than $2 \times 10^6$ cells/g.

The roasted meat, prepared in advance under inadequate sanitary conditions, stored at improper temperature, and served without further heating, was associated in this *Clo. perfringens* gastroenteritis outbreak. The most likely sequence of events was as follows: The raw beef (or the facilities in the kitchen) served as the source of the bacterium (most probably spores). Roasting the large-size meat did not kill the spores. Subsequently, during storage for three days under improper refrigeration, it took a long time for the temperature in the large roasts to drop below 10°C. Thus, the spores germinated and the cells multiplied rapidly in the protein-rich food and reached a high population before the temperature reached below 10°C. When the roast was served, it had a high number of *Clo. perfringens* cells. Following consumption of the roast, the cells sporulated, releasing enterotoxins and producing the gastroenteritis symptoms in 2–18 hours, which lasted for approximately 12 hours. This outbreak could have been avoided by proper sanitation, proper cooking, proper and quick refrigeration (10°C or below), and warming the roast above 60°C before serving.

## *Bacillus cereus*[7]

### Importance

*Bacillus* species are widely distributed in nature and food. The genus *Bacillus* currently consists of at least 56 genera and 545 species.[8] The *Bacillus cereus* group (*Bac. cereus*, *Bac. anthracis, Bac. mycoides*, *Bac. thuringiensis*, *Bac. pseudomycoides*, *Bac. weihenstephanensis*, and *Bac. medusa*) and several other enterotoxin producing *Bacillus* are of public health significance.[9] *Bac. anthracis* causes fatal anthrax in humans and animals. In humans, the disease can be the cutaneous form, respiratory, or gastrointestinal form. The incidence of foodborne gastroenteritis by *Bac. cereus* origin is relatively high in some European countries. In contrast, the incidence is quite low in the United States, where *Bac. cereus* was recognized as a causative gastroenteritis agent in 1969. Between 1973 and 1987, 58 outbreaks involving 1123 cases were reported. Both the incidence of outbreaks and number of cases per outbreak were low, and no fatalities were reported. In 2009 and 2010, a total of 25 outbreaks were reported resulting in 427 cases in the United States.[2] The CDC now estimates about 63,000 Americans suffer from *Bac. cereus* food poisoning each year.[3] However, as the symptoms are not severe and last only for approximately 12 hours, many cases may not be reported.

### Characteristics

The cells are Gram-positive motile rods, which form endospores in the middle of the cells (Figure 27.2). Cells are sensitive to pasteurization. Spores can survive the high heat treatment used in

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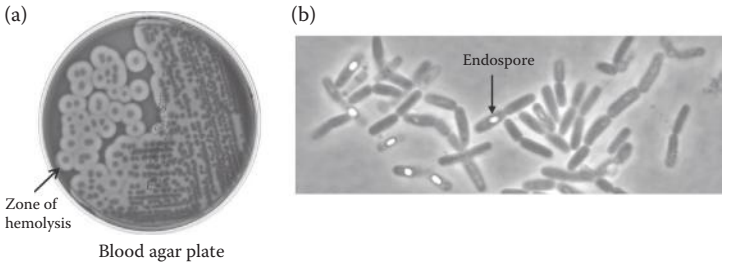



**Figure 27.2 (See color insert.) (a) *Bacillus cereus* colonies on blood agar plate showing zone of hemolysis and (b) microscopic picture of cells showing endospores.**

many cooking procedures. *Bac. cereus* is aerobic but can also grow under some degree of anaerobic environment. The cells can multiply in a temperature range of 4°C–50°C with the optimum at approximately 35°C–40°C. Other parameters of growth are pH of 4.9–9.3, $A_W$ of 0.95 and above, and NaCl concentration below 10%. Most *Bacillus* spp. in the *Bac. cereus* group are motile except *Bac. anthracis*, and they form large colonies on agar plates.

## Habitat

Spores and cells of *Bac. cereus* are common in soil and dust and can be readily isolated in small numbers from many foods, which include both raw and finished products. Intestinal tracts of 10% of healthy adult humans have *Bac. cereus* under normal conditions.

## Toxins and Toxin Production

*Bac. cereus* produces multiple toxins, including emetic, diarrheagenic, cytotoxin K, enterotoxin FM, and enterotoxin T. Among these, emetic and diarrheagenic toxins are thought to be the most important and are associated with specific types of symptoms. The emetic toxin is responsible for vomiting. It consists of cyclic peptide and the molecular mass is about 1.2 kDa and is also known as cereulide. It is highly heat stable and is active at a pH range of 2–11. The diarrheagenic toxin is 38–43 kDa heat-labile toxin. Diarrheagenic toxin is comprised of hemolytic B and L complex (HBL) and/or nohemolytic eneterotoxin (NHE). In addition, Enterotoxin T, Enterotoxin FM, Cytotoxin K, hemolysins, and phospholipases are produced by *Bac. cereus*.[10] Toxin is produced during the exponential/stationary phase or in the intestine upon lysis of bacterial cells. They occur in the intestinal tract but can also occur in foods. Thus, the cases can also be regarded as food poisoning as in staphylococcal food poisoning.

## Disease and Symptoms[7]

In general, a large number of cells ($10^6$–$10^8$/g) need to be ingested to produce gastroenteritis. As stated above, two types of enterotoxins produce two types of symptoms. Emetic toxin is also called cereulide and is responsible for severe vomiting resembling staphylococcal food poisoning. The toxin binds to receptor 5-$HT_3$ in the stomach and stimulates the vagus nerve to initiate vomiting. The mode of action for the diarrheagenic toxin is not well understood, but it induces diarrhea by stimulating the cAMP system. As a result, $Na^+$, $Cl^-$, and $H_2O$ are lost from epithelial cells, resulting in an electrolyte imbalance. In the diarrheal form, symptoms occur 6–12 hours

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following consumption of a food containing the viable cells. Symptoms include abdominal pain, profuse watery diarrhea, and perhaps nausea, but no vomiting or fever. Recovery is usually within 24 hours. These symptoms in many respects are similar to those produced by *Clo. perfringens*.

In the emetic form, the symptoms occur one to five hours following ingestion of a food containing the toxin and viable cells. As the toxin is heat stable, once the toxin is formed in cells, heating food containing a large number of cells before eating can still cause disease. Symptoms are nausea and vomiting; abdominal pain and diarrhea may also be present. Symptoms last for approximately 24 hours and are self-limiting. In some respects, the symptoms are similar to those of staphylococcal gastroenteritis.

## Food Association

Many types of food can contain small numbers of cells and spores of *Bac. cereus*. Consumption of these foods does not cause the disease. However, when these foods are abused to facilitate spore germination and cell multiplication, the population of live cells can reach the high levels necessary for the disease. In the diarrheal outbreaks, a variety of foods, including vegetables, salads, meats, pudding, casseroles, sauces, and soups, has been implicated, mostly because of their improper cooling. However, in the emetic form, outbreaks mostly involve rice, pasta, and sometimes other starchy foods. Table 27.2 presents a list of foods associated with *Bac. cereus* gastroenteritis outbreaks in the United States from 1973 to 1987. Although many foods are listed, the most predominant was fried rice in Chinese restaurants (Chinese rice syndrome). Investigations revealed that, following boiling for preparation, the rice is kept at room temperature for a long time. The surviving (or contaminated) spores germinate, and the cells multiply to high levels before the rice is served.

**Table 27.2 Food Types Associated with *Bac. cereus* Gastroenteritis from 1973 to 1987 in the United States**

| *Food Type* | *No. of Outbreaks* | *%* |
|---|---|---|
| Chinese food | 24 | 41.5 |
| Mexican food | 5 | 8.6 |
| Beef | 3 | 5.2 |
| Chicken | 1 | 1.7 |
| Turkey | 1 | 1.7 |
| Vegetables | 3 | 5.2 |
| Finfish | 1 | 1.7 |
| Shellfish | 2 | 3.4 |
| Ice cream | 1 | 1.7 |
| Others | 8 | 13.8 |
| Unknown | 9 | 15.5 |

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### Prevention

The predominant contributing factors associated with *Bac. cereus* gastroenteritis, in order of relative importance, are improper holding temperature, contaminated equipment, inadequate cooking, and poor personal hygiene. The heat treatment normally used in food preparation, except for pressure-cooking, may not destroy *Bac. cereus* spores. The most important control measure is to keep food at a temperature at which the spores do not germinate and cells do not grow. This can be achieved by uniform quick chilling of the food to approximately 4°C–5°C or holding the food above 60°C. Quick chilling can best be accomplished by storing a food in a shallow container, no more than 2–3 in. (5–6 cm) thick. Because *Bac. cereus* cells, given sufficient time, can grow and produce toxins at refrigerated temperatures (≥4°C), a food should not be stored at low temperatures for long periods of time. This means that preparation of a food well in advance should be avoided. Because cells can get in a food through cross-contamination, proper sanitary measures should be adopted while handling a food. Finally, as live cells are necessary for the symptoms, there should be uniform reheating of a suspected food to above 75°C before serving. However, heating may not destroy heat-stable toxins associated with emetic symptoms.

### Detection Method

*Bac. cereus* can be enumerated by surface plating on an agar medium containing mannitol, egg yolk, and polymyxin B (as a selective agent), such as Phenol Red Mannitol agar (PRM), Mannitol-Egg Yolk-Polymyxin agar (MYP), *B. cereus* base agar (BCA), and *Bacillus cereus* chromogenic agar media (BACARA). Colonies surrounded by precipitation resulting from lecithinase of the cells are indicative of *Bac. cereus*. *Bac. cereus* is hemolytic and produces a zone of hemolysis on a blood agar plate (Figure 27.2). *Bac. cereus* toxins could be detected by ELISA and cytotoxicity assays rapidly using mammalian cells.[11] Molecular methods employing PCR targeting toxin genes are also used for detection of *Bac. cereus*.

## *Vibrio cholerae*

### Importance

*Vibrio* species belong to the family *Vibrionaceae* and are usually found in aquatic environments, including seawater, fish, sea plankton, coral reefs, and chitinous sea animals. They can also be found on the body surface and in the gastrointestinal tract of marine animals. The genus *Vibrio* contains more than 50 species. *Vib. cholerae* cause a disease called cholera, which is a noncontagious disease but can cause large epidemics with high mortality. In the 19th century, both epidemic and isolated cholera cases were recorded in the United States. Since 1911, the disease was thought to be eradicated. Most of the outbreaks in the 20th century were recorded in Asian countries. However, in 1973, a cholera case was recorded in Texas. Between 1973 and 1987, six outbreaks involving 916 cases with 12 deaths were recorded, mostly in the coastal states of the United States. Contaminated seafoods (cooked crab, finfish, and raw oysters) were involved in these cases. In 1991 and 1992, a large cholera epidemic that started in Peru spread to many South American countries and affected approximately 640,000 people, with 5600 deaths. It was also introduced into the United States by travelers from these countries, who either ate contaminated foods before entering the United States or brought contaminated concealed foods to the United States. In addition to the O1 strains, *Vib. cholerae* non-O1 strains have also been involved in

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cholera cases (also designated as non-O1 gastroenteritis) in the United States and other countries. Non-O1 strains were previously thought to be incapable of causing large epidemics, but in 1992, a non-O1 serotype, non-O139, was involved in large epidemics in Bangladesh and India. In 2011, CDC reported that annually 84 people are infected by foodborne *Vib. cholerae* each year in the United States without any fatality.[3]

## Characteristics

*Vib. cholerae*, like other vibrios, is a gram-negative motile, curved rod, and often carry a single polar flagellum. The species has many serogroups. Strains in O1 and O139 are associated with epidemic cholera. The non-O1/non-O139 are rarely toxigenic and also seldom involved in outbreak. The O1 serotype is further characterized by biotype and serotype.[12] The type currently associated with cholera epidemics worldwide is of the El Tor biotype. The O1 serotype is again classified into Inaba, Ogawa, and Hikojima. O1 serotype does not have a capsule while O139 carries a capsule. Non-O1 serotypes do not agglutinate with antibodies prepared against O1 antigens. Also, non-O1 serotypes, similar to O1 serotypes, are not sensitive to trimethoprim-sulfamethoxazole with furazolidone. Both types are sensitive to heat and are killed by the temperature used for cooking. Improper heating (at lower temperatures for a shorter time) may not be able to kill all the cells present in a food. The optimum temperature of growth is between 30°C and 37°C. The growth rate is very rapid, even at room temperature. The cells do not multiply in contaminated live crabs, oysters, or fish. However, in cooked seafoods, rapid growth can occur at 25°C–35°C. Alkaline foods facilitate rapid growth. Survival of cells is better in cooked foods at 5°C–10°C.

## Habitat

Cholera is a human disease. The disease results from the ingestion of infective doses of *Vib. cholerae* cells through food and water contaminated with feces of humans suffering from the disease. Chronic carriers are rare and may not be important in large epidemics. Marine environments may serve as long-term reservoirs. Both serotypes, especially non-O1, have been isolated from water in the U.S. Gulf Coast states (Florida, Louisiana, and Texas), in Chesapeake Bay, and along California coasts. Seafoods (crab, oysters, and finfish) harvested from these areas can carry contamination and provide the infective dose. Contaminated water can also be the source of the disease.

## Toxins and Toxin Production[12]

The pathogenic strains (O1 and O139 serotypes) harbor a cholera toxin prophage (CTXΦ) that carries the genes that encode the cholera toxin (CT), which is responsible for the major clinical symptoms of the disease. CT is an 85-kDa cytotoxic protein, heat labile, and it is also called A-B toxin. The B subunit binds to a specific cell receptor (GM1) on host enterocytes, and the toxin is internalized, stimulates adenylate cyclase, leading to elevated intracellular cAMP levels, and resulting in a major loss of water and electrolytes in profuse secretory diarrhea. There is a massive secretion of water along with chloride, potassium, and bicarbonate in the lining of the intestine. In addition, bacteria also produce pili called toxin coregulated pilus (TCP) and chitin binding protein that help colonization in the gut. The non-O1 and O139 serotypes produce a cytotoxin and a hemolysin but seldom cause outbreak. Following ingestion of *Vib. cholerae* cells in sufficient numbers, the cells colonize the small intestine and multiply rapidly and produce toxins. When the cells die and lyse, the toxins are released into the intestine.

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## Disease and Symptoms

*Vib. cholerae* is not contagious. A person must consume a large number of viable cells through contaminated food or water to contract the disease. Fecal-oral infection is also possible. The infective dose for cholera is approximately $10^6$ viable cells per person, but it varies with age and health. An infection dose of $10^2$–$10^3$ has been reported for outbreaks in Bangladesh. The incubation period ranges from one to five days, but is usually two days. After consumption, bacteria reach the intestine and colonize in the epithelial cell lining with the help of TCP. Bacteria then produce CT, and the toxin activates the adenylate cyclase, resulting in fluid loss. The toxin does not cause any cell damage or visible pathological lesions. The diarrhea caused by non-O1/O139 is milder than cholera and they are most common in the United States. The symptoms include the sudden onset of profuse watery diarrhea and vomiting. Loss of fluid results in dehydration. Other symptoms in severe cases are painful muscle cramps and clouded mental status. Many infected persons may not have any symptoms or have mild to moderate diarrhea. Treatments consist of rapid replacement of fluids, along with electrolytes, and administration of proper antibiotics. In addition to diarrhea, the non-O1 toxins also cause infection of soft tissues and septicemia.

## Food Association

Food can serve as a source of *Vib. cholerae* if it is contaminated directly with human feces from the patient or previously contaminated water. The handling of food by a person suffering from the disease can also contaminate food because of poor personal hygiene. In addition, food originating from natural reservoirs of the causative bacteria can be contaminated and spread cholera. The natural reservoirs include marine and brackish water environments. Testing of samples of water, oysters, crabs, and shrimp from U.S. coastal states has indicated the presence of both O1 and non-O1 serotypes. Table 27.3 lists the types of contaminated foods involved in the disease. In the Gulf Coast incidents, consumption of raw seafoods (oysters and shrimp) and partially cooked crabs (not

**Table 27.3 Contaminated Food Associated with Some Cholera Outbreaks in the United States**

| | *Gulf Coast (1973–1992)* | | *South American Countries (1991–1992)* | |
|---|---|---|---|---|
| *Food Type* | *Outbreak* | *Cases* | *Outbreak* | *Cases* |
| Raw oysters | 4 | 4 | 2[a] | 3 |
| Crab | 4 | 32 | 4 | 14 |
| Fish and shrimp[c] | 1 | 2 | 2 | 77 |
| Cooked rice | 1 | 16 | — | — |
| Seafood[c] | — | — | 2 | 3 |

[a] In one incident, raw clams were consumed.
[b] Improperly cooked crab.
[c] Some items were raw.

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enough to kill *Vib. cholerae* cells) were mainly involved. These foods were probably contaminated with *Vib. cholerae* cells from the water from which they were harvested. Seafood, eaten either raw or improperly cooked, was responsible for the incidents from South American countries too. These foods either had *Vib. cholerae* when harvested or became contaminated after harvest. One of the most important aspects of these data is to recognize how foods from one country can be involved in a disease in another country.

## Prevention

The spread of the disease can be prevented or reduced by adopting proper hygienic measures. These include provision for properly treated municipal water, decontamination of other water by boiling or chemical treatment, and proper disposal of sewage. The infected persons may be treated with antibiotics to enhance recovery, along with replacing body fluids. Unexposed people can be vaccinated to protect them from the disease. Seafood should not be harvested from polluted water or from water found to harbor *Vib. cholerae*. Finally, seafoods should not be eaten raw. The time-temperature of heat treatment of a suspected food should be enough to kill the pathogen. An example of this is the survival of *Vib. cholerae* in contaminated crabs boiled for approximately eight minutes; a 10-minute boiling time is probably necessary to ensure killing.

## Detection Method

Isolation of *Vib. cholerae* from a sample is achieved by an initial preenrichment in alkaline peptone water, followed by streaking on a selective agar medium plate (such as thiosulfate citrate bile salt sucrose agar).[13] Suspected colonies (yellow) are biochemically and serologically tested for confirmation. The toxin is detected by immunoassay or bioassay (Chapter 42). The toxin gene can be identified by PCR.[14]

## Analysis of an Outbreak

Between March 31 and April 3, 1991, eight people developed profuse watery diarrhea after eating crabmeat transported from Ecuador; of these, five also had vomiting and three had severe leg cramps. The symptoms developed one to six days after consuming the crabmeat. The stool samples of some patients yielded *Vib. cholerae* O1, biotype El Tor, serotype Inaba (the same serotype involved in the cholera epidemic in South American countries, including Ecuador). No crabmeat samples were available for analysis.

Investigations revealed that the crabs were purchased at a fish market in Ecuador, then boiled, shelled, wrapped in foil, and transported by air, unrefrigerated in a plastic bag, to the United States on March 30. The meat was delivered to a private residence, refrigerated overnight, and served as a salad on March 31 and April 1.

The probable sequence of events was as follows. The live crabs were contaminated with *Vib. cholerae* harvested from contaminated water, and cooking was not sufficient to kill all the cells. The viable cells subsequently grew in the crabmeat at nonrefrigerated temperatures to reach the high population necessary to cause the disease. Other possibilities include post-heat contamination of the meat during shelling by one or more people with the disease in mild or asymptomatic form or from the contamination of equipment or the water used. Consumption of the crabmeat caused the disease. Preventing the harvest of crabs from contaminated water, proper time and temperature of cooking the crabs, proper sanitation in preparing crabmeat, refrigerating crabmeat

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following picking, heat treatment before preparing salad: Any one or more of these steps could have been used to avoid this incident. (Note: It is illegal to bring food into the United States from other countries without prior permission of regulatory agencies.)

## Enterotoxigenic *Escherichia coli* (ETEC)[15,16]

### *Importance*

One pathogenic *Esc. coli* subgroup that correlates well with toxico-infection belongs to the enterotoxigenic *Esc. coli* (ETEC) type. They produce diarrheal diseases when ingested in large numbers through contaminated foods and water. The symptoms are more like those in cholera. The incidence is high in many developing countries and is directly related to poor sanitation.

### *Characteristics*

Many serotypes in both subgroups are involved in human gastroenteritis. They are gram-negative small curved rods, nonsporulating, and motile (nonmotile strains can be present). The strains are facultative anaerobes and can grow effectively in both simple and complex media and many foods. Growth occurs between 10°C and 50°C with optimum at 30°C–37°C. Some strains can grow below 10°C. Rapid growth occurs under optimum conditions. Growth-limiting factors are low pH (below 5.0) and low $A_W$ (below 0.93). The cells are sensitive to low-heat treatment, such as pasteurization.

### *Habitat*

All strains in the ETEC subgroup can establish in the small intestine of humans without producing symptoms. The carriers can shed the organisms in feces and can contaminate food and water directly or indirectly. Many animals, including domestic ones, can also harbor different serotypes and contaminate soil, water, and food. In animals, some serotypes may not produce disease symptoms.

### *Toxins and Toxin Production*

The strains in the ETEC subgroup produce two types of enterotoxins: One is heat labile (LT) and the other is heat stable (ST). A strain can produce either LT or ST or both. LT toxin is an antigenic protein (Figure 26.3), similar to cholera toxin (CT) produced by *Vib. Cholerae*. LT has two serogroups, LT-I (86 kDa) and LT-II, which are antigenically distinct. LT induces fluid secretion by epithelial cells of the small intestine. ST is a heat-stable 2 kDa peptide and is nonantigenic. It also increases fluid secretion by intestinal cells but through a different mode of action. In addition to LT and ST enterotoxins, ETEC serotypes can also produce additional factors that enable the cells to colonize, multiply, and initiate infection. The genetic determinants of the enterotoxins in ETEC are plasmid linked and can be transferred to other *Esc. coli* strains.

The production of enterotoxins by ETEC strains is influenced by media composition, culture age, and alteration of a culture during growth. Optimum production occurs in a nutritionally rich medium at pH 8.5. Aeration of broth facilitates good toxin production. The toxins are generally

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detected in a growing culture within 24 hours at 35°C. However, the toxin can be produced by the cells growing at 25°C–40°C.

### Disease and Symptoms

ETEC strains are regarded as the cause of traveler's diarrhea, a nonfatal diarrheal disease. Ingestion of a high level ($10^{6-9}$ cells) of viable cells of the organisms by adults is necessary for the symptoms to occur within 24–72 hours. Symptoms include mild to severe diarrhea that lasts for 24–30 hours. In severe cases, dehydration, prostration, and shock may accompany the diarrhea. Not all individuals show symptoms; those who develop symptoms may shed the organisms in feces after recovery.

### Food Association

Many types of foods, including meat products, fish, milk and dairy products, vegetables, baked products, and water have been associated with gastroenteritis of *Esc. coli* origin in many countries. Direct or indirect contaminations of these foods (and water) with fecal materials, along with improper storage temperature and inadequate heat treatment, were involved in these incidences.

### Prevention

The most important factor in the prevention of gastroenteritis in humans by pathogenic *Esc. coli* is to prevent contamination of food and water, directly or indirectly, by fecal matters. This can be achieved by developing effective sanitation in water supplies and treating and disposing sewage. The other factor is to prevent contamination of food from poor personal hygiene by people who shed the pathogen. Finally, one needs to recognize that even if the pathogen is present in very small initial numbers in a food, temperature abuse of the food can facilitate multiplication of cells to the high levels necessary for the disease symptoms. Thus, food should be refrigerated or eaten quickly, preferably after reheating.

### Detection Methods

The detection methods used involve selective enrichment of the sample (food, water, and feces), isolation of pathogens on selective agar medium, and biochemical characterization of suspected isolates (Chapter 42). Confirmation tests to detect toxins involve one or more serological tests (ELISA). Other methods to detect toxins include injecting test material into the ligated ileal loop of infant mice or exposing Y-1 adrenal cells to toxin (for LT) or both.

## Conclusion

Foodborne pathogens in this group include several gram-positive spore-formers and gram-negative rods. The cells of spore-formers, when consumed through contaminated foods, sporulate in the gastrointestinal (GI) tract and release the toxin. In contrast, following consumption, gram-negative rods multiply rapidly in the GI tract and die off, releasing toxins. The symptoms, mainly enteric, are associated with the toxins. They are usually required in very high numbers to cause the diseases. Proper sanitation and refrigeration can be used to reduce the incidence.

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**QUESTIONS**

1. List and discuss five important factors that differentiate foodborne toxico-infection from (a) food intoxication and (b) food infection.
2. List the important characteristics of *Clo. perfringens* gastroenteritis (symptoms, suspected foods, and toxin production).
3. Discuss the methods to be adopted to prevent foodborne *Clo. perfringens* gastroenteritis.
4. What are the differences in the two types of toxins associated with *Bac. cereus* gastroenteritis? What is the sequence of events that leads to this disease?
5. Discuss the importance of cholera as a foodborne disease. List the various serotypes that are associated with cholera.
6. Discuss the factors and foods involved in the cholera outbreaks in the United States since the 1970s.
7. Explain the mechanism of disease production by cholera toxins.
8. Discuss the methods to be implemented to prevent foodborne *Esc. coli* gastroenteritis.
9. Explain the nature of toxins involved in foodborne toxico-infection by *Clo. perfringens*, *Bac. cereus*, *Vib. cholerae* O1 and non-O1, and ETEC.
10. Explain the terms in relation to foodborne diseases: Banquet disease, Chinese rice disease, seafood disease, traveler's diarrhea, and infant diarrhea.
11. From an outbreak incidence of cholera or ETEC (instructor may provide or get from the Internet), discuss the sequence of events that caused the disease. What could have been done to prevent the incidence?
12. List four factors that could be associated with a high incidence of foodborne gastroenteritis, such as traveler's diarrhea, in many developing countries.

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# Chapter 28

# Opportunistic Bacterial Pathogens, Molds and Mycotoxins, Viruses, Parasites, and Fish and Shellfish Toxins

## Introduction

Besides the foodborne bacterial pathogens discussed in Chapters 25–27, several other bacterial species are suspected of having the potential to cause foodborne illnesses. Normally, they are not pathogenic to humans, but strains in these species have been known to produce toxins. Thus, consumption of foods contaminated with these bacterial species and strains may cause illness, especially under certain circumstances, such as if they are consumed in extremely high numbers or the individuals are either very young or not in normal physical condition or immunologically challenged. These bacterial species and strains are considered opportunistic pathogens. A brief discussion on the characteristics of some of these species, their association with foods, and their disease-producing potential are discussed in this chapter. In addition, diseases caused by molds and mycotoxins, enteroviruses, prion proteins (causes bovine spongiform encephalopathy, BSE), parasites, and fish and shellfish toxins are discussed.

## Opportunistic Bacterial Pathogens

### Aeromonas hydrophila[1]

#### *Characteristics*

This genus includes 24 species, including *Aeromonas hydrophila*, *Aer. caviae*, *Aer. veronii* biovar sobria, *Aer. popoffii*, etc. They are Gram-negative motile rods. The strains are found in both saltwater



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and freshwater environments and are pathogenic to fish. They are also found in the intestinal contents of humans and animals. Human can acquire *Aeromonas* from wild animals, pets, the environment, water, and from foods. Their growth temperature ranges between 3°C and 42°C with an optimum between 15°C and 20°C; a few strains can grow at 1°C. They are facultative anaerobes, but grow better in an aerobic environment. Pasteurization effectively kills the cells. Factors such as pH (below 4.5), NaCl (above 4%), and low temperature (below 3°C) can reduce their growth.

### Food Association

Because of the nature of its normal habitat, *Aer. hydrophila* is found in many foods, especially in foods of animal origin. It may be isolated from milk, finfish, seafood, red meat, and poultry; in some foods, it occurs at a level of $10^5$ cells/g or /mL. The strains have been implicated in the spoilage of foods. Because of the psychrotrophic nature, they can grow in foods at refrigerated temperatures and, even from a low initial load, can reach a high population with time during storage. Their presence in high numbers in food can be controlled by heat treatment, preventing post-heat contamination, and using one or more of the growth-limiting parameters, such as low pH and low $A_W$.

### Disease-Causing Potential

Aeromonads are pathogenic to amphibians, fish, and reptiles. It causes ulcerative stomatitis in snakes and lizards, "red leg" disease in frogs, septic arthritis in calves, and septicemia in dogs. Some species of *Aeromonas* have been suspected to cause gastroenteritis (diarrhea) in humans, especially when consumed in large numbers and when individuals have impaired health. Diarrhea may last up to 14 days. Other symptoms associated with *Aeromonas* infection include dysentery, ulcerative colitis, haemolytic anemia, and renal dysfunction. Several virulence factors (cytotoxic enterotoxins, hemolysins, adhesins, and siderophores) and enzymes (protease, DNase, RNase, lecithinase, chitinase, gelatinase) may be responsible for pathogenesis. No confirmed cases of foodborne illness caused by *Aer. hydrophila* have been reported. Several incidences of gastroenteritis from the consumption of food and water contaminated with this species were reported. Many strains of *Aer. hydrophila*, especially those isolated from foods, were found to produce cytotoxins and hemolysins. It is not definitely known whether these toxins can cause gastroenteritis in humans.

## Plesiomonas shigelloides[2]

### Characteristics

*Plesiomonas shigelloides* is a facultative anaerobic, motile, nonsporulating, and Gram-negative rod. The species has many characteristics similar to those of *Aeromonas* spp. and was previously classified as *Aer. shigelloides*. The organism is isolated from the intestinal contents of humans and warm- and cold-blooded animals. It is found in fresh and brackish water and in fish and oysters harvested from water. Most strains grow between 8°C and 45°C with optimum growth at 25°C–35°C. The cells are killed by pasteurization. Low temperatures (below 10°C), low pH (below 4.5), and NaCl (above 5%) can be used, especially in combinations of two or more, to reduce growth.

### Food Association

*Ple. shigelloides* strains are isolated from foods of aquatic origin, such as fish and shellfish. They are present in higher frequencies and levels in oysters collected during warmer months and from

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muddy beds. Also, because of fecal contamination, they can be present in raw foods of animal, bird, and plant origin. In heat-treated foods, their presence indicates either improper heating or post-heat contamination, or both. They can grow rapidly in most foods under optimum growth conditions. They are not expected to grow in foods held in refrigeration (3°C–4°C or below) even for a long time. Foods with low pH (4.5), low $A_W$ (0.95), and salt (5%) discourage their growth.

### *Disease-Causing Potential*

*Ple. shigelloides* strains were implicated in many human gastroenteritis outbreaks associated with contaminated drinking water. In many cases, the organisms were isolated from both the implicated water and stools of affected people. The typical symptoms are diarrhea, nausea, and abdominal pain; many can also have vomiting, fever, and chills. The incubation period ranged from 24 to 50 hours, and the symptoms lasted for one to nine days. Several incidents of gastroenteritis from the consumption of seafood, oysters, crabs, and fish were suspected to be the result of *Ple. shigelloides*. In some instances, the organisms were isolated both from foods and stools of patients. In these cases, foods were eaten either raw or after being improperly cooked or stored.

It is difficult to confirm a direct involvement of *Ple. shigelloides* in human foodborne gastroenteritis. *Ple. shigelloides* was isolated in stools from both unaffected people and individuals suffering from gastroenteritis. Thus, these strains are considered as opportunistic pathogens, affecting individuals with less resistance, such as the young, old, and sick. In addition to gastroenteritis, several strains are associated with bacteremia and septicemia. Several studies demonstrated that *Ple. shigelloides* strains produce cholera-like enterotoxins and heat-stable and heat labile enterotoxins that may be responsible for diarrhea.

## *Non*-Escherichia coli *coliforms*

Coliform groups include species from genera *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter*, all belonging to the family *Enterobacteriaceae* (see Table 30.1) and thus sharing some common characteristics. Previously, *Escherichia coli* strains (both nonpathogenic and pathogenic) were thought to mainly inhabit the intestinal tract of humans and warm-blooded animals and birds, and most species in the other three genera were thought to be mainly of nonintestinal origin. However, other studies have shown that species and strains from *Klebsiella*, *Enterobacter*, and *Citrobacter* (together referred to as non-*Esc. coli* coliforms) can colonize the human gut and produce potent enterotoxins. In several acute and chronic cases of diarrhea, they were isolated from stools and the intestinal tract. Some isolates of *Enterobacter cloacae*, *Klebsiella pneumonia*, and *Citrobacter* spp. were found to produce enterotoxins similar to heat-labile or heat-stable toxins of enterotoxigenic *Esc. coli*. In enterotoxigenic *Esc. coli*, these traits are plasmid linked. The ability of non-*Esc. coli* coliforms to produce enterotoxins similar to those of pathogenic *Esc. coli* strains probably results from the intergeneric (horizontal gene transfer) transfer of plasmids encoding these phenotypes.

Non-*Esc. coli* coliforms are normally present in raw food materials as well as in some pasteurized foods because of post-heat contamination. They can grow in many foods if the growth parameters are not limiting. Some strains can grow at refrigerated temperatures. Temperature abuse during storage can also facilitate their rapid growth in a food. The significance of their presence in a food may need to be reevaluated. The ability of food isolates to produce enterotoxins may be included in test protocols.

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### *Toxigenic Physchrotrophic* Bacillus *Species*[3–5]

Cells and spores of many *Bacillus* species can be present in non-heat-treated foods. Also, most heat-treated foods can contain their spores. In refrigerated foods, spores of psychrotrophic *Bacillus* spp. can germinate and outgrow, and the vegetative cells can multiply. With time, they can be present in high numbers.

Spoilage of some refrigerated foods has been recognized, such as pasteurized milk, resulting from growth of several *Bacillus* spp. The psychrotrophic *Bacillus* spp. isolated from foods includes strains of *Bacillus cereus*, *Bac. mycoides*, *Bac. circulans*, *Bac. lentus*, *Bac. polymyxa*, and *Bac. pumilus*. An isolate is considered a psychrotroph if it grew at ≤6°C. Many isolates of these species produced enterotoxins. However, the capability of these toxins to cause gastroenteritis was not examined. This aspect may need further consideration as more and more low-heat-processed foods are stored at refrigeration temperature for long times.

Some mesophilic *Bacillus* species, such as several *Bac. licheniformis* strains, are toxigenic and are suspected to have the potential to cause foodborne disease, especially in foods stored or temperature abused at ≥10°C.

## Diseases Caused by Viruses and Prion[6]

### *Importance*[7]

Enteric viruses are responsible for most foodborne illnesses in the United States and most other countries. These viruses include Norovirus, Hepatitis A virus, Astrovirus, Rotavirus, and Sapovirus. Data collected from 2000 to 2008 by the CDC indicate that foodborne viruses are responsible for about 5.5 million cases annually while bacterial foodborne illnesses are responsible for about 3.6 million illnesses in the United States. Among the foodborne pathogens, most (58%) illnesses are caused by norovirus followed by non-typhoidal *Salmonella* (11%), *Clostridium perfringens* (10%), *Campylobacter* (9%). However, unlike bacterial pathogens, viruses are difficult to detect and recover from a contaminated food; for some viruses, suitable methods to isolate them from a food have not been developed and for some other viruses, very little research has been conducted to develop methods to detect them in foods. Also, unlike bacteria, human enteric viruses do not multiply in food systems; some may die off rapidly under various conditions of food storage and preservation. Thus, seldom is a food routinely examined for contamination by enteric viruses. To study enteric viruses, a mammalian cell culture is used. Depending on the viruses, a cell culture system exists for most viruses while there is now a suitable cell culture available for Norovirus.

Foodborne viral infections can occur only from enteric pathogenic human viruses. Before the 1940s, polio viruses transmitted through raw milk were considered the only virus involved in foodborne viral infections. In recent years, hepatitis A virus (HAV), norovirus (NoV), Sapovirus, Rotavirus, and hepatitis E virus (HEV) have been associated with foodborne infections. Among them, hepatitis A and norovirus are more predominant and are discussed below.

### *Food Association*

Food contaminated with the fecal matter of infected people directly (from food handlers) or indirectly (via sewage and polluted water) is the main source of both hepatitis A and NoV outbreaks. Infected food handlers, even without symptoms, can contaminate ready-to-eat food with fecal

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matter. Vegetables (salads) can be contaminated with polluted water. Shellfish (oysters, clams, mussels, and cockles) harvested from water polluted with sewage and eaten raw or improperly heated before eating have been implicated in many outbreaks of both types of viruses. The virus can survive in shellfish for a long time. Depuration (in tanks filled with disinfected saline water) or relaying (kept in unpolluted water in the sea) for self-cleaning of the viruses from the digestive tracts of the shellfish may not be very effective for hepatitis A or NoV. In the United States, the major cause of foodborne viral disease outbreaks is the contamination of ready-to-eat foods because of poor personal hygiene and contaminated equipment used with foods served at delicatessens, cafeterias, and restaurants.

## Prevention

The two major preventative methods of foodborne virus infections are to kill the viruses in contaminated foods and to adopt good sanitation and personal hygiene habits to control contamination. Proper heat treatment, such as pasteurization, is enough to kill the viruses. Steaming lightly to open the shellfish may not be an effective heat-treatment procedure. Hydrostatic pressure processing, currently being used to open the shellfish, can also destroy the viruses if the pressure used is above 300 MPa. As indicated before, depuration and relaying may not be effective. Sanitation, using oxidative agents, such as hypochlorite, can kill viruses in contaminated equipment or in water used in food processing. Good personal hygiene and keeping suspected individuals away from handling ready-to-eat foods will also be important to control viral foodborne infections. Vaccination against hepatitis A is available and is used to control the disease.

## Detection Methods[6]

Because enteric viruses do not multiply in food and are usually present in food in relatively low numbers, their detection from a contaminated food poses difficulties. Initially, the viruses are concentrated from a suspected food. For identification of hepatitis A virus, specific cell culture procedures and immunological methods have been developed. The reverse transcription-polymerase chain reaction (RT-PCR) nucleic acid amplification method is effective in detecting both hepatitis A and NoVs and is commonly used. In one such method, the viral RNA is amplified with hepatitis A or Norovirus-specific primers by RT-PCR, using a viral RNA internal standard control. By this method, a total of $10^{2-3}$ viruses were detected in suspected food samples (e.g., ham, turkey, and roast beef). Enzyme immunoassays (EIA) are also used using antibodies to viral capsid proteins and now genome sequencing is used for identification.

## Norovirus[8–10]

Norovirus (NoV) infection is characterized by self-limiting vomiting and diarrhea and occurs all year round but peaks in winter months. It was originally isolated from an outbreak in school children in Norwalk, Ohio (USA) in 1968, and the virus was not identified until 1972 by Dr. Albert Z. Kapikian who named it "Norwalk virus." Now it is called norovirus. Norovirus cases cause approximately 21 million illnesses annually in the United States. It is a nonenveloped virus with an icosahedral symmetric structure and is very small (27–38 nm). It is a single-stranded RNA virus and belongs to the family of *Caliciviridae*. Norovirus has been grouped into five genogroups (GI-GV), and genogroups GI, GII, and GIV are known to infect humans. The method to cultivate

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NoV in cell cultures has not yet been successful; thus various surrogate viruses, murine norovirus (MNV) and feline calcivirus (FCV), are used as a model to study NoV.

Norovirus transmission occurs primarily from person to person through contacts or aerosolized vomit, water, and foods contaminated with feces or vomit. Outbreaks are generally associated in an environment in which large numbers of people are present in close contact, such as in hospitals, cruise ships, hotels, restaurants, nursing homes, daycare centers, prisons, military installments, and sports stadiums. Fresh produce, including lettuce, tomato, melons, green onions, strawberries, raspberries, peppers, fresh-cut fruits, and salads, and food handlers, processors, and irrigation water are also involved in norovirus transmission. Bivalve shellfish, such as oysters, clams, and mussels, are also the source of foodborne norovirus. The infectious dose of norovirus is very low: about 10 viral particles. NoVs infect the mature enterocytes covering the intestinal villi leading to massive cell damage and malabsorption, leading to diarrhea. The gastrointestinal symptoms include explosive projectile vomiting, nausea, cramps, diarrhea, chills, headache, dehydration, and a high-grade fever. Adults are more susceptible to norovirus than children and the incubation period is 24–48 hours. Symptoms appear within 12–24 hours after ingestion and last for one to two days. The disease is self-limiting and the viruses are excreted in the feces of infected persons (100 billion virus particles/gram of feces) for a prolonged period of time. NoV is relatively stable in increasing concentrations of chlorine and temperatures from freezing to 60°C (140°F).

## *Hepatitis A Virus*[6,11]

Hepatitis A is a nonenveloped, single-stranded RNA virus and belongs to the *Picornaviridae* family and *Hepatovirus* genus. HAV virus is 27–32 nm in diameter and has icosahedral symmetry. It is grown in mammalian cell cultures but requires a long adaptation period. Annually, 1.4 million people suffer from HAV infection worldwide. The virus remains stable in the environment for at least a month and may be resistant to heating and disinfectant, such as chlorine.

Because it is of enteric origin, HAV is excreted in very high numbers in human feces. HAV also do not multiply outside the human body, including in contaminated foods, because it requires live human cells to multiply. HAV transmission occurs through contaminated food, water, food handlers, or direct contact with an infected person. An infectious dose of the virus is very low, about 10–100 virus particles. Following ingestion of HAV through contaminated food (or other sources), an individual may or may not develop symptoms. The viruses rapidly move to the liver and invade hepatocytes. In affected individuals, symptoms occur after approximately four weeks with a range of two to seven weeks. The general symptoms in the early stage involve fever, malaise, nausea, vomiting, abdominal discomfort, and inflammation of the liver leading to jaundice at a later stage. In a jaundiced patient, the feces are pale colored and the urine becomes dark. Anorexia, vomiting, malaise, and fever are manifested in the hepatitis patients, and virus particles are shed in large numbers in feces ($10^9$ particles per gram). Symptoms may last for one to two weeks or longer. The viruses are shed via feces, generally during the last half of the incubation period. Severity of the disease is strongly associated with age. Young children display an asymptomatic form of the disease while young adults and adults show symptomatic disease.

## *Bovine Spongiform Encephalopathy (BSE)*[12]

Transmissible spongiform encephalopathy (TSE) is also known as bovine spongiform encephalopathy (BSE) or mad cow disease, scrapie in sheep, Creutzfeldt-Jakob (CJD) or variant CJD (vCJD), and kuru in humans. In the early 1950s, in the eastern highlands of Papua New Guinea, kuru

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was spreading among humans as a result of cannibalism of the brain tissues of infected relatives. The disease was characterized by degenerative brain with a spongy appearance, and the victims suffered from rapid physical and mental abnormalities, culminating in paralysis, coma, and death. The incubation period is about 2–10 years. It became a major concern in the early 1990s when the disease was detected in cattle, and the wasting of brain tissue resulted in abnormal behavior in cattle and was thus called mad cow disease. Though there is no human case directly linked to the consumption of contaminated beef, finding the organism in the late 1990s and early 2000 in Canada and the United States caused a major beef embargo among developed countries with huge economic impacts.

Consumption of infected beef products with brain, lymph nodes, or neurons can potentially cause this disease in humans. It has been documented that cattle likely acquire this from meat and bone meal (MBM) consisting of sheep offal or beef or dairy cattle. In 1997, the FDA banned the use of proteins derived from mammalian tissues in feeding to ruminants to prevent transmission.

The infective agent for BSE is not a virus but a protenaceous infectious particle called prion protein (PrP) that is resistant to most treatments and found primarily in the central nervous system, including brain and neurons. The monomeric form of the protein is 22–36 kDa while the abnormal or infective molecule is a macromolecular aggregate with molecular mass greater than 400 kDa. The PrP is resistant to protease and accumulates in the neural cells causing vacuolation (a spongy appearance), cell death, and disrupting brain function.

The psychiatric symptoms include depression, withdrawal, anxiety, paranoid delusions, head and neck pain, and progressive dementia. The mean duration of suffering is about 14 months. In the terminal stage, the patient becomes bed bound and also becomes a kinetic mute (a state in which a person is not able to or will not move or make sounds). There is no diagnostic test available for testing live animals. Postmortem analyses of brain tissues for spongy appearance and immunoassays (Western blot or ELISA) are used to detect BSE in cattle after slaughter.

Prion is highly resistant to heat and certain chemicals. It can withstand heat treatments of 160°C for 24 hours, 360°C for one hour, and autoclaving at 121°C for one hour. Prion is also resistant to chemical treatments, such as 0.5% sodium hypochlorite for one hour, 3% hydrogen peroxide for one hour, and ethanol. However, complete inactivation is possible by autoclaving at 132°C for 1.5 hour, and treatment with 1 M sodium hydroxide at 20°C for one hour or sodium hypochlorite (2% chloride) for one hour at 20°C. Prevention strategies include (i) stopping feeding of beef cattle with animal proteins derived from other animals and (ii) identifying and discarding infected cattle. Currently the European Union and the United States have banned such practices.

## Molds and Mycotoxins[13,14]

### *Importance*

Infectious molds can cause diseases, such as ringworm, athlete's foot, etc., and is termed as mycoses. Inhalation of mold spores can lead to allergic diseases in humans. However, many strains of molds, while growing in a suitable environment (including in foods), produce metabolites that are toxic to humans, animals, and birds and are grouped as mycotoxins. Consumption of foods containing mycotoxins causes mycotoxicosis. They are secondary metabolites and not proteins or enteric toxins. Many are carcinogens and, when consumed, can cause cancer in different tissues in the body, such as hepatocarcinoma (liver cancer). Some cause toxicity of organs (liver, kidney) by modulating the cell signaling pathway and programmed cell death. Incidence of mycotoxicosis

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in humans has not been recorded in recent years in many countries, at least in the developed countries. This is because their presence in many foods are critically regulated and evaluated. Incidences of mycotoxicosis have been recorded in some developing countries in recent years. Some of the well-known mycotoxicosis incidences in humans in the past include ergotism from the consumption of bread made from rye infected with *Claviceps purpurea* in Europe between the 14th and 16th centuries, yellow rice disease from the consumption of rice infested with toxigenic strains of several *Penicillium* species in Japan during the 17th century, and alimentary toxic aleukia from the ingestion of grains infested with toxigenic strains of *Fusarium* species in Russia in the early 20th century. In recent years, several incidences were reported in animals and birds, which include the death of thousands of turkeys from liver necrosis in the 1960s in England following feeding peanut meal in which *Aspergillus flavus* grew and produced the toxin aflatoxin.

## Characteristics

### Organisms

Toxigenic species and strains of molds from many genera are known to produce mycotoxins. Some of the toxigenic strains from several species and genera and the toxins they produce include *Asp. flavus* and *Asp. parasiticus*, which both produce aflatoxins; *Asp. nidulans* and *Asp. virsicolor* produce sterigmatocystin; *Fusarium verticilioides* produces fumonisin; *Fus. graminearum* produces deoxynivalenol-DON; *Fusarium* spp. produces Zearalenone; *Penicillium viridicatum* produces ochratoxin; *Pen. patulum* produces patulin; *Pen. roqueforti* produces roquefortin; and *Claviceps purpurea* produces ergot alkaloids. The toxigenic strains cannot be differentiated from nontoxigenic strains just from the morphological characteristics (Figure 28.1). It is necessary to grow a strain under suitable conditions and test the material for the presence or absence of a mycotoxin.

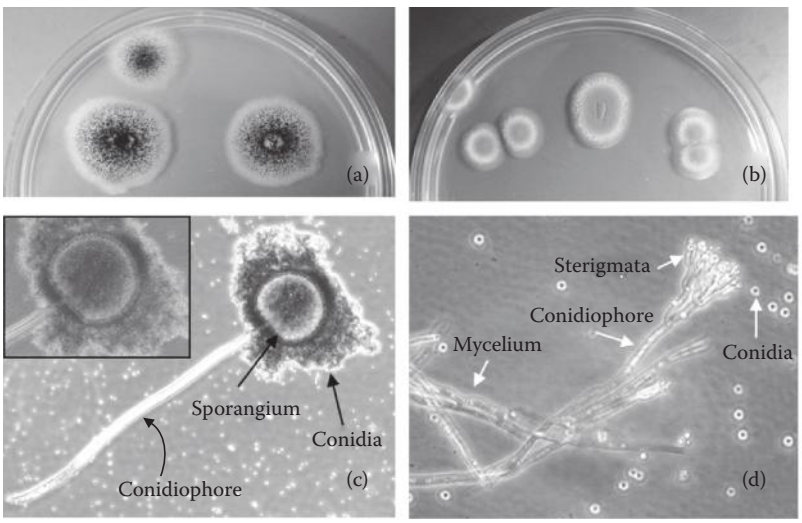



**Figure 28.1** (See color insert.) Photographs of molds. Panel (a) and (b) showing growth of *Aspergillus niger* and *Penicillium citrinum* on agar plates. Panel (c) and (d) are light microscopic photograph of *Aspergillus niger* (c) and *Penicillium citrinum* (d) (magnification 400×).

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This is particularly important for the mold strains from different genera that are used in food production.

### Growth and Mycotoxin Production

In general, molds grow best in humid and warm environments. They are aerobic and thus need air for growth. They can grow, though slowly, at very low $A_W$ (0.65), low temperatures (refrigerated temperatures), and low (pH 3.5). These conditions are often used to extend the shelf life of many foods. Unless other methods (such as vacuum packaging) are used, they can grow in these foods and, if toxigenic, can produce toxins in the foods. The optimum temperature and water activity required for various molds and their toxin production is summarized in Table 28.1.

### Habitat

The spores are present in soil, dust, and the environment. Many foods can have viable spores or mycelia, especially before a heat treatment.

## Toxins and Toxin Production

Mycotoxins include a large number of toxins produced by different toxigenic species and strains of molds. Many have not yet been identified. Some of the toxins have been listed before. There is more than one chemical type (Figure 28.2). An example of this is aflatoxin, which has two major types, B1, B2, G1 and G2, and M1, and each has several subtypes. Aflatoxin B1 is considered the most potent.

Mycotoxins are produced by the toxigenic mold strains as secondary metabolites. Toxin production, in general, is directly related with the growth rate by a mold strain. In microbiological media suitable for growth of molds, *Aspergillus flavus* strains are capable of producing optimum concentrations of aflatoxin at 33°C (91.4°F), pH 5.0, and $A_W$ 0.99.

**Table 28.1 Mold Growth and Mycotoxin Production**

| *Mold* | *Mycotoxin* | *Optimum Temperature (°C)* | *Optimum Water Activity ($A_W$)* |
|---|---|---|---|
| *Aspergillus flavus, Asp. parasiticus* | Aflatoxin | 33 | 0.99 |
| *Aspergillus ochracius* | Ochratoxin | 30 | 0.98 |
| *Penicillium verucosum* | Ochratoxin | 25 | 0.90–0.98 |
| *Aspergillus carbonarius* | Ochratoxin | 15–20 | 0.85–0.90 |
| *Fusarium verticilloides* | Fumonosin | 10–30 | 0.93 |
| *Fusarium verticilloides* | Deoxynivalenol-DON | 11 | 0.90 |
| *Fusarium graminearum* | Zearalenone | 25–30 | 0.98 |
| *Penicillium expansum* | Patulin | 0–25 | 0.95–0.99 |

*Source:* Adapted from Murphy, P.A. et al., *J. Food Sci.*, *71*, R51–R65, 2006.

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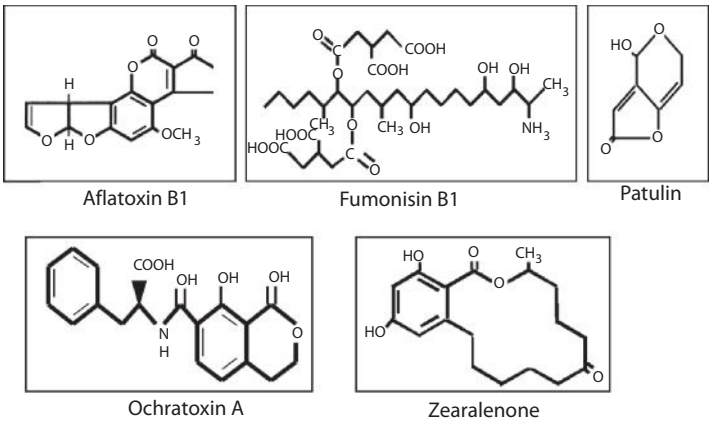



**Figure 28.2 Chemical structure of selected mycotoxins.**

## *Food Association*

The growth of toxigenic mold strains and the presence of specific mycotoxins have been detected in many foods. These include corn, wheat, barley, rye, rice, beans, peas, peanuts, bread, cheeses, dry sausages, spices, apple cider, grain meals, dough cassava, cottonseeds, and spaghetti. Consumption of mycotoxin-contaminated food can cause mycotoxicosis in humans. Feeding moldy products to food animals (including moldy silage) and birds can also produce foods of animal origin (milk, eggs) that are contaminated with mycotoxins. Many of the mycotoxins are resistant to heat used in the normal preparation of foods. Thus their elimination by heating is not used as a means to remove them from foods. In the United States, the regulatory agencies have set limits of aflatoxins in peanut butter (<20 ppb) and milk (<2 ppb).

## *Symptoms and Prevention of Mycotoxicosis*[15]

Mycotoxins, if consumed at high dosage, cause acute to chronic diseases, and they are mutagenic, carcinogenic, and teratogenic. The symptoms may range from skin irritation, vomiting, immunosuppression, birth defects, cancer, liver cancer, hepatotoxicity, liver hemorrhage, kidney damage (nephrotoxicity), neurotoxicity, brain and lung hemorrhage, and death. In preventing human mycotoxicosis, the contamination of food with toxigenic mold strains (or all molds, unless necessary) should be reduced. This is relatively difficult to achieve, but proper packaging can be used to reduce the incidence. Heat treatment, where possible, can also reduce the load by killing the molds and their spores. Preventing growth in food (and feeds) should be a major consideration in reducing the incidence of human mycotoxicosis. This can be achieved by using anaerobic packaging, reducing $A_W$ where possible to ≤0.6, freezing, and by using specific preservatives against mold growth. A product in which molds have grown should not be consumed. Although trimming of foods showing mold growth is a common practice, one cannot be sure that the remaining portion is free of mycotoxins. Under commercial operations, removal of some mycotoxins (aflatoxins) from foods or food ingredients has been studied. Generally, solvent extraction methods have been used. Finally, treatment of some foods or food ingredients with suitable chemicals to inactivate mycotoxins, such as aflatoxins, has also been studied. Some chemicals (e.g., ammonia, hydrogen peroxide, and sodium hypochlorite) were found to inactivate aflatoxins.

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### *Detection Methods*[16,17]

Several methods have been studied to detect mycotoxins and, more particularly, aflatoxins in food. These include solvent extraction of a suspected food sample, thin layer chromatographs of the extract, high pressure liquid chromatography (HPLC), and visualizing it under a UV light or a fluorescent light. Chemical tests and analysis by mass spectral methods are used for identification of the specific type of mycotoxins. In recent years, immunoassays and immunosensors are used for rapid and sensitive detection of mycotoxins from food (see Chapter 42).

## Biogenic Amines

Different types of amines can form in many protein-rich foods by the decarboxylation of amino acids by the specific amino acid decarboxylases of microorganisms. Some of these amines are biologically active and cause illness when consumed in considerable amounts. Examples of biogenic amines are histamine (produced from L-histidine), putrescine, cadaverine (breakdown of lysine by lysine decarboxylase), and tyramine (produced from L-tyrosine). Many bacteria found in foods can produce very active histidine or tyrosine decarboxylases and, if free L-histidine and L-tyrosine are present, can convert them to histamine and tyramine, respectively. Some food spoilage bacteria and even several lactobacilli can have very active histidine and tyrosine decarboxylases. The protein-rich foods associated with biogenic amine poisoning include cheeses ripened for a long time, mold-ripened cheeses, fermented sausages stored for a long time, and different types of fish. Histamine poisoning of fish origin also called scombriod poisoning is further discussed under fish and shellfish toxins.

## Toxins Associated with Fish and Shellfish[18–21]

Fish and shellfish are responsible for food poisoning as a result of the consumption of varieties of toxins originating from harmful species of algae from marine, brackish, and fresh-water environments. Fish accumulates toxins upon feeding on toxic algae, and the increased consumption of fish and shellfish has resulted in large numbers of food poisoning cases. Toxins are of two types: diarrheagenic and neurotoxic, and they are commonly classified as paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), ciguatera fish poisoning (CFP), azaspiracid shellfish poisoning (AZP), and amnesiac shellfish poisoning (ASP) (Table 28.2). In addition, scombroid toxin, which results from bacterial breakdown of fish proteins, also causing severe allergic response are also discussed.

### *Scombroid Poisoning*

Scombroid poisoning, also known as histamine fish poisoning, is an allergy-like form of food poisoning and was initially recognized to occur from the ingestion of spoiled fish of scombroid groups (tuna, mackerel, and bonito). However, it is now recognized that the illness can occur from the consumption of other types of spoiled fish, such as mahi mahi, bluefish, yellowtail, amberjack, herring, sardines, and anchovies. Most of these fish have a high level of free L-histidine in their tissues. In addition, the breakdown of muscles by autolytic proteases and bacterial proteases produced during their growth in fish release histamine. Histidine decarboxylase produced by

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**Table 28.2 Fish and Shellfish Toxins Associated with Food Poisoning**

| *Type of Poisoning* | *Toxin(s) Involved [Microbial Source]* | *Fish and Shellfish Source* | *Symptoms* | *Symptom Onset Time* |
|---|---|---|---|---|
| Scombroid | Histamine [Multiple bacterial species] | Tuna, mahi mahi, bonita, marlin, bluefish, wahoo, mackerel, and salmon | Severe headache, dizziness, nausea, vomiting, allergic response: flushed skin, urticaria, and wheezing | Min to 1 h |
| Ciguatera fish poisoning (CFP) | Ciguatoxin [Algae: *Gambierdiscus toxicus*] | Coral reef fish: amberjack, snappers, grouper, goat fish, barracuda, sea bass, surgeon fish, ulua, and papio | Abdominal pain, diarrhea, vomiting, paresthesias (tingling, burning, numbness), cold-to-hot sensory reversal, weakness, and myalgias | 30 min– 4h |
| Puffer fish poisoning (PFP) | Tetrodotoxin | Ocean sunfishes, porcupine fishes, and fugu (puffer fish) | Paresthesias, headache, vomiting, diaphoresis, respiratory paralysis, circulatory collapse, and death | 10–45 min |
| Paralytic shellfish poisoning (PSP) | Saxitoxins [Algae: *Alexandrium catenella*] | Mussels, clams and oysters | Vomiting, diarrhea, facial paresthesias, and respiratory paralysis leading to death | 5–30 min |
| Neurotoxic shellfish poisoning (NSP) | Brevetoxins [Algae: *Karenia brevis*] | Mussels and clams | Diarrhea, vomiting, abdominal pain, myalgias, paresthesias, and ataxia | 30 min–3 h |
| Amnesic shellfish poisoning (ASP) | Domoic acid (neurotoxin) [Algae: *Pseudonitzschia pungens*] | Mussels, clams, crabs, and anchovies | Vomiting, diarrhea, headache, myoclonus, loss of short-term memory, confusion, disorientation, seizures, coma, and hemiparesis | 15 min to >35 h |

*(continued)*

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**Table 28.2 Fish and Shellfish Toxins Associated with Food Poisoning (Continued)**

| *Type of Poisoning* | *Toxin(s) Involved [Microbial Source]* | *Fish and Shellfish Source* | *Symptoms* | *Symptom Onset Time* |
|---|---|---|---|---|
| Diarrhetic shellfish poisoning (DSP) | Okadaic acid, Dinophysistoxins, Pectenotoxins, Yessotoxin [Algae: *Dinophysis fortii*] | Mussels, clams, and scallops | Diarrhea, nausea, vomiting, and abdominal pain | 30 min to 6 h |
| Azaspiracid shellfish poisoning (AZP) | Azaspiracid-1 [Algae: *Azadinium spinosum*] | Mussels | Diarrhea, nausea, vomiting, and abdominal pain | 30 min to 6 h |
| Cyanotoxin | Nodularin and Cylindrospermopsin [Cyanobacterial blooms] | Crayfish | Hepatotoxicity, gastroenteritis | |

*Source:* Adapted and summarized from Kalaitzis, J.A. et al., *Toxicon, 56,* 244–258, 2010; Tsabouri, S. et al., *Ped. Allergy Immunol., 23,* 608–615, 2012.

bacteria growing in fish catalyzes L-histidine to histamine. The most important bacterial species associated with histamine production in fish is *Morganella morganii*. Several other species are also found, namely, *Klebsiella pneumoniae*, *Proteus* spp., *Enterobacter aerogenes*, *Hafnia alvei*, and *Vibrio alginolyticus*. In fermented fish products, several *Lactobacillus* spp. have also been implicated in histamine production.

The symptoms of histamine poisoning include gastrointestinal (nausea, vomiting, abdominal cramps, and diarrhea), neurological (tingling, flushing, palpitations, headache, burning, and itching), cutaneous (hives, rash), and hypotension. Generally, high levels of histamine in fish are necessary to produce the symptoms. The FDA hazard action level is currently set at 50 ppm in fish. A histamine level of 20 ppm indicates temperature abuse in products. Depending on the histamine concentrations and susceptibility of an individual, symptoms can occur very quickly; generally within one hour. Histamine poisoning is not fatal. However, the reactions can be severe in individuals allergic to histamine.

Histamine poisoning from fish is a worldwide problem. The frequency of incidence is probably directly related to the popularity of fish and the methods used in preservation from harvest to consumption. In the United States, between 1973 and 1987, there were 202 incidents affecting 1216 people. The frequency of incidence seemed to increase as there were 83 incidents between 1983 and 1987. Finfishes were involved in 81 of 83 incidents; 62.6% occurred with fish served in restaurants, and 26.5% occurred at home. The most important factor was improper holding temperature of fish (which caused the fish to decompose). Seasonal variation is not apparent, probably because fish is eaten in the United States throughout the year.

As the consumption of fish increases, the incidence of histamine poisoning is expected to rise. The most important control measure will be to store fish by freezing or by refrigeration at <1°C for a limited time. In addition, proper sanitation to reduce contamination with histamine-producing bacteria will be important. Cleaning fish soon after catching can also reduce the autolytic effect

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of muscle tissues. Histamine is heat stable; once formed, cooking cannot inactivate it. Thus, if there is a doubt that a fish is spoiled, the best way to avoid histamine poisoning is to not eat it. An antihistamine is used to alleviate histamine poisoning.

### *Ciguatera Fish Poisoning (CFP)*

Finfish, especially those feeding on reef and mud (such as sea bass, grouper, and snapper), while feeding on the toxic algae *Gambierdiscus toxicus* and several others, accumulate toxin in the muscle tissues. Liver, intestines, and roe accumulate more toxin than muscle tissues. Consumption of these fish results in disease with gastrointestinal and neurological symptoms developing within a few hours. The gastrointestinal symptoms are nausea, vomiting, cramps, and diarrhea and last for a short time. Neurological symptoms include tingling and numbness of the lips and tongue, dryness of mouth, chills, sweating, blurred vision, and paralysis. The disease can be fatal under severe conditions.

Foodborne disease resulting from ciguatoxin occurs in many countries, especially where reef fishes are consumed. Worldwide, 25,000 people are affected annually by ciguatera toxin. In the United States, between 1973 and 1987, there were 234 confirmed incidents affecting 1052 individuals without any fatalities; 232 were from the consumption of finfish harvested from unsafe places. Among the 87 incidents between 1983 and 1987, 69 occurred at home and only four occurred in food service establishments.

Immunoassays and liquid chromatographic methods have been used to detect ciguatoxin, and therefore monitoring fish for the presence of this toxin before consumption is possible. Incidence of the disease can be reduced by avoiding consumption of large fish from the reef, particularly their inner organs.

### *Paralytic Shellfish Poisoning (PSP)*

Scallops, clams, and mussels feeding on *Alexandrium catenella* and related toxic species of algae accumulate a heat-stable toxin known as saxitoxin in the tissues. Consumption of these shellfish produces paralytic poisoning. The common neurological symptoms include tingling and numbness of lips and fingertips, drowsiness, poor coordination, incoherent speech, and dryness of the throat. In extreme cases, respiratory failure and death can result. The symptoms appear within one hour and subside within a few days.

Occurrence of paralytic shellfish poisoning is sporadic and depends on the growth (bloom) of the toxic algae, as influenced by water temperature (approximately 8°C), salinity, runoff, the presence of nutrients, and others. Shellfish growing in shallow water tend to accumulate more toxins. In the United States, the incidence of paralytic shellfish poisoning is quite low. Between 1973 and 1987, there were only 21 incidents affecting 160 people. Between 1983 and 1987, only two incidents were reported. This has been possible because of monitoring systems of shellfish for toxins and closing contaminated shellfish beds for harvesting.

### *Neurotoxic Shellfish Poisoning (NSP)*

NSP is caused by brevetoxin produced by dinoflagellate *Karenia brevis*. Growth of the algae produces red tide, and feeding of the algae by shellfish causes the toxin to be accumulated in the muscle. Consumption of these shellfish produces neurological symptoms as described for paralytic shellfish poisoning but less severe. The symptoms appear very quickly (30 minutes to three hours)

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and generally subside in a few hours. The incidence is very low in the United States because of effective monitoring systems. Sometimes a satellite is used to monitor ocean color, including red tide. *Karenia brevis* bloom is a recurring problem in Gulf of Mexico.

### *Diarrhetic Shellfish Poisoning (DSP)*

DSP is associated with two types of toxins, okadaic acid and dinophysistoxins produced by the dinoflagellate species of *Dinophysis* and *Prorocentrum*. Gastrointestinal disorders from the consumption of scallops, mussels, and clams that feed on the toxic algae *Dinophysis fortii* or other species were reported in Japan and the Netherlands. The gastrointestinal disorders include diarrhea, nausea, vomiting, and abdominal pain. The duration of the symptoms is short. The toxin is heat stable, and thus cooking does not eliminate it. The incidence of the disease can be reduced by removing the digestive organs of the shellfish because they tend to accumulate the majority of the toxins.

### *Azaspiracid Shellfish Poisoning (AZP)*

AZP is associated with the consumption of mussels, which accumulate Azaspiracid toxin. This toxin is produced by a dinoflagellate *Azadinium spinosum*. The symptoms are similar to diarrhetic shellfish poisoning (DSP) and are manifested as diarrhea, nausea, vomiting, and abdominal pain.

### *Amnesic Shellfish Poisoning (ASP)*

Amnesic shellfish poisoning (ASP) results from consumption of mussels, clams, crabs, and anchovies. The neurotoxin is domoic acid, which is produced by algae, *Pseudonitzschia pungens*. The neurological and gastrointestinal symptoms include vomiting, diarrhea, headache, disorientation, seizures, coma, and hemiparesis (lack of voluntary movement of limbs and fingers similar to patients suffering from stroke). In addition, there is a short-term memory loss and confusion associated with ASP toxicity.

### *Puffer Fish Poisoning (PFP)*

PFP is associated with consumption of puffer fish (also known as fugu), ocean sunfishes, and porcupine fishes. The toxin involved is tetrodotoxin, a neurotoxin that blocks the sodium channel in neurons and symptoms include tingling, burning, numbness, headache, vomiting, diaphoresis, respiratory paralysis, circulatory collapse, and death. Symptoms appear with 10–45 minutes. Puffer fish poisoning is most prevalent in Asian countries with an estimated 50 deaths annually in Japan. Repeated cases have also been reported from Singapore and Bangladesh.

## Parasites[22–24]

Included in this group are several intestinal and tissue helminths (roundworms, flatworms, and tapeworms) and protozoa that are known to cause human illness and where a food association has either been confirmed or suspected. They can stay in the gastrointestinal (GI) tract and produce gastrointestinal symptoms. Some invade body tissues and produce specific problems. Several of these organisms and the diseases they produce are briefly described here.

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## Trichinella spiralis[25]

*Trichinella spiralis* is a roundworm and can be present in high frequencies in pigs and causes trichinosis or trichinellosis in humans and animals. Eight species of the genus *Trichinella* are recognized as zoonotic pathogens worldwide. It is also found quite frequently among game animals. A person is infected by consuming raw or insufficiently cooked meat of infected animals. The infected meat contains encysted larvae. Once the meat is consumed, the cysts dissolve in the GI tract, releasing the larvae, which then infect the GI tract epithelium. Here, the parasites mate and deposit larvae in the lymphatic system. Through lymphatic circulation, the larvae infect other body tissues. In the United States, between 1973 and 1987, 128 incidents of trichinosis infecting 843 people were recorded. Although many types of foods were involved, the highest incidence (55) was from pork. Almost all incidences were a result of improper cooking.

The symptoms of trichinosis appear in 2–28 days following ingestion. Initial symptoms are nausea, vomiting, and diarrhea, followed by fever, swelling of eyes, muscular pain, and respiratory difficulties. In extreme cases of infection, toxemia and death may result.

The prevention methods consist of not raising pigs on garbage and cooking meat thoroughly [140°F (60°C) and above]. Microwave cooking may not be effective in killing all the larvae. Freezing meat for 20 days at –20°C (home freezer) or in liquid nitrogen for several seconds also kills the parasite. Irradiation is generally effective in inactivating *Trichinella* in meats.

## Anisakis simplex

The nematode *Anisakis simplex* is a parasite in many fish, particularly of marine origin. Human infection, called anisakiasis, results from the consumption of raw, infected fish. Inadequately cooked, brined, or smoked fish has also been implicated in some cases. Symptoms appear after a few days and generally include irritation of throat and digestive tract. Anisakiasis is predominant in countries where raw fish is consumed. Proper cooking, salting, or freezing at –20°C for three days can be used as a preventative measure.

## Taenia *Species*

Taeniasis is a tapeworm disease caused by *Taenia solium* from pork and by *Taenia saginata* from beef. The illness results from the ingestion of raw or improperly cooked meat contaminated with larvae (taeniasis) or the adult tapeworms (cysticercosis). Symptoms appear within a few weeks and are digestive disorders and severe abdominal pain. If an organ is infected by the larvae, especially a vital organ, the consequences can be severe.

## Toxoplasma gondii[26]

The zoonotic infection toxoplasmosis is a tissue protozoan disease transmitted to humans from the consumption of undercooked and raw meat and raw milk contaminated with oocysts of *Toxoplasma gondii* (Figure 28.3). *Toxoplasma gondii* was first reported in 1908, and its lifecycle was not discovered until 1970. Drinking water contaminated with cat feces was thought to be responsible for several outbreaks worldwide. Unwashed hands after contact with pet cats, fruits and vegetables washed in contaminated water, and eating of undercooked meat (pork, lamb) are considered to be potential sources for this parasite. Cats serve as the primary host. Cats become infected by consuming infected prey. The cyst in a cat is dissolved in the digestive tract and

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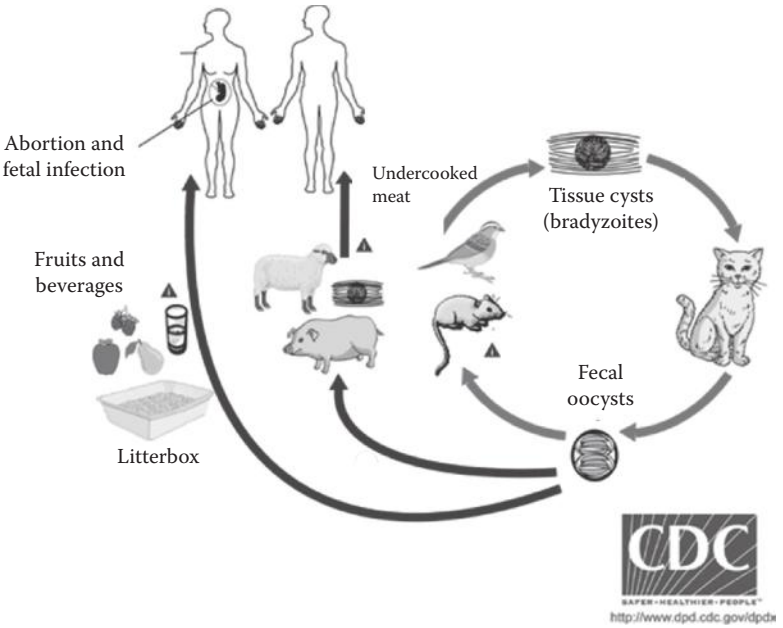



**Figure 28.3 Life cycle of *Toxoplasma gondii*. (Adapted from CDC, http://www.dpd.cdc.gov/dpdx/HTML/Toxoplasmosis.htm.)**

multiplies. It is then localized in muscle tissue, tissue cysts (bradyzoites). Contamination of food with feces, especially from cats, can transmit the oocyst to humans and localize them in skeletal muscle, myocardium, the brain, and the eyes. In many people, it does not cause any problems. However, in individuals with low resistance or who are immunocompromised, the symptoms are generally flu-like, accompanied by joint and muscle pain with fever and headache. It can cause fatal deformation and be life-threatening in immunocompromised people. Toxoplasma infection in the early stage of pregnancy can lead to spontaneous abortion. Toxoplasma infection can cause severe neurologic or ocular disease in the fetus. Preventative measures include cooking meat to a minimal internal temperature of 70°C or freezing at –20°C.

## Giardia lamblia

Giardiasis is caused by the intestinal protozoan *Giardia lamblia*. Giardiasis has been considered as a food- and waterborne disease in many parts of the world. In the United States, between 1973 and 1987, five incidents infecting 131 individuals were confirmed. The incidence is much higher in countries with inadequate sanitation facilities and improper water supplies. Contaminated raw vegetables; foods such as salads and sandwiches, contaminated with water containing the causative agent; and poor personal hygiene are considered the major causes of the disease. The infective dose is 10–100 cysts and the incubation period is one to two weeks. The main symptoms are acute or chronic diarrhea and abdominal pain. The oocysts and cysts are excreted in the feces of the affected individuals. Wild animals, such as beavers, can carry the protozoa and contaminate water. Giardia is resistant to chlorine treatment that is applied to water supplies. Improved sanitary conditions and personal hygiene are important to reduce the incidence.

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## Cryptosporidium parvum

*Cryptosporidium parvum* is an intestinal protozoa and causes cryptosporidiosis in humans through the consumption of contaminated food and water with the oocysts. It can also be present in the GI tract of domesticated and wild animals and contaminate food and water. The infective dose is thought to be very low, ~10 oocysts, and the incubation period is 2–10 days. The major symptoms of infection in humans are watery diarrhea, abdominal pain, fever, muscle aches, dehydration, and, in some debilitated individuals, such as AIDS patients, death. *Cryptosporidium* caused the largest waterborne outbreak in Milwaukee, Wisconsin (USA), involving 403,000 cases in 1993. Since then, there have been four outbreaks in the United States from apple cider, chicken salad, and green onions. These foods were contaminated with oocysts by the people infected with the protozoa because they had poor personal hygiene. It is a significant cause of waterborne protozoan disease in humans. The oocysts are resistant to disinfectants normally used to treat water.

## Cyclospora cayetanensis

*Cyclospora cayetanensis* is a coccidian intestinal protozoan and is considered a newly emerging foodborne pathogen in the United States. However, the pathogenic nature of the protozoan has been recognized in many other countries for a long time. Humans and some primates are the only natural hosts. Consumption of food and water contaminated with the oocysts and subsequent infection results in the disease. The infective dose is unknown but the incubation period is typically one week. The disease usually lasts for two weeks. The main symptoms are watery diarrhea, cramps, nausea, vomiting, fever, and fatigue. Since 1995, 80 outbreaks have been reported in the United States from the consumption of contaminated raspberries, lettuce, and basil. In the late 1990s, contaminated raspberries from Guatemala caused widespread outbreaks in 20 states and again in 2004; Guatemalan snow peas also caused an outbreak in the United States.

# Conclusion

Several bacterial species, normally regarded as nonpathogenic, can cause enteric foodborne disease in susceptible individuals. With the rise in susceptible individuals, there may be more cases of foodborne diseases in the future. An increase in the consumption of seafood, some raw, has been regarded as the main cause in the increase of foodborne diseases of fish and shellfish toxins. Similarly, an increase in the consumption of raw vegetables (salads) and fruits has been related to the increase in foodborne diseases caused by different parasites. Proper selection of food, sanitation, and processing and preservation methods can be used to reduce their incidence in food.

### QUESTIONS

1. Define an opportunistic pathogen. Briefly explain how *Aer. hydrophila* can be considered an opportunistic pathogen.
2. Briefly discuss the possibility of causing foodborne gastroenteritis by *Ple. shigelloides*.
3. Name three noncoliforms that may be associated with human foodborne gastroenteritis. What evidence can be given to justify them as the causative agents for gastroenteritis?
4. Discuss the importance of psychrotrophic *Bacillus* spp. as a possible causative agent for foodborne gastroenteritis. Why can the consumption of pasteurized milk stored in the refrigerator for a long time be associated with gastroenteritis caused by psychrotrophic *Bacillus* spp.?

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5. List the enteric viruses implicated in foodborne disease outbreaks. Why are foodborne diseases of viral origin not always detected?
6. How are foods contaminated with hepatitis A virus, and what control measures should be adopted to reduce the incidence?
7. What methods are used to detect incidence of foodborne disease of viral origin?
8. What is the causative agent of mad cow disease, and how does this protein differ from the normal protein?
9. Describe the prevention strategies for BSE.
10. Discuss the role of microorganisms in histamine poisoning from the consumption of some ripened cheeses, old fermented sausages, and fish. Name the important microbial species that can be associated with scombroid poisoning. Suggest the preventative methods.
11. Why is ciguatera poisoning more prevalent with foods served at home? How can one prevent the incidence?
12. List the types of shellfish poisoning. What are their major symptoms?
13. List the major causes of trichinellosis in humans. How can the incidence be reduced?
14. Discuss the reasons for foodborne anisakiasis, taeniasis, and toxoplasmosis in humans and explain how their incidence can be reduced.
15. List four protozoan diseases transmitted through foods in humans. Discuss the importance of intestinal protozoan diseases of food origin in humans. Which foods are more often associated with these diseases and how can they be reduced?
16. Explain these terms: Mycotoxins, mycotoxin-producing strain of *Penicillium* species, aflatoxin, and growth characteristics of molds.
17. List five foods that directly or indirectly can be contaminated with mycotoxins. Discuss mycotoxin concerns in foods and food ingredients imported to the United States from other countries.

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# Chapter 29

# New and Emerging Foodborne Pathogens

## Introduction

A comparison of the lists of bacteria and viruses that were known to be foodborne pathogens in the past and those confirmed as foodborne pathogens currently reveals quite an astonishing picture. Many that were not considered or known to be foodborne pathogens in the past have later been implicated in foodborne illnesses. These pathogens, following their recognition, are generally designated as new or emerging foodborne pathogens. Table 29.1 presents a list of pathogens recognized as new foodborne pathogens during the past 30 years in the United States. Before 1959, four bacterial species were considered as foodborne pathogens: *Staphylococcus aureus*; *Salmonella enterica* serovars, including Typhi and Paratyphi; *Clostridium botulinum* Types A and B; and *Shigella* spp. In the 1960s, *Vibrio cholerae* non-O1 and *Clo. botulinum* Type E were added to the list of bacteria, along with hepatitis A virus. Between 1971 and 1980, *Vibrio parahaemolyticus*, enteropathogenic *Escherichia coli* (O124:H17), *Yersinia enterocolitica*, *Vib. cholerae* O1, *Campylobacter jejuni*, and *Vib. vulnificus* were confirmed either in foodborne disease outbreaks or sporadic cases. The food association of *Clo. botulinum* with infant botulism was also recognized. In the 1980s and 1990s, *Listeria monocytogenes*, enterohemorrhagenic *Esc. coli* O157:H7, enterotoxigenic *Esc. coli* O27:H20, Norovirus, and *Campylobacter*, *Cryptosporidium*, and *Cyclospora* became the new pathogens. *Salmonella* enterica ser. Enteritidis, which was earlier involved in foodborne outbreaks in a relatively low frequency, became a major causative agent of foodborne salmonellosis. In recent years, the possible role of several pathogens, namely, *Clostridium difficile*, *Cronobacter sakazaki*, *Helicobacter pylori*, *Mycobacterium paratuberculosis*, and a few enteric viruses in foodborne/waterborne diseases has been suspected. Similar situations involving the sudden emergence of new foodborne pathogens with time also exist in other countries.

Are they really "new" foodborne pathogens? If a pathogen uses a food as a vehicle for its transmission to humans for the first time and causes the specific illness, then it is probably not new. Rather, it is appropriate to recognize that it probably has caused foodborne illnesses before but has not been confirmed. Because of changes in several situations, its direct involvement in a foodborne illness has probably been suspected before and identified later for the first time. In this section, the changes in several situations that have probably led to the discovery of a new foodborne pathogen are discussed.



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**Table 29.1 New Pathogenic Bacteria and Viruses Associated with Confirmed Foodborne Diseases**

| *Before 1959* | *1959–1970* | *1971–1980* | *1981–1990* | *1991–2013* |
|---|---|---|---|---|
| *Sta. aureus* | *Clo. perfringens* (1959)[a] | *Vib. parahaemolyticus* (1971) | *Esc. coli* O157:H7 (1982) | *Esc. coli* O104:H4 (2011) |
| *Salmonella* serovar (Typhi and Paratyphi included) | *Bac. cereus* (1959) | Enteropathogenic *Esc. coli* (O124:H17; 1971) | *Lis. monocytogenes* (1983) | Nipah virus (1998) |
| *Clo. botulinum* (Type A, B) | *Vib. Cholera* (non-O1; 1965) | *Esc. coli* O27:H20 (1983) | *Cam. coli* | |
| *Shigella* spp. | *Clo. botulinum* (Infant botulism; 1976)<br>*Clo. botulinum* (Type E; mid-1960s) | *Vib. vulnificus* (1976) | Viruses (1982) | |
| | Hepatitis A (1962) | *Yer. enterocolitica* (1976) | *Salmonella* Enteritidis[b] | |
| | | *Vib. cholera* (O1;1978) | *Cronobacter sakazaki* (1988) | |
| | | *Cam. jejuni* (1979) | | |

[a] Year first confirmed in foodborne outbreak.
[b] Recognized as a major cause of salmonellosis of egg origin.

A critical analysis of the data in Table 29.1 reveals three important observations: (1) a pathogen that has not been confirmed in the past or at present can emerge as a foodborne pathogen in the future; (2) a pathogen that currently is involved in a few sporadic foodborne illnesses or outbreaks may, in the future, become a major cause of foodborne outbreaks (such as in *Salmonella* Enteritidis); and (3) conversely, a pathogen considered to be the major cause of a foodborne disease in the past or at present can, in the future, become associated with fewer incidences (e.g., in the United States, there were 26 staphylococcal food intoxication outbreaks in 1976 affecting 930 people; in 1987, there was only one outbreak with 100 cases). The reasons for these changes can be attributed to many factors, some of which include changes in our food consumption pattern, modern innovation in food processing and retailing technologies, changes in agriculture and animal husbandry practices, climate change, an increase in food imports from many countries resulting from changes in the trade barrier, an increase in international travel, an increase in numbers of elderly people and immunocompromised people, and better knowledge of pathogens and regulations for testing foods. Some are discussed here.

## Associated Factors[1]

Several factors could be attributed to the emergence or reemergence of foodborne pathogens and a convergence model has been proposed to describe this phenomenon.[2] Fundamentally,

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**Table 29.2 Factors Responsible for Emergence and Reemergence of Foodborne Disease Outbreaks**

| |
|---|
| • Intensive agricultural practices to produce cheaper foods: Farming practice with large numbers of animals and birds in close quarters help emerge and spread diseases. |
| • Increased interaction of humans with domestic animals and wildlife and their products and intrusion of wildlife habitats. |
| • Contamination of water, an environmental common, can spread pathogens across farms, regions, states, and nations. |
| • Climate change and temperature rise help bring new vectors and help emerge pathogens with greater adaptation and stress response. |
| • Rapid population growth and an aging population with compromised immune system. |
| • Increasing global import and export of fruits and vegetables, ethnic foods, and meats. |
| • Changing eating habits and consumption of raw or lightly cooked foods and a demand for exotic foods, such as bush meats. |
| • Increased demand for a high-protein diet in countries that are becoming economically affluent for more meats and fish products. |

*Source:* Adapted from Newell, D.G. et al., *Int. J. Food Microbiol.*, 139, S3–S15, 2010; With kind permission from Springer Science+Business Media: *Microbial Food Safety: An Introduction*, 2012, 3–12, Oyarzabal, O.A. and Backert, S., Eds.

the convergence model takes into account the complex interaction among humans, pathogens, domestic and wild animals, and the environment, some of which are summarized in Table 29.2.

## *Better Knowledge of Pathogens*

There are more than 200 known microbial, chemical, or physical agents that can cause foodborne illness when ingested. The Centers for Disease Control and Prevention (CDC) estimates that foodborne agents affect 48 million people, 128,000 hospitalizations, and 3,000 deaths annually in the United States, of which 9.4 million cases are attributed to 31 known pathogens while the etiological agents for 38.4 million cases, that is, 90% of cases are not known.[3] In general, among etiological agents, bacterial and viral pathogens are the major causes of foodborne illnesses. Many of these with unknown etiology are considered to be of bacterial, viral, parasitic, or other origins, such as mushroom and marine biotoxins, metals, and other inorganic toxins, or emerging pathogens for which no diagnostic tests have been developed. In a reported outbreak, when the food and environmental samples and samples from the patients are available, they are examined for the presence of the most likely or most common pathogens. Thus, in many incidents, the samples will usually not be tested for pathogens that are not considered at that time to be a foodborne pathogen. This is generally the situation with reported sporadic cases or small outbreaks. In case of a large outbreak or a similar type of outbreak occurring frequently or if the incident results in death or severe consequences, the samples are generally tested for other suspected pathogens along with the common foodborne pathogens. Most of the new foodborne pathogens were discovered this way. In the United States, the association of *Yer. enterocolitica*, *Cam. jejuni*, *Lis. monocytogenes*, *Cronobacter sakazaki* and *Esc. coli* O157:H7 in foodborne outbreaks has been confirmed this way.

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In the future, other pathogens will be recognized as "new" pathogens in the same way. This is probably true for several pathogens, such as *Aeromonas hydrophila*, *Edwardsiella* spp., *Plesiomonas shigelloides*, and other opportunistic pathogens, as well as *Helicobacter pylori*, *Mycobacterium paratuberculosis*, *Clostridium difficile*, and several enteric viruses, which have been suspected from circumstantial evidence to be foodborne pathogens but have not yet been directly confirmed.

One of the major reasons for testing available food samples for new pathogens, other than those tested routinely, is our current expanded knowledge about the characteristics of many pathogens. Much information is available about different pathogens with respect to their physiological, biochemical, immunological, and genetic characteristics, as well as their pathogenicity, habitat, and mode of disease transmission. Recent knowledge of entire genome sequences of several pathogens and advances in molecular biology techniques have greatly aided these studies. This information has helped develop new, effective, and specific methods to isolate and identify foodborne pathogens from samples. Many of the identification techniques are specific, rapid, and less involved, even in the presence of large numbers of associated bacteria. This aids in testing, economically and effectively, a large number of samples in a relatively short span of time for many pathogens, which includes those routinely tested and those that are probably suspected. Availability of ready-to-use kits from commercial sources has advanced this approach. It is anticipated that more new or emerging pathogens will be added to the list of pathogens of concern in coming years similar to a new strain of enteroaggregative *Escherichia coli* (EAEC) O104:H4 that caused one of the largest outbreaks in Germany and other countries in Europe in 2011 due to consumption of sprouts (see below).

## *Improvement in Regulatory Actions*

In recent years, at least in most developed countries, the local, state, and federal regulatory agencies have been highly active in reporting a foodborne outbreak and taking quick actions to identify the etiological agents. The number of reported outbreaks has increased in the United States from an average of 240 per year in the 1960s to an average of 530 per year in the 1980s. In 2009, there were 675 outbreaks and in 2010, 852 outbreaks. Norovirus was at the top of the list, responsible for 42% and *Salmonella* was second with 30% of outbreaks in 2009 and 2010.[4] This increase is a result, in large part, of an increase in reporting. Once a new foodborne pathogen is identified, its frequency of occurrence in foods, mode of transmission in foods, and growth and survival in foods under conditions of processing, storage, and handling at different stages from production to consumption are determined. After this information becomes available, regulatory agencies develop effective methods to either prevent or reduce its presence in the food ready for consumption. This is accomplished by developing proper methods of sanitation during processing and handling of foods and preservation. For some pathogens, methods to reduce their presence in raw foods or even in live animals (such as for *Esc. coli* O157:H7) are being studied. Finally, some improved efforts have been and are being made by regulatory agencies to educate consumers (especially at home) and food handlers (especially at food service facilities) about the means by which one can reduce the incidence of contamination (such as for *Salmonella* from uncooked chicken or eggs) resulting from cross-contamination and improper cooking or cooling of foods. Public education will need increased focus in order to reduce the incidence of foodborne diseases by pathogens, including those that are and will become newly emerging. Because foods served at home and at food service facilities have been implicated in high frequencies of foodborne disease outbreaks, including those with unknown etiology, people involved in handling food in these two places need to be aware of the means by which the incidences can be prevented or reduced.

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The efforts by regulatory agencies have probably paid off for several pathogens in the United States with reduced numbers of foodborne illnesses and mortalities reported in 2011. Recent data show foodborne agents affect 48 million people, 128,000 hospitalizations, and 3,000 deaths, which are much lower than the 1999 estimates.[5] To improve safety efforts even further, the Food Safety Modernization Act (FSMA) was enacted in 2011.[6] The major emphases in this Act include (i) preventive control plans, (ii) mandatory produce safety standards: science-based food safety approach, (iii) mandatory inspection for high risk products, (iv) product tracing, (v) performance standards, (vi) third-party certification, (vii) certification for high-risk foods, (viii) increased inspection authority, (ix) mandatory recall authority when the samples show evidence for pathogen contamination and death associated with the food, and (x) suspension of registration of the offending food producer.

Current efforts to identify the incidence of foodborne diseases by establishing FoodNet and proper education of consumers and food handlers by regulatory agencies in the United States will be important factors in reducing the incidence of foodborne diseases by currently known pathogens and new pathogens of the future. Similar efforts should be undertaken in other developed and developing countries to overcome problems associated with foodborne pathogens.

## *Changes in Lifestyle and Food Habits*

In this category, several factors can be included that have contributed to the emergence of new foodborne pathogens. One of the most important factors is the increase in traveling, especially international traveling. A person arriving in a foreign country or returning from it can bring a new foodborne pathogen into a country where it was not recognized previously. *Esc. coli*, associated with traveler's diarrhea and *Vib. cholerae* non-O1 (new serotype) were probably introduced to the United States this way.

Another important factor is changes in food habits. In the United States, increased consumption of seafoods, some of which are eaten raw, have resulted in an increase in foodborne disease outbreaks by *Vib. parahaemolyticus*, *Vib. vulnificus*, *Vib. cholerae*, and Hepatitis A, all of which were not always recognized as foodborne pathogens. Similarly, consumer preferences for low-heat-processed foods with a long shelf life at refrigerated temperatures and fewer preservatives have enhanced the chances of psychrotrophic pathogens (e.g., *Lis. monocytogenes* and *Yer. enterocolitica*) becoming important foodborne pathogens. Several food preferences, such as consumption of raw milk, soft cheese made with unpasteurized milk, and undercooked hamburgers, provided the right consequences for *Cam. jejuni*, *Lis. monocytogenes*, and *Esc. coli* O157:H7, respectively, to cause foodborne disease outbreaks. Similarly, preference for some imported foods, especially the ready-to-eat type, ethnic foods, and also exotic meats such as bush meats, consisting of wild animals, such as monkeys, elephants, gorillas, crocodiles, anteaters, bats, etc., can introduce a new pathogen.[7] Data collected by the CDC from 2005–2010, show that imported foods from 15 countries were responsible for 39 outbreaks resulting in 2348 illnesses in the United States.

How a change in food consumption patterns can change the profile of foodborne diseases is best exemplified by an increase in foodborne diseases from the increase in consumption of minimally processed ready-to-eat foods. According to a survey by Information Resource, Inc., convenience food continues to be the priority for U.S. consumers. The data show the food preference as follows: ready-to-eat, 77%; heat-and-eat, 76%; packaged for on-the-go consumption, 65%; and no utensils required, 64%. In addition to these, the preference for foods that are more natural has encouraged the production of large varieties of minimally processed foods. Many of these are fresh or given a low-heat treatment and expected to have a shelf life of 3–10 weeks or more

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at refrigerated temperature. Chapter 21 gives examples of many psychrotrophic bacteria being associated with the spoilage of minimally processed foods. Most of these spoilage bacteria gain access to the products as post-heat contaminants, generally in low numbers; during long storage, they multiply to reach the spoilage-detection levels. Many of them are new spoilage bacteria or bacteria not previously suspected to be spoilage bacteria. A similar situation can exist for the presence of pathogens in minimally processed foods. The storage temperature of these products ranges from 4°C to 15°C or more (during display). Psychrotrophic and many mesophilic pathogens can multiply during storage and from a very low contamination level (e.g., ca. 1/25 g) can reach the hazard potential level.

Since 1990, there has been an increase in consumption of ready-to-eat raw vegetables (salads) and fruits (fruit juices). Surprisingly, foodborne disease incidence from these sources has increased (Table 29.3). Before the 1990s, many of these vegetables and fruits were not considered to be associated with so many different types of foodborne diseases, many of which are thought to be caused by emerging pathogens. Large quantities of this produce come from many countries; often it is difficult to determine the source of production because of complexities involved in international trade. The major sources of contamination are the lack of field sanitation, use of poor-quality water for washing, transportation in dirty trucks, and lack of sanitation in handling the produce. Controlled studies showed that many pathogens can not only survive but also grow in fresh vegetables and fruits and fruit juices at refrigerated and ambient temperatures and many of the pathogens are of the emerging type (Table 29.4).

Many minimally processed ready-to-eat foods of animal origin, especially low-heat-processed meat products, have been implicated in foodborne disease outbreaks. Because most of the products

**Table 29.3 Fresh Produce Associated with Some Foodborne Diseases in Recent Years in the United States**

| *Vegetables* | *Pathogen* | *Fruits* | *Pathogen* |
|---|---|---|---|
| Sprouts | *Salmonella, Esc. coli* O157:H7, O104:H4 | Cantaloupe | *Salmonella, Lis. monocytogenes* |
| Lettuce | *Esc. coli* O157:H7, *Shigella* spp., Hepatitis A virus | Watermelon | *Salmonella*, Norwalk-like |
| Cabbage | *Lis. monocytogenes* | Strawberries | Hepatitis A virus, *Cyclospora* |
| Celery | Norovirus, *Lis. monocytogenes* | Raspberries | Hepatitis A virus, *Cyclospora* |
| Carrots | Enterotoxigenic *Esc. coli* | Apple cider | *Esc. coli* O157:H7, *Salmonella*, *Cryptosporidium* |
| Tomatoes | *Salmonella*, Hepatitis A virus | Orange juice | *Salmonella* |
| Scallions (green onion) | *Shigella* spp., Hepatitis A virus | Coconut milk (frozen) | *Vib. cholerae* O1 |
| Spinach | *Esc. coli* O157:H7 | | |
| Snow peas | Cyclospora | | |

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**Table 29.4 Growth Potential of Pathogens in Vegetable and Fruit Products**

| *Pathogens* | *Produce (Temperature)* |
|---|---|
| *Shigella* | Lettuce, melon, cabbage (20°C–22°C) |
| *Salmonella* | Tomatoes, melons, sprouts (20°C–23°C) |
| *Esc. coli* O157:H7 | Melons, cider, lettuce, cucumber (8°C–25°C) |
| *Yer. enterocolitica* | Different produce (4°C) |
| *Lis. monocytogenes* | Different produce (4°C) |
| *Bac. cereus*[a] | Vegetable purees (broccoli, peas, carrots, and potatoes; 10°C) |
| *Cam. jejuni* | Melon |
| Enterovirus[b] | Different produce (4°C–23°C) |

*Source:* Remaining data adapted from Sagoo, S.K. et al., *J. Food Prot.*, *66*, 403–409, 2003.

[a] Supplied by Dr. S.R. Tatini (University of Minnesota, St. Paul).
[b] Survived.

are refrigerated, the predominant pathogen was found to be *Lis. monocytogenes* (Table 29.5). As these products were given heat treatment (at pasteurization or higher temperature), the pathogens, namely, *Lis. monocytogenes*, *Salmonella*, and *Esc. coli* O157:H7, could not come from the raw meat and other ingredients. They are, in all probability, post-heat contaminants. All the products are handled extensively following heating and before repackaging (in most cases). The pathogens

**Table 29.5 Pathogens Isolated from Minimally Processed (Cooked) Foods of Animal Origin**

| *Products* | *Pathogens* |
|---|---|
| Roast and corned beef | *Lis. monocytogenes, Salmonella* |
| Ham and luncheon meat | *Lis. monocytogenes, Salmonella* |
| Comminuted products: hot dogs, wieners, bratwurst, salami, bologna, sausage, etc. | *Lis. monocytogenes, Salmonella* |
| Jerky | *Lis. monocytogenes, Salmonella, Esc. coli* O157:H7 |
| Poultry products: whole, fried parts; nuggets; sliced, etc. | *Lis. monocytogenes, Salmonella* |
| Meat salads: different types | *Lis. monocytogenes, Salmonella* |
| Fully cooked meat patties | *Esc. coli* O157:H7 |
| Dry and semidry sausages: pepperoni, salami, summer sausage, etc. | *Lis. monocytogenes, Salmonella, Esc. coli* O157:H7, staphylococcal enterotoxins |
| Cheeses: soft, blue-veined, mold-ripened | *Lis. monocytogenes* |

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contaminated the products during that time from the sources with which the products came in contact. Consumption of the contaminated products can result in foodborne disease by pathogens, many of which are new and emerging.

### New Food-Processing Technology

Because of economic reasons, one of the objectives of food processors is to produce a product in large volumes and at a faster rate in a centralized plant. Handling a food in large volumes has its disadvantages; accidental contamination of the product by a pathogen can cause a foodborne disease outbreak among large numbers of people over a large area. If the outbreak is a result of a pathogen previously unrecognized (or recognized from sporadic incidences), the chance of its being isolated and identified is much higher. An outbreak affecting 220 people from the consumption of chocolate milk produced by a processor led to the examination for other possible suspects, along with those routinely tested, and resulted in the identification of *Yer. enterocolitica* as a new foodborne pathogen.

For faster production, equipment may be designed without much prior consideration of possible microbiological problems, particularly with foodborne pathogens. An incident of listeriosis from the consumption of hot dogs contaminated with *Lis. monocytogenes* is an example. *Lis. monocytogenes* strains were isolated from many types of foods, including ready-to-eat hot dogs and other processed meat products. Epidemiological investigations have also suspected that sporadic listeriosis in humans could be caused by the consumption of foods contaminated with *Lis. monocytogenes*, including hot dogs. In 1989, for the first time in the United States, a case of human listeriosis was linked to eating ready-to-eat hot dogs because the same serotype (1/2a) of *Lis. monocytogenes* was isolated from the person who ate the hot dogs, from the remaining refrigerated hot dogs in the opened package, and from hot dogs from unopened retail packages produced in the same processing facility. Subsequently, regulatory agencies tested products, line samples, and environmental samples from processing facilities to determine the sources of *Lis. monocytogenes* contamination (Table 29.6). Of the seven retail samples from seven lots produced during a 35-day period, six had *Lis. monocytogenes* 1/2a serotype. Among the line samples collected at different phases of production, *Lis. monocytogenes* 1/2a was found in high frequency in products following peeling and before packaging. (Following peeling, the products traveled via the conveyor belt before going for packaging.) Among the environmental samples (swabs) tested, the same serotype was isolated from the cooler floor but, more importantly, from the conveyor belt that the heat-treated products came in contact with following peeling. From the analysis of the data, the conveyor belt, attached to the peeler, was suspected to be the main source of product contamination. The porous conveyor belt in use at this processing plant is difficult to clean (see Chapter 22). In designing conveyor belts, the possibility of microbiological problems needs to be considered. Use of conveyors that do not have porous belts or large numbers of small jointed segments (such as small links) may help to efficiently sanitize and control microbiological problems.[8]

## Miscellaneous Factors

### Genetic Exchange

Several other factors may have contributed to the emergence of new pathogens in foodborne disease outbreaks.[9] Some of these remain speculative, whereas others have been confirmed. One of the

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**Table 29.6 Isolation of *Listeria monocytogenes* Serotype 1/2a from the Product of Environmental Samples in a Processing Plant[8]**

| *Samples Tested* | *No. Positive/No. Tested* |
|---|---|
| Packaged products[a]: retail | 21/42 |
| **Line Samples during Product Manufacture** | |
| During stuffing | 0/8 |
| After cooking | 1/12 |
| After cooling | 0/13 |
| After peeling and before packaging | 15/21 |
| **Environmental Samples (Swabs)** | |
| Cooking room | 0/3 |
| Cooler floor | 1/1 |
| Cooler rack and elements | 0/2 |
| Peeler area | 0/3 |
| Conveyor belt | 1/1 |
| Other equipment | 0/6 |

[a] The products were cooked at an internal temperature of 75°C for 2 to 3 hours. *Lis. monocytogenes* is not expected to survive if the schedule is followed properly. Contamination occurred following heating and peeling and before packaging.

possible reasons for the increase in foodborne gastroenteritis by pathogenic *Esc. coli* strains is the ability of pathogenic Enterobacteriaceae to transfer plasmids among themselves that encode for toxin production and colonization in the digestive tract. Such a plasmid transfer in the environment from a pathogenic strain into a nonpathogenic strain will enable the new variant strain to establish in the intestine and produce illness, and a food can be a vehicle through which the pathogen can be consumed. Bacteriophage encoded virulence factors, such as Shiga-toxin (Stx), can be transferred from Shiga-toxigenic *Esc. coli* to other *Esc. coli*. For example, the 2011 outbreak in Germany caused by *Esc. coli* O104:H4 is an enteroaggregative *Esc. coli* that has possibly acquired bacteriopophage encoded *stx* gene and became a highly virulent strain. (See *Esc. coli* O104:H4 description below).

## *Antibiotic Resistance[10]*

There are some concerns that the use of unrestricted antibiotics in animals for therapeutic and growth-promoting purposes has provided the antibiotic-resistant pathogens with a better chance of competing with the sensitive microorganisms and establishing themselves in the environment. In the absence of competition, these pathogens can predominate and, through contamination of a food, emerge as new foodborne pathogens. Such an assumption was made against some sanitary practices used in the processing and handling of foods. Some of the practices are designed to overcome problems from

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pathogenic and spoilage bacteria traditionally suspected to be present in a food. Elimination of these predominant bacteria may allow minor as well as poorly competing bacteria to become significant in the absence of competition and subsequently cause problems. A 2011 outbreak of *Salmonella* in Heidelberg with ground turkey meat made 136 persons sick. This organism is resistant to several common antibiotics; thus treatment failure in patients resulted in increased hospitalizations.

### Stress Adaptation

In recent years, consumption of several low-pH foods, such as orange and apple juices, and fermented sausages, has been implicated in foodborne diseases caused by *Esc. coli* O157:H7, *Salmonella*, and *Lis. monocytogenes*. These pathogens are normally sensitive to low pH (pH ≤ 4.5) and are expected to die off rapidly during storage at refrigerated temperatures. Studies showed that the pathogens isolated from these foods are acid-resistant variants and are thus able to survive well in a low-pH product (see Chapter 10). A stress response can enable pathogens to survive in food through many processing conditions and make the food potentially hazardous.

### Farming Practices

There is also some speculation that present methods of raising and feeding food animals and birds may have given some pathogens a better chance to become established as carriers in the animals and birds. Food from these sources has a greater chance of being contaminated with these pathogens. The occurrence of *Esc. coli* O157:H7 in hamburger meat prepared from culled dairy cows, spinach, sprouts and the high incidence of *Salmonella* Enteritidis in eggs and chickens could be a result, in part, of the way they are raised now. In 2006, an outbreak of *Esc. coli* O157:H7 involving about 200 cases with three mortalities was linked to the consumption of spinach. It was speculated that the irrigation water was the likely source that was contaminated with cattle manure indicating the requirement for improved agricultural practices to prevent such outbreaks. Modern farming practices may be responsible for a *Lis. monocytogenes* outbreak with cantaloupe in 2011, which resulted in 139 cases, 29 deaths, and one miscarriage. A *Salmonella* Enteritidis outbreak with eggs in 2010 resulted in 1900 cases.

### Aging and Immunocompromised Population

Finally, people who are immunocompromised can easily become ill from consuming a food contaminated with a pathogen even at a low dose level. For example, *Lis. monocytogenes* is fatal or can cause severe infection in immunocompromised people, neonates, the elderly, and pregnant women. People with normal resistance will have no problem with the same food. This is particularly true for opportunistic pathogens. Some, such as *Aer. hydrophila* and *Ple. shigelloides*, are currently implicated indirectly as possible foodborne pathogens. In the future, people with low immunity may be found to become ill from consuming a food contaminated with an opportunistic pathogen, which then will become a new foodborne pathogen.

## Other Emerging Foodborne Pathogens of Concern

There are several more pathogens[9,11] that may cause a widespread problem in the future, such as *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*), Hepatitis E virus, Shiga-toxin and

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non-Shiga toxin producing *Escherichia coli*, *Helicobacter pylori*, *Aeromonas* species (Chapter 29), nematode *Gnathostoma*, bovine spongiform encephalopathy (BSE) (Chapter 29), and Nipah virus, some of which are discussed briefly.

## Cronobacter sakazaki[12–14]

*Cronobacter sakazakii* (formerly *Enterobacter sakazakii*) is considered an emerging opportunistic pathogen that primarily infects infants, but now it has been implicated to cause disease in adults. *Cro. sakazaki* is a gram-negative, facultative anaerobic, nonsporulating, motile rod-shaped bacterium and is the member of the Enterobacteriaceae family. Six species of *Cronobacter* have been identified. *Cro. sakazaki* produces a yellow pigmented colony on a tryptic soy agar (TSA) plate and can grow at a temperature range of 6°C–45°C with an optimum temperature of 37°C–43°C. In general, these organisms are thermotolerant and can adapt well to osmotic desiccation stress. They can survive in infant cereal with an $A_W$ range of 0.30–0.83 for up to 12 months. *Cronobacter* has been associated with a number of outbreaks involving powdered infant formula (PIF). *Cronobacter* spp. has also been isolated from varieties of foods, including rice, bread, milk, cheese, dried foods, spices, tea, meats, water, and vegetables. These organisms are also found in food factories, including a powdered infant formula production facility and livestock facilities.

The pathogenesis of *Cro. sakazaki* is not fully understood; however, the bacterium is known to translocate from the gastrointestinal tract to the blood where it multiplies in large numbers and then crosses the blood brain barrier to reach to cerebrospinal fluid. In infants, it causes bacteremia, necrotizing enterocolitis, neonatal meningitis, and memingoencephalitis. The mortality rate in infants is 50%–80%. Recently, it has been shown that adults, especially the immunocompromised adults and the elderly are susceptible to this infection, showing clinical symptoms of pneumonia, sepsis, foot ulcers, wound infections, and osteomyelitis. For isolation of this pathogen, FDA and ISO methods are used that involve preenrichment and/or selective enrichment before plating on TSA or chromogenic media. Yellow-pigmented colonies are further confirmed by biochemical testing and by PCR methods. Various control strategies have been proposed that include, strict hygienic practices during PIF production, monitoring of the processing environments, raw materials, and final product testing. Thermal treatment, including rehydration of PIF with water at 70°C or higher, can reduce counts by 4 logs. In addition, irradiation, bacteriophages, bacteriocins, and plant phenolics have been proposed for inactivation of *Cronobacter* spp. in foods.

## Clostridium difficile[15,16]

*Clostridium difficile* is a gram-positive, rod-shaped, spore-forming obligate anaerobe. It is traditionally considered a nosocomial, hospital-acquired human pathogen and is responsible for antibiotic associated diarrhea (AAD). It was first reported in 1978 by John Bartlett and his colleagues. Since 2003, *Clo. difficile* infection (CDI) cases have risen dramatically and caused numerous fatalities in both North America and Europe. In 2007, there were 3875 deaths reported in the United States. *Clo. difficile* colonizes human and animal intestines and has been isolated from many domestic and wild animals: pigs, calves, poultry, horses, donkeys, dogs, cats, seals, snakes, rabbits, and ostrich. This organism has been isolated primarily from ground meats (pork, beef, and poultry) at a prevalence rate of 3%–12% or higher. It has been also isolated less frequently from raw vegetables (cucumber, onions, radishes, carrots, mushrooms, and ready-to-eat salads) and milk. The ubiquitous nature of this organism (resulting from spore formation), fecal contamination of the soil, and its presence in food processing and the packaging environment are thought to be responsible for

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food contamination. *Clo. difficile* association with meat animals and routine isolation of this organism from meats provide a strong evidence for its involvement as a foodborne pathogen.

*Clo. difficile* produces two toxins; Toxin A and Toxin B. Toxin A is an enterotoxin and causes diarrhea, and Toxin B induces cytotoxicity and damages the mucus membrane. The clinical symptoms include mild self-limiting diarrhea, cramps, and abdominal pain to life-threatening pseudomembranous colitis, megacolon, and intestinal perforation. Enzyme immunoassay specific for toxins and PCR methods are used as diagnosis and detection tools. For food-sample testing, culture methods employing suitable enrichment broth and solid agar media supplemented with antibiotics, cefoxitin, and cycloserine or moxalactam and norfloxacin are used for pathogen isolation.

## Escherichia coli *O104:H4*[17]

A Shiga toxin-producing *Escherichia coli* (STEC) serovar O104:H4 caused the largest outbreak in Germany, France, and other countries in 2011 resulting from consumption of fenugreek sprouts imported from Egypt. A total of 3842 cases were reported, of which 2987 suffered from gastroenteritis (bloody diarrhea) with 18 deaths, and 855 people exhibited hemolytic uremic syndrome (HUS) with severe kidney damage and 35 deaths. These symptoms are characteristics of enterohemorrhagic *Escherichia coli* (EHEC) that produce Shiga-toxin (Stx) and the prototype serovar is *Esc. coli* O157:H7 (see Chapter 26). However, the serovars O104:H4 is a member of enteroaggregative *Esc. coli* (EAEC), which expresses aggregative adherence fimbriae (AAF) required for attachment, but most serovars in this group do not produce Stx. The isolate O104:H4 found in this outbreak is a Shiga toxin–producing EAEC that has acquired the *stx* gene by a bacteriophage and has been considered a newly evolved EAEC. In addition, this bacterium contained several virulence genes that were encoded in the phage (prophage state) and in a plasmid (pAA). Whole genome sequencing of this bacterium further revealed that it contains a colonization factor and virulence genes that are common in both EHEC and EAEC, resulting in a highly virulent pathogen. The pathogenesis of this *Esc. coli* O104:H4 was thought to be a result of increased adhesion, colonization, and damage of intestinal epithelial cells followed by increased dissemination of Stx to blood circulation and the kidney. This may explain why high numbers of HUS patients were associated with this outbreak. This epidemic O104:H4 strain was sensitive to antibiotics carbapenems, which were recommended for treatment but were resistant to all penicillin and cephalosporin antibiotics.

## *Hepatitis E Virus*[18]

Hepatitis E virus (HEV), a RNA virus, is responsible for acute viral hepatitis exhibiting a mild jaundice. The high-risk group of people who are most susceptible to HEV infection include patients with chronic liver disease, pregnant women, and young children. HEV is a member of *Hepeviridae* family, and there are four genotypes; genotype 1 and 2 are associated with human infection, and genotype 3 and 4 are associated with humans, pigs, birds, and other species. The incubation period for this disease is about 60 days. It is primarily a waterborne virus and caused several outbreaks in developing countries in Asia, Latin America, and Africa. Travelers may carry the virus from endemic zones to different parts of the world. Sporadic outbreaks are reported in the developed countries including the United States. Though it is a waterborne virus, several recent outbreaks were associated with consumption of deer meat and raw or undercooked swine liver in Japan. It is now considered a zoonotic disease. Furthermore, HEV has been routinely isolated from swine from Canada, Korea, Japan, Spain, the Netherlands, New Zealand, and the United States, implying potential future outbreak of HEV with the consumption of undercooked pork meat.

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## *Nipah Virus*[19,20]

The first Nipah virus (NiV) outbreak was reported in Malaysia in 1998–1999, which affected 276 people with a 39% fatality rate, and the infection was transmitted through sick pigs. The NiV was amplified at large numbers in the respiratory tracts and facilitated the spread of infection of farm workers. NiV is a RNA virus and is a member of genus *Henipavirus* within the family of *Paramyxoviridae*. It was originally isolated from a patient from the Sungai Nipah Village in Malaysia, and fruit bats serve as a natural reservoir. Interaction of fruit bats with swine and humans led to increased numbers of outbreaks in Malaysia. Bats transmit viruses through saliva or urine to the fruits, and pig farms located near the bat habitat acquire the organisms and aid in zoonotic transmission of the disease (Figure 29.1). Domestic animals foraging may eat virus-laden, partially eaten fruits and may transmit the disease to humans. NiV is also transmitted through the sap of date palm trees when fruit bats feed on sap at night, and this led to numerous epidemic and sporadic outbreaks in Bangladesh and parts of India. Date palm tree sap (juice) is used for making molasses. The virus survives well in the sap, and unheated sap will transmit the virus to humans. Person-to-person transmission also occurs. A Nipah virus outbreak was also reported in Cambodia and Thailand.

The virus causes high fever and encephalitis and may also cause acute respiratory tract infection. The mortality rate is very high (75%). Culling of infected swine helped reduce NiV cases in Malaysia. Avoiding unprocessed sap will also help prevent viral infection. Serologic testing for antibody titer in human sera and the reverse transcriptase PCR assay have been used for diagnosis and detection from human urine, cerebrospinal fluid, and oral swabs.

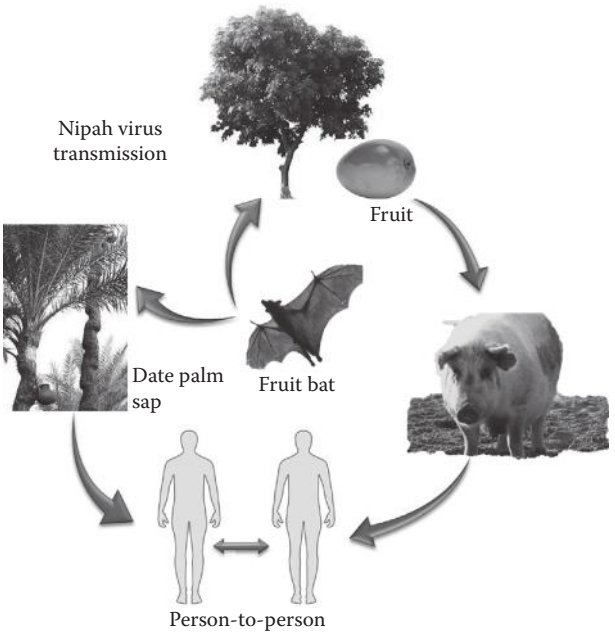



**Figure 29.1 Nipah virus transmission pathway.**

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## Conclusion

The designation of new and emerging foodborne pathogen may be inappropriate because many of the so-called new pathogens have been contaminating foods for a long time. For one reason or another, their presence in food has not been examined. Several socioeconomic patterns and lifestyles of consumers have increased the chances of such pathogens being involved in foodborne outbreaks. Scientific understanding and advancement have helped to recognize these chances. In the future, more pathogens, especially viruses, will be in this "new" group, and foods will have to be tested for them. Concerns of outbreaks with fresh produce and fruits need greater attention because these products are minimally processed, and they present greater opportunity for emergence of pathogens including protozoan parasites.

### QUESTIONS

1. Define a new foodborne pathogen.
2. List the new foodborne pathogens that have emerged in the United States in the past decade.
3. List the possible factors that can help identify a new foodborne pathogen.
4. Discuss the factors that could be involved in the emergence of a new foodborne pathogen.
5. Discuss how international travel could be a factor in the emergence of a new foodborne pathogen. Give an example.
6. Discuss how new technology can be a factor in increasing the chances of identifying a new foodborne pathogen. Give an example.
7. Why are some pathogens that are recognized as major causes of foodborne illnesses in one country no longer found to be the same in another country? Explain with appropriate examples.
8. "Some opportunistic pathogens of today may be confirmed as new foodborne pathogens of tomorrow." Justify the statement with an example.
9. Describe how Nipah virus is transmitted to humans.
10. What is the major source of *Cronobacter sakazaki* and what are typical symptoms associated with this disease?
11. What genetic properties made the *Esc. coli* O104:H4 serovar highly virulent?

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# Chapter 30

# Indicators of Bacterial Pathogens

## Introduction

All pathogenic microorganisms implicated in foodborne diseases are considered enteric pathogens except *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium botulinum* (except in the case of infant botulism), *Clo. perfringens*, and toxicogenic molds. This means they can survive and multiply or establish in the gastrointestinal (GI) tract of humans, food animals, and birds. A food contaminated directly or indirectly with fecal materials from these sources may theoretically contain one or more of these pathogens and can thus be potentially hazardous to consumers. To implement regulatory requirements and ensure consumer safety, it is necessary to know that a food is either free of some enteric pathogens, such as *Salmonella* serovars and *Escherichia coli* O157:H7 or contains low levels of some other enteric pathogens, such as *Yersinia enterocolitica* and *Vibrio parahaemolyticus*. The procedures used to isolate and confirm a pathogen from a food involve several steps, take a relatively long time, and are costly. Some of the new tests involving molecular biology techniques require high initial investment and highly skilled technicians. In a modernized, large commercial operation, involving procurement of ingredients from many countries, processing of different products, warehousing, distribution over a large area, and retail marketing, it is not practical or economical to test the required number of product samples from each batch for all the pathogens or even those that are suspected of being present in a particular product. Instead, food samples are examined for the number (or level) of groups or a species of bacteria that are of fecal (enteric) origin, usually present in a higher density than pathogens but usually considered to be nonpathogenic. Their presence is viewed as resulting from direct or indirect contamination of a food with fecal materials and indicates the possible presence of enteric pathogens in the food. These bacterial groups or species are termed indicators of enteric pathogens. Although *Sta. aureus*, *Clo. botulinum*, *Clo. perfringens*, and *Bac. cereus* can be present in the fecal matter of humans and food animals, they, along with toxigenic molds, are not considered classical enteric pathogens. Their presence in a food is not normally considered to be due to because of fecal contamination, and the indicators of enteric pathogens are not very effective for the purpose. To determine the presence of these microorganisms and their



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toxins, specific methods are recommended for their detection and identification. The total aerobic plate count (TAPC), also known as mesophilic aerobic bacteria (MAB) or standard plate count (SPC), is not an indicator of the possible presence of pathogens. Instead, it is an indicator of the microbiological quality of a food as well as a measure of the level of sanitation used in the handling (processing and storage) of a food, such as cooked, ready-to-eat foods, pasteurized milk, and spices.

## Criteria for Ideal Indicators

Two types of microbiological indicator assays are used: index organisms and indicator organisms.[1,2] An index organism is an organism, a group of organisms, or their metabolic byproducts that is predictive of the presence of a pathogen. However, this system is not as effective as an indicator organism, which can indicate the presence of a pathogen. Several criteria were suggested for selecting a bacterial group or species as an indicator of enteric foodborne pathogens.[1–3] Some are listed with brief explanations:

1. The indicator should preferably contain a single species or a few species with some common and identifiable biochemical and other characteristics in order to be able to identify them from the many different types of microorganisms that might be present in a food.
2. The indicator should be of enteric origin, that is, it should share the same habitat as the enteric pathogens and should be present when and where the pathogens are likely to be present.
3. The indicator should be nonpathogenic so that its handling in the laboratory does not require safety precautions as required for pathogens.
4. The indicator should be present in the fecal matter in much higher numbers than the enteric pathogens so that they can be easily detected (enumerated or isolated) even when a food is contaminated with small amounts of fecal matter.
5. The indicator should be detected (enumerated or isolated) and identified within a short time, easily, and economically, so that a product, following processing, can be distributed quickly, and several samples from a batch can be tested.
6. The indicator should be detected by using one or more newly developed molecular biology techniques for rapid identification.
7. The indicator should be detected (enumerated or isolated) even in the presence of large numbers of associated microorganisms, which can be achieved by using compounds that inhibit growth of associated microorganisms but not of the indicator.
8. The indicator should have a growth and survival rate in a food the same as that of the enteric pathogens. It should not grow more slowly or die off faster than the pathogens in a food. If it dies off more rapidly than the pathogen, then, theoretically, a food can be free of the indicator during storage but can still have pathogens.
9. The indicator should not suffer sublethal injury more (in degree) than the pathogens do when exposed to physical and chemical stresses. If the indicator is more susceptible to sublethal stresses, it will not be detected by the selective methods used in the enumeration, and a food may show no or very low acceptable levels of the indicator even when the pathogens are present at higher levels.
10. The indicator should preferably be present when the pathogens are present in a food; conversely, it should be absent when the enteric pathogens are absent. Unless such correlations exist, the importance of an indicator to indicate the possible presence of a pathogen in a food reduces greatly.

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11. There should preferably be a direct relationship between the level of an indicator present and the probability of the presence of an enteric pathogen in a food. This will help set up regulatory standards or specifications for an indicator limit for the acceptance or rejection of a food for consumption. For this criterion, it is very important to recognize whether the high numbers of an indicator in a food have resulted from a high level of initial contamination (and a greater chance for the presence of a pathogen) or from their growth in the food from a very low initial contamination (in which case a pathogen may not be present even when the indicator is present in high numbers).

It is apparent that no single bacterial group or species will be able to meet all the criteria of an ideal indicator. Several bacterial groups or species satisfy many of these criteria. The characteristics, advantages, and disadvantages of some of the important and accepted indicator bacterial groups and species (of enteric pathogens) are described here.[1–3]

## Coliform Group

### *Coliforms*

In the coliform group, coliforms, fecal coliforms, and *Esc. coli* are discussed. They are not separate, as both fecal coliforms (mostly *Esc. coli*) and *Esc. coli* belong to coliforms. Depending on the situation, food, water, and environmental samples are examined for one or more of the three.

#### *Organisms and Sources*

The term "coliform" does not have taxonomic value; rather, it represents a group of species from several genera, namely, *Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter*, and probably *Aeromonas* and *Serratia*. The main reason for grouping them together is their many common characteristics. They are all gram-negative, non-spore-forming rods; many are motile, are facultative anaerobes resistant to many surface-active agents, and ferment lactose to produce acid and gas within 48 hours at 32°C or 35°C. Some species can grow at higher temperatures (44.5°C), whereas others can grow at 4°C–5°C. All are able to grow in foods except in those that are at pH ≤ 4.0 (a few that are acid resistant can grow or survive) and $A_W \leq 0.92$. All are sensitive to low-heat treatments and are killed by pasteurization.

They can be present in the feces of humans and warm-blooded animals and birds. Some can be present in the environment and contaminate food. Thus, some *Klebsiella* spp. and *Enterobacter* spp. are found in soil, where they can multiply and reach high population levels. Some are found in water and plants.[1,4–6]

#### *Occurrence and Significance in Food*

Coliforms are expected to be present in many raw foods and food ingredients of animal and plant origin. In some plant foods, they are present in very high numbers because of contamination from soil. Because they can grow in foods, some even at refrigerated temperatures, a low initial number can reach a high level during storage. The occurrence of some coliforms of nonfecal origin and their ability to grow in many foods reduce the specificity of coliforms as an indicator of fecal contamination in raw foods. In contrast, in heat-processed (pasteurized) products, their presence is

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considered post-heat-treatment contamination from improper sanitation. In heat-processed foods, their presence (even in small numbers) is viewed with caution. Thus, in heat-processed foods, their specificity as an indicator is considered favorably (more as an indicator of improper sanitation than fecal contamination). Several selective media have been recommended to determine coliform numbers in food samples. These are selective-differential media, and they differ greatly in their recovery ability of coliforms (Chapter 42). The results are based on the ability of most coliforms to ferment lactose and produce gas and are available in one to two days. The presence of sublethally stressed or injured cells can considerably reduce the recovery in selective media. Several other factors, such as high temperature of melted agar media in pour plating, high acidity of a food, and presence of lysozymes (egg-based products) in a food, can further reduce the enumeration of stressed cells. Modified detection methods have been developed to recover injured coliforms (see Chapters 10 and 42). Even with some disadvantages, coliforms are probably the most useful and most extensively used indicators.[1,4–7]

## *Fecal Coliforms*

### *Organisms and Sources*

Fecal coliform bacteria also constitute a group of bacteria and include those coliforms whose specificity as fecal contaminants is much higher than that of coliforms. This group includes mostly *Esc. coli*, along with some *Klebsiella* and *Enterobacter* spp. Nonfecal coliforms are eliminated by using a high incubation temperature (44.5°C ± 0.2°C or 45.0°C ± 0.2°C) for 24 hours in selective broths containing lactose. Lactose fermentation, with the production of gas, is considered a presumptive positive test.[1,7]

### *Occurrence and Significance in Food*

Some fecal coliforms are present in raw foods of animal origin. They can be present in plant foods from contaminated soil and water. High numbers can be a result of either gross contamination or growth from a low initial level, probably because of improper storage temperature. Their presence in heat-processed (pasteurized) foods is probably because of improper sanitation after heat treatment.

In raw foods that are to be given heat treatment, their presence, even in high numbers (~$10^3$/g or mL) is not viewed gravely; if the numbers go higher, some importance is given to contamination of fecal matter, improper sanitation, and possible presence of enteric pathogens. A need for corrective measures becomes important. In contrast, in heated products and ready-to-eat products (even raw), their presence, especially above a certain level, is viewed cautiously for possible fecal contamination and presence of enteric pathogens. A food can be accepted or rejected based on the numbers present. This group is extensively used as an indicator in foods of marine origin (shellfish) and in water and wastewater.[1,7]

## Escherichia coli

### *Organisms and Sources*

In contrast to either coliforms or fecal coliforms, *Esc. coli* has a taxonomic basis.[1] It includes only the *Escherichia* spp. of the coliform and fecal coliform groups. *Esc. coli* strains conform to the general characteristics described for coliform groups. Biochemically, they are differentiated from

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other coliforms by the indole production from tryptone, methyl red reduction resulting from acid production (red coloration), Voges–Proskauer reaction (production of acetyl-methyl carbinol from glucose), and citrate utilization as a C-source (IMViC) reaction patterns. *Esc. coli* Type I and Type II give IMViC reaction patterns, respectively, of + + – – and – + – –. The – + – – reaction pattern of *Esc. coli* Type II could also be a result of slow or low production of indole from tryptone (or peptone). The IMViC tests are conducted with an isolate obtained after testing a food sample for the coliform group or fecal coliform group. However, there is a concern now about the adequacy of these reaction patterns to identify *Esc. coli* types.

Initially, *Esc. coli* types were used as indicators of fecal contamination and the possible presence of enteric pathogens (in food) with the considerations that they are nonpathogenic and occur normally in the GI tract of humans, animals, and birds in high numbers. However, it is now known that some variants and strains of *Esc. coli* are pathogenic (e.g., *Esc. coli* O157:H7). None of the methods mentioned previously are able to differentiate pathogenic from nonpathogenic *Esc. coli* strains; this can be achieved only through specific tests designed to identify different pathogenic *Esc. coli* strains. This discussion emphasizes the value of *Esc. coli* as an indicator.[1] The significance and importance of pathogenic *Esc. coli* have been discussed previously (Chapters 26 and 27).

### *Occurrence and Significance in Food*

*Esc. coli* is present in the lower intestinal tract of humans and warm-blooded animals and birds. Its presence in raw foods is considered an indication of direct or indirect fecal contamination. Direct fecal contamination occurs during the processing of raw foods of animal origin and because of poor personal hygiene of food handlers. Indirect contamination can occur through sewage and polluted water. In heat-processed (pasteurized) foods, its presence is viewed with great concern. Its value as an indicator of fecal contamination and the possible presence of enteric pathogens is much greater than that of coliform and fecal coliform groups. However, the time to complete the tests (IMViC) is relatively long (ca. five days). Some direct-plating methods have been developed that give an indication of *Esc. coli* in a shorter time. There are several other inadequacies of *Esc. coli* as an indicator. *Esc. coli* strains may die at a faster rate in dried, frozen, and low-pH products than some enteric pathogens, and some enteric pathogens can grow at low temperatures (0°C–2°C) at which *Esc. coli* strains can die. In addition, *Esc. coli* strains can be injured by sublethal stresses in higher degrees than some enteric pathogens and may not be effectively detected by the recommended selective media unless a prior resuscitation (repair) step is included.

## *Enterobacteriaceae* Group

The methods recommended for detecting coliforms, fecal coliforms, and *Esc. coli* are based on the ability of these bacterial species to ferment lactose to produce gas and acid. In contrast, some enteric pathogens do not ferment lactose, such as most *Salmonella* serovars. Thus, instead of only enumerating coliforms or fecal coliforms in a food, enumeration of all the genera and species in the *Enterobacteriaceae*[1] family is advocated. Because this family includes not only coliforms but also many genera and species that are enteric pathogens, enumeration of the whole group can be a better indicator of the level of sanitation, possible fecal contamination, and possible presence of enteric pathogens (Table 30.1). In European countries, this concept has been used to a certain degree. The method includes the enumeration of organisms from colony-forming units in a selective-differential agar medium containing glucose instead of lactose.[1]

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**Table 30.1 Genera, Habitat, and Association to Foodborne Illnesses of the *Enterobacteriaceae***

| *Genera*[a] | *Main Habitat* | *Associated with Foodborne Illness*[b] |
|---|---|---|
| *Escherichia* | Lower intestine of humans and warm-blooded animals and birds | Only the pathogenic strains |
| *Shigella* | Intestine of humans and primates | All species |
| *Salmonella* | Intestine of humans, animals, birds, and insects | All are considered pathogenic |
| *Citrobacter* | Intestine of humans, animals, birds; also soil, water, and sewage | Can be opportunistic |
| *Klebsiella* | Intestine of humans, animals, birds; also soil, water, and grain | Can be opportunistic |
| *Enterobacter* | Intestines of humans, animals, birds; widely distributed in nature, mostly plants | Can be opportunistic |
| *Erwinia* | Mostly in plants | No association |
| *Serratia* | Soil, water, plants, and rodents | Can be opportunistic |
| *Hafnia* | Intestines of humans, animals, birds; also soil, water, and sewage | No association |
| *Edwardsiella* | Cold-blooded animals and water | No association |
| *Proteus* | Intestines of humans, animals, birds; also soil and polluted water | Can be opportunistic |
| *Providencia* | Intestines of humans and animals | Can be opportunistic |
| *Morganella* | Intestines of humans, animals, and reptiles | Can be opportunistic |
| *Yersinia* | Intestines of humans and animals; also environment | Some species or strains are pathogenic |
| *Obesumbacterium* | Brewery contaminant | No association |
| *Xenorhabdus* | Nematodes | No association |
| *Kluyvera* | Soil, sewage, and water | Can be opportunistic |
| *Rahnella* | Fresh water | No association |
| *Tatumella* | Human respiratory tract | No association |

[a] Some are of nonfecal origin.

[b] The pathogenic species and strains in the indicated genera are confirmed with foodborne and waterborne illnesses. Some species and strains in some genera, indicated as opportunistic, are suspected of being foodborne pathogens.

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This concept is criticized because many species in the *Enterobacteriaceae* are not of fecal origin; many are found naturally in the environment, including plants, and those that form typical colonies because of glucose fermentation in the selective medium do not all belong to this family. However, in heat-processed foods (all are sensitive to pasteurization) and ready-to-eat foods, their presence in high numbers should have public health significance.

## *Enterococcus* Group

### *Characteristics and Habitat*

The genus *Enterococcus* is relatively new and includes many species that were previously grouped as fecal streptococci and other streptococci.[7–10] They are gram-positive, non-spore-forming, nonmotile cocci or coccobacilli, catalase negative, and facultative anaerobes. They can grow between 10°C and 45°C, and some species can grow at 50°C. Some require B vitamins and amino acids for growth. Some can survive pasteurization temperatures. In general, they are more resistant than most coliforms to refrigeration, freezing, drying, low pH, NaCl, and water. They are found in the intestinal tracts of humans and warm- and cold-blooded animals, birds, and insects. Some can be species specific, whereas others can be present in humans, warm-blooded animals, and birds. Among the currently recognized species, several are found in the intestines of humans and food animals and birds, including *Enterococcus faecalis*, *Ent. faecium*, *Ent. durans*, *Ent. gallinarum*, *Ent. avium*, and *Ent. hirae*. Many have been found in vegetation, processing equipment, and processing environments. Once established, they can continue to multiply in the equipment and environment and are often difficult to completely remove. They are found in sewage and water, especially polluted water and mud. They probably do not multiply in water but can survive longer than many coliforms. They can grow in most foods.

### *Occurrence and Significance in Food*

*Enterococcus* can enter different foods through fecal contamination or through water, vegetation, or equipment and processing environments and may not be of fecal origin. In this respect, its value as an indicator of fecal contamination and possible presence of enteric pathogens in food is questionable. Also, the ability of some strains to survive pasteurization temperatures (being thermodurics) reduces their value as an indicator. On the other hand, their better survivability in dried, frozen, refrigerated, and low-pH foods and water can make them favorable as indicators. Currently, their presence in high numbers, especially in heat-processed (pasteurized) foods, can be used to indicate their possible presence in high numbers in raw materials and improper sanitation of the processing equipment and environment. They have been used to determine the sanitary quality of water in shellfish beds and are considered to be better as indicators than coliforms for shellfish. Some strains have also been associated with foodborne gastroenteritis, probably as opportunistic pathogens.[8–11]

## Miscellaneous Indicator Organisms[2]

1. *Staphylococcus aureus* has been used to indicate the extent of human handling in such matrices as foods from food service establishments and fermented meat and dairy products (fermentation failures).

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2. *Listeria* species have been used to indicate the effectiveness of sanitation programs in food production facilities for ready-to-eat refrigerated products.
3. *Salmonella enterica* has been used to indicate control of enteric bacteria in raw meat and poultry.
4. Coliphages have been used to indicate the effectiveness of enteric virus control in water and shellfish.
5. The thermophilic bacilli (such as *Anoxybacillus flavithermus* and *Geobacillus* spp.) have been used to indicate poor hygienic practices during dairy processing. Growth of these indicators in milk can also cause product defect and off flavor.[12]

## Conclusion

In food microbiology, the concept of indicator bacteria was introduced to measure the sanitary quality of pasteurized foods (e.g., milk). Its purpose was to indicate the possible presence of enteric pathogens (e.g., *Salmonella*) in the food. Over time, the suitability of several bacterial groups has been considered and this aspect has been discussed in this chapter. It is apparent that selection of a suitable indicator and its level may change with food types and for raw, processed, and ready-to-eat foods.

### QUESTIONS

1. Discuss the need of using indicator bacteria for enteric pathogens in food.
2. List, with brief explanations, six criteria that should be considered in selecting an indicator of enteric pathogens.
3. Which foodborne pathogens are generally not considered enteric? Why? What could be done to determine their possible presence in a food?
4. What are some similarities and differences in characteristics between coliforms and fecal coliforms? What is the significance of their presence in high numbers ($\geq 10^4$/g or /mL) and low numbers ($\leq 10^1$/g or mL) in a raw food, in a pasteurized food, and in a ready-to-eat food?
5. Why are fecal coliforms considered a better indicator of enteric pathogens than are coliforms?
6. What are the advantages and disadvantages of using *Esc. coli* as an indicator of enteric pathogens in food? "An indicator should be present when an enteric pathogen is present in a food." Why might this statement not be valid for *Esc. coli* as an indicator?
7. List the conditions under which coliforms, fecal coliforms, and *Esc. coli* have questionable values as indicators of enteric pathogens.
8. What are the justifications of using the Enterobacteriaceae family as indicators? What are the disadvantages of using this group as an indicator of enteric pathogens?
9. List some characteristics of enterococci that put them in an advantageous position over coliforms as indicators of enteric pathogens. What are the disadvantages of using enterococci as indicators?
10. Oyster-harvesting waterbeds in rivers are often closed because of high levels of indicator bacteria. Explain the advantages and disadvantages of using fecal coliforms and enterococci as sanitary indicators in water samples, mud, and oyster meat.

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# VI CONTROL OF MICROORGANISMS IN FOODS

It is apparent from the materials presented in the previous chapters that although some microorganisms are desirable for the production of bioprocessed food, many are undesirable because they can cause food spoilage and foodborne diseases. For efficient production of bioprocessed food, the objectives are to stimulate growth and increase the viability of desirable microorganisms.

In contrast, with respect to spoilage and pathogenic microorganisms, the objective is to minimize their numbers or completely eliminate them from food. Several methods, individually or in combination, are used to achieve these goals by (1) controlling access of the microorganisms in foods, (2) physically removing the microorganisms present in foods, (3) preventing or reducing the growth of microorganisms and germination of spores present in foods, and (4) killing microbial cells and spores present in foods.

The influence of intrinsic and extrinsic factors necessary for optimum microbial growth and the range in which each factor supports microbial growth have been discussed in Chapter 6. To control growth and kill microorganisms in a food, some of these factors are used, but only beyond the range that supports growth and induces germination of spores but can induce injury and death (Chapter 10). Some of these controlling methods are discussed here.

Irrespective of the methods used, it is important to recognize that a control method is more effective when a food has fewer microbial cells and when the cells are in the exponential growth phase and are injured. Also, spores are more resistant than vegetative cells, and gram-negative cells are generally more susceptible than gram-positive cells to many control methods. Finally, bacteria, yeasts, molds, bacteriophages, and viruses differ in sensitivity to the methods used to control them. Different species and strains of the same species also differ in sensitivity to these control methods.

The following control or preservation methods are discussed in this section:

Chapter 31: Control of Access of Microorganisms: Cleaning, Sanitation, and Disinfection
Chapter 32: Control by Physical Removal

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Chapter 33: Control by Heat (Thermal Processing)
Chapter 34: Control by Low Temperature
Chapter 35: Control by Reduced Water Activity and Drying
Chapter 36: Control by Low pH and Organic Acids
Chapter 37: Control by Modified Atmosphere (or Reducing O-R Potential)
Chapter 38: Control by Antimicrobial Preservatives and Bacteriophages
Chapter 39: Control by Irradiation
Chapter 40: Control by Novel Processing Technologies
Chapter 41: Control by a Combination of Methods (Hurdle Concept)

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# Chapter 31

# Control of Access of Microorganisms: Cleaning, Sanitation, and Disinfection

## Introduction

The internal tissues of plants and animals used as foods are essentially sterile. However, many types of microorganisms capable of causing food spoilage and foodborne diseases enter foods from different sources. This has been discussed in Chapter 3. It is impossible to prevent access of microorganisms in food from these sources. However, it is possible to control their access to food in order to reduce the initial load and minimize microbial spoilage and health hazard. This is what regulatory agencies advocate and food processors try to achieve through sanitation.

When and how sanitation was introduced in food-handling operations is not clearly known. However, the consequences of changes in food consumption and production patterns during the late 19th and early 20th centuries and the understanding of the scientific basis of food spoilage and foodborne diseases may have helped enhance food sanitation. An outcome of the Industrial Revolution was an increased population in cities and urban areas, which needed processed foods for convenience and a stable supply. The foods produced by available processing techniques were not safe. With the knowledge of microbial association with food spoilage and foodborne diseases, food-processing techniques to reduce spoilage and ensure the safety of foods were studied. It was recognized during this time that microorganisms can get into food from various sources but that proper cleaning and sanitation during handling of foods could reduce their levels, and a food with a lower level of microorganisms could be processed and preserved more effectively to ensure stability and safety than a food with a high initial microbial load. Thus, sanitation became an integral part of food-processing operations.

In recent years, more foods than ever are being processed in both developed and developing countries. In addition, particularly in some countries, such as the United States, many centralized processing facilities are producing foods in large volumes. In these centralized plants, many types of raw materials and finished products are handled at a rapid rate. This has been possible because of the availability of needed processing technologies capable of handling large volumes. Some of



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the food-contact machineries are extremely complex and automated and require special methods for effective sanitation.

Although our understanding of the mechanisms by which microorganisms contaminate foods and the means by which they can be intervened have increased, the volume of foods spoiled and the incidence of foodborne diseases remain high. This indicates the need for more effective methods to control microbial access to foods through efficient sanitation and disinfection (see Appendix C: HACCP).

## Objectives of Sanitation

The main objective of sanitation is to minimize the access of microorganisms in food from various sources at all stages of handling.[1–4] Because the microbial sources and level of handling vary with each food of plant and animal origin and fabricated foods, the methods by which microorganisms contaminate foods differ.

Proper sanitation helps reduce the microbial load to desired levels in further processed food. An example of this is that a low microbial level in raw milk produced through effective sanitation makes it easier to produce pasteurized milk that meets the microbial standard. Also, proper sanitation helps produce food that, when properly handled and stored, will have a long shelf life. Finally, proper sanitation helps reduce the incidence of foodborne diseases.

## Factors to Consider

To minimize the access of microorganisms in foods, the microbiological quality of the environment to which a food is exposed (food-contact surfaces), and the ingredients added to a food should be of good microbiological quality. To achieve these goals, several factors need to be considered, which are briefly discussed here.[1,4]

### *Plant Design*

At the initial designing stage of a food-processing plant, an efficient sanitary program has to be integrated in order to provide maximum protection against microbial contamination of foods. This includes both the outside and the inside of the plant. Some elements to consider are specific floor plan, approved materials used in construction, adequate light, air ventilation, direction of air flow, separation of processing areas for raw and finished products, sufficient space for operation and movement, approved plumbing, water supply, a sewage disposal system, waste treatment facilities, drainage, soil conditions, and the surrounding environment. Regulatory agencies have specifications for many of these requirements and can be consulted at the initial stage of planning to avoid costly modifications.

### *Quality of Water, Ice, Brine, and Curing Solution*

Water is the most important entity in food manufacturing operations. Water is used as an ingredient in many foods and is also used in some products after heat treatment. The microbiological quality of this water, especially if the foods are ready-to-eat types, should not only be free from pathogens (as in drinking water) but also be low (if not free) of spoilage bacteria, such as

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*Pseudomonas* spp. This is particularly important for foods kept at low temperatures for extended shelf life. The ice used for chilling unpackaged foods also should not contaminate a food with pathogenic and spoilage bacteria or viruses. Water used for chilling products, such as chicken at the final stage of processing, can be a source of cross-contamination of a large number of birds from a single bird contaminated with an enteric pathogen. Similarly, the warm water used to defeather chickens can be a source of thermoduric bacteria.

Brine and curing solutions used in products, such as ham, bacon, turkey-ham, and cured beef brisket can be a source of microbial contamination. To reduce this, brine and curing solutions should be made fresh and used daily. Storing brine for extended periods before use may reduce the concentration of nitrite through formation and dissipation of nitrous oxide and may reduce the shelf life of the products.

## Quality of Air

Some food-processing operations, such as spray drying of nonfat dry milk, require large volumes of air that come into direct contact with the food. Although the air is heated, it does not kill all the microorganisms present in the dust of the air and thus can be a source of microbial contamination of foods. The installation of air inlets to obtain dry air with the least amount of dust and filtration of the air is important to reduce microbial contamination from this source.

## Training of Personnel

A processing plant should have an active program to teach the plant personnel the importance of sanitation and personal hygiene to ensure product safety and stability. The program should not only teach how to achieve good sanitation and personal hygiene but also monitor the implementation of the program. People with an illness and infection should be kept away from handling the products. Some kind of incentive to the workers might help make the program efficient.[4,5]

## Equipment

The most important microbiological criterion to be considered during the designing of food-processing equipment is that it should protect a food from microbial contamination. This can be achieved if the equipment does not contain dead spots where microorganisms harbor and grow or that cannot be easily and readily cleaned in place or by disassembling. Some of the equipment, such as meat grinders, choppers, or slicers and several types of conveyor systems, may not be cleaned and sanitized very effectively and therefore serve as a source of contamination to a large volume of product. This is particularly important for products that come in contact with equipment surfaces after heat treatment and before packaging.[1,4]

## Cleaning of Processing Facilities

Cleaning is used to remove visible and invisible soil and dirt from the food-processing surroundings and equipment. The nature of soil varies greatly with the type of food processed, but chemically, it consists of lipids, proteins, carbohydrates, and some minerals. Although water is used for some cleaning, to increase efficiency of cleaning and to break down fatty micelles, heat, chemical agents, or detergents are used with water. In addition, some form of energy with the liquids, such as spraying, scrubbing, or turbulent flow, is used for better cleaning.

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Many types of detergents are available, and they are selected based on the need. The effectiveness of a cleaning agent to remove soil from surfaces depends on several characteristics, such as efficiency of emulsifying lipids, dissolving proteins, and solubilizing or suspending carbohydrates and minerals. In addition, a detergent should be noncorrosive, safe, rinsed easily, and compatible, when required, with other chemical agents. The detergents frequently used in food-processing facilities are synthetic, which can be anionic, cationic, or nonionic. Among these, anionic detergents are used with higher frequency. Examples of anionic detergents include sodium lauryl sulfate and different alkyl benzene sulfonates and alkyl sulfonates. Each molecule has a hydrophobic or lipophilic (nonpolar) segment and a hydrophilic or lipophobic (polar) segment. The ability of a detergent to remove dirt from a surface is attributed to the hydrophobic segment of a molecule. They dissolve the lipid materials of the soil on the surface by forming micelles with the polar segments protruding outside in the water. The concentration of a detergent at which micelle formation starts is called the critical micelle concentration (CMC), which varies with the detergent. The concentration of a detergent is used above its CMC level. Generally, this is approximately 800–900 ppm but could be 1000–3000 ppm if skin contact does not occur (as in the clean-in-place or CIP method) or where heavy-duty cleaning is required.

The frequency of cleaning depends on the products being processed and the commitment of the management to good sanitation. From a microbiological standpoint, prior microbiological evaluation of a product can give an indication about the frequency of cleaning necessary in a particular facility. Cleaning of the equipment is done either after disassembling the equipment or by the CIP system. Because of its efficiency and lower cost, CIP cleaning has become popular. The system uses detergent solutions at a high pressure. Because microorganisms can grow in some detergent solutions, they preferably should be prepared fresh (not exceeding 48 hours).[1]

## *Sanitation of Food-Processing Equipment*

Efficient cleaning can remove some microorganisms along with the soil from the food-contact surfaces, but it cannot ensure complete removal of pathogens. To achieve this goal, food-contact surfaces are subjected to sanitation after cleaning. The methods should effectively destroy pathogenic microorganisms as well as reduce total microbial load. Several physical and chemical methods are used for sanitation of food-processing equipment.

Physical agents used for sanitation of food-processing equipment include hot water, steam, hot air, and UV irradiation. UV irradiation is used to disinfect surfaces and is discussed in Chapter 39. Hot water and steam, although less costly and efficient for destroying vegetative cells, viruses, and spores (especially steam), can be used only in a limited way.

Chemical sanitizers are used more frequently than physical sanitizers. Several groups of sanitizers are approved for use in food-processing plants. They vary greatly in their antimicrobial efficiency. Some of the desirable characteristics used in selecting a chemical sanitizer are effectiveness for a specific need, nontoxicity, noncorrosiveness, no effect on food quality, ease of use and rinse, stability, and cost effectiveness. Important factors for antimicrobial efficiency are exposure time, temperature, concentrations used, pH, microbial load and type, microbial attachment to surface, formation of biofilm, and water hardness. Microbial attachment has been discussed separately (see Chapter 7). The mechanisms of antimicrobial action and the advantages and disadvantages of some of the sanitizers used in food-processing plants are briefly discussed here. Some sanitizers, designated as detergent sanitizers, can both clean and sanitize. They can be used in a single operation instead of first using detergent to remove the soil and then using sanitizers to control microorganisms. They are also discussed here.[4,6]

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### Chlorine-Based Sanitizers

Some of the chlorine compounds used as sanitizers are liquid chlorine, hypochlorites, inorganic or organic chloramines, and chlorine dioxide. Chlorine compounds are effective against vegetative cells of bacteria, yeasts and molds, spores, and viruses. Clostridial spores are more sensitive to chlorine compounds than the bacilli spores. The antimicrobial (germicidal) action of chlorine compounds is a result of the oxidizing effect of chlorine on the –SH group in many enzymes and structural proteins. In addition, damage to membrane, disruption of protein synthesis, reactions with nucleic acids, and interference with metabolisms have been suggested.

The germicidal action of liquid chlorine and hypochlorites is produced by hypochlorous acid (HOCl). It probably enters the cell and reacts with the –SH group of proteins. HOCl is stable at acid pH and is thus more effective; at alkaline pH, it dissociates to $H^+$ and $OCl^-$ (hypochlorite ions), which reduces its germicidal effectiveness. They are also less effective in the presence of organic matter. Chloramines (inorganic or organic), such as Chloramine T, release chlorine slowly, but they are less active against bacterial spores and viruses. They are effective, to some extent, against vegetative cells at alkaline pH. Chlorine dioxide is more effective at alkaline pH and in the presence of organic matter.

Chlorine compounds are fast acting against all types of microorganisms, less costly, and easy to use. However, they are unstable (especially at higher temperatures and with organic matter), corrosive to metals, can oxidize food (color, lipid), and are less active in hard water.

### Iodophores

Iodophores are prepared by combining iodine with surface-active compounds, such as alkylphenoxypolyglycol. Because of the surface-active compounds, they are relatively soluble in water. Iodophores are effective against gram-positive and gram-negative bacteria, bacterial spores, viruses, and fungi. Their germicidal property is attributed to elemental iodine ($I_2$) and hypoiodous acid, which oxidize the –SH group of proteins, including key enzymes. They are more effective at acidic pH and higher temperatures. In the presence of organic matters, they do not lose germicidal property as rapidly as chlorine does. However, their effectiveness is reduced in hard water.

They are fast acting, noncorrosive, easy to use, nonirritating, and stable. However, they are expensive, less effective than hypochlorites against spores and viruses, can cause flavor problems in products, and react with starch.

### Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) can be used as detergent sanitizers because they have cleaning properties along with germicidal abilities. However, they are principally used as sanitizers. They are synthesized by reacting tertiary amines with alkyl halides or benzyl chloride (Figure 31.1). The general structure is where R1, R2, R3, and R4 represent alkyl and other groups. The cationic group is hydrophobic, and the anionic group is hydrophilic. QACs can act as bactericides in high concentrations and when used in solution. However, they form a film on the equipment surface, in which state (low concentrations) they are bacteriostatic. They are more effective against gram-positive bacteria than many gram-negative bacteria, bacterial spores, fungi, and viruses. The antimicrobial action is produced by the denaturation of microbial proteins and destabilization of membrane functions. They are more effective against microorganisms at acidic pH and higher temperature. Their effectiveness is not greatly reduced in the presence of organic matters. However, they are less effective in hard water.

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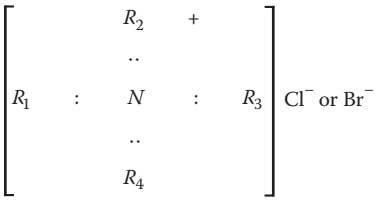



**Figure 31.1 The general structure of quaternary ammonium compound.**

QACs are advantageous as sanitizers because they are highly stable, noncorrosive, nonirritating, nontoxic, show a residual bacteriostatic effect (however, they need to be rinsed before the use of the equipment), and show a detergent effect. The disadvantages are high cost; low activity against many gram-negatives, spores, and viruses; incompatibility with anionic synthetic detergents; and have a rinsing requirement before use because of film formation on equipment surfaces. Some gram-negative bacteria, such as *Pseudomonas* spp., can grow in diluted QAC solutions.

### $H_2O_2$

$H_2O_2$ is a very effective germicide and kills vegetative cells, spores, and viruses. The use of $H_2O_2$ solutions in food (milk and liquid egg) is discussed in Chapters 17 and 38. Its use as a sanitizer is briefly described here. The U.S. Food and Drug Administration has approved its use for sanitation of equipment and containers used in the aseptic packaging of foods and beverages. Equipment and container surfaces can be sterilized in 15 minutes with a 30%–50% solution; the treatment time can be reduced if the temperature of the solution is raised to 150°F–160°F (65.6°C–71.7°C). Use of $H_2O_2$ in the vapor phase can also be effective in killing microorganisms on food-contact surfaces. Organic materials greatly reduce the germicidal effect of $H_2O_2$.

## *Decontamination and Sanitization of Fruits and Vegetables*

Fruits and vegetables have been responsible for large numbers of outbreaks and illnesses in recent years (see also Chapter 24). Fruits and vegetables generally are eaten raw or minimally processed, that is, they did not receive a treatment step for reduction of pathogens, spores, or toxins to a safe level; thus, these products are highly vulnerable to spoilage and pathogen contamination.[7,8] The FDA guidelines emphasized (i) prevention of entry of pathogens into produce over disinfecting them as a corrective strategy, (ii) adoption of good agricultural and management practice (GAMP), (iii) prevention of entry of pathogens into the supply chain, (iv) minimizing contamination through water, (v) management of animal manure as fertilizers, and (v) workers' hygiene and sanitation practice to improve produce safety.[7] Recently, there is an increased emphasis on the application of on-farm HACCP practice to reduce pathogens on produce.[9] Generally, proper washing and sanitization are employed to increase shelf life and product safety. Freshly harvested produce is routinely washed to remove soils, pesticide residues, insects, plant debris, and microorganisms. For disinfection of produce, several chemical sanitizers have been tried, such as chlorine, chlorine dioxide, bromine, iodine, trisodium phosphate, quaternary ammonium compounds, acids, hydrogen peroxide, and ozone. Some of these, including chlorine (50–200 ppm), ozone (0.1–2.5 ppm), chlorine dioxide (0.5–5 ppm), and peroxyactic acid (<80 ppm), are permitted on

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produce for washing and decontamination.[8,10,11] In addition, plant essential oils are also used at various concentrations to disinfect produce surfaces.[12] Studies have shown that sequential washing of alfalfa seeds, lettuce, and carrots with aqueous chlorine dioxide, ozone, and plant essential oils allowed for a several-fold reduction in *Esc. coli* O157 counts in those products.[13,14]

Applications of chemicals in gaseous form or in the vapor phase to decontaminate products are drawing much attention because of their efficacy in improving shelf life and product safety. Chemical sanitizers, such as chlorine, iodophors, or $H_2O_2$, are widely used in liquid form to decontaminate equipment and have been discussed above. In this section, the application of chemicals in gaseous or in vapor phase to decontaminate fruits and vegetables are discussed.

## Chlorine

Chlorine is the most commonly used sanitizing agent, and it is used as sodium hypochlorite (50–200 ppm) at a pH < 8.0 with a contact time of one to two minutes. A pathogen reduction of 1–3 log CFU/g is commonly achieved with chlorine treatment.[15] In fresh-cut produce, components from tissue may neutralize chlorine action before it can exert its antimicrobial action on microbes.

Chlorine dioxide gas ($ClO_2$) is a water-soluble neutral compound of chlorine, and it disinfects by oxidation (see above).[8,16] It has 3.5 times more oxidative capacity than chlorine and is less reactive to organic compounds. The $ClO_2$ gas is unstable, and it is generated by reacting chlorine gas ($Cl_2$) with sodium chlorite ($2NaClO_2$), thus generating $2ClO_2$ gas and salt (2NaCl) when needed. $ClO_2$ is permitted (Approval No. 21 CFR 173.300) up to 5 ppm in water for rinsing whole fresh fruits and vegetables by the FDA in 1998. Application of $ClO_2$ gas for 10–30 minutes caused more than a 5 log reduction of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on the surface of various products, such as green peppers, strawberries, apples, and cantaloupes, while maintaining the acceptable quality of the products. $ClO_2$ gas also reduced *Salmonella* by 4.50 log in 10 minutes on almonds;[17] however, direct use of $ClO_2$ gas on these produce has not become official yet.

## Ozone

Ozone is a strong oxidizer and a colorless gas with a pungent odor and kills spoilage or pathogenic microorganisms by oxidizing key cellular enzymes.[18] It has been used to treat drinking water to kill pathogens. Ozone is unstable and decomposes quickly; thus, it has to be generated on-site before use. Treatment of inoculated fruits and vegetables, such as black pepper, carrots, lettuce, and blackberries, with ozone resulted in more than a 5 log reduction in *Esc. coli* O157:H7 counts and 3–6 log reduction in *Salmonella*, *Penicillium*, and *Aspergillus* species. It has been also shown that the shelf life of strawberries, raspberries, and grapes could be doubled with the application of ozone at 2–3 ppm for a few hours per day. In recent years, ozone has been used to disinfect produce rinse water for reuse in the produce industry as an environmentally friendly alternative to chlorine.

Other gases or vapors, such as $H_2O_2$, allyl isothiocyanate gas, acetic acid vapor, and natural plant volatiles, are shown to be effective in reducing the spoilage and pathogenic microorganisms in varying degrees.

Application of ozone as a preservative has been approved. Acetic acid and $H_2O_2$ are part of the approved GRAS list; however, there is no regulation in the use of these and other plant oil–derived gases/vapors on food products.

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### Microbiological Standards, Specifications, and Guidelines

Microbiological standards, specifications, and guidelines are useful in keeping the microbial load of foods at acceptable levels by various methods, one of which is by controlling their access to foods. Microbiological standards of food are set and enforced by regulatory agencies to increase consumer safety and product stability. A standard dictates the maximum microbial level that can be accepted in a food. With proper sanitation and quality control, this level is generally attainable. Some examples are maximum acceptable levels of standard plate counts (SPCs) of Grade A raw milk, 100,000/mL; pasteurized Grade A milk, SPC 20,000/mL; and coliforms ≤ 10/mL. However, very few foods have microbiological standards. Instead, many foods and food ingredients have microbiological specifications. A specification indicates the maximum permissible microbial load for the acceptance of a food or food ingredient. It should be attainable and agreed on by the buyers and sellers of the products. It is not set up or enforced by regulatory agencies. In the United States, the military has microbiological specifications for foods purchased outside for army rations. For example, dried whole egg has the following specifications: aerobic plate count (APCs), 25,000/g; coliforms, 10/g; and *Salmonella*, negative in 25 g. The specifications discourage mixing of a microbiologically poor-quality product with a good-quality product. Microbiological guidelines are generally set either by regulatory agencies or food processors to help generate products of acceptable microbiological qualities. A guideline is set at a level that can be achieved if a food-processing facility uses good cleaning, sanitation, and handling procedures. It also helps detect if a failure has occurred during processing and handling, and thus alerts the processor to take corrective measures.

## Conclusion

Spoilage and pathogenic microorganisms enter into food from different sources. One of the major objectives to produce a safe food with a desirable shelf life is to minimize the access of microorganisms in food from various sources. This can be achieved by proper plant design, training personnel, designing equipment that can be sanitized effectively, and establishing an efficient cleaning and sanitation procedure. Many cleaning and sanitizing chemicals are available commercially. The aim will be to select agents that are suitable for a specific purpose. Adaptation of an efficient and approved procedure (by regulatory agencies) helps meet the required microbiological standards and specifications. Because fresh produce–related outbreaks are on the rise, chemical sanitizers are now used to disinfect these commodities to reduce foodborne illness. Chapter 32 describes methods used to remove microorganisms from food to keep their initial load at a low level.

**QUESTIONS**

1. Discuss the objectives of food sanitation.
2. List five factors that are important in reducing microbial access to foods.
3. Describe how water, ice, brine, and curing solution can contribute to the microbial load in a food.
4. List the objectives of using cleaning agents in food-processing facilities. Describe the properties of a desirable detergent for use in a food-processing plant.

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5. Discuss the functions of sanitizers for use in food. Describe the advantages and disadvantages of the following agents as sanitizers: hot water, steam, UV light, hypochlorite, iodophores, QACs, and $H_2O_2$.
6. Define microbiological standards, microbiological specifications, and microbiological guidelines. Explain briefly how they help to keep microbial load in a food at an acceptable level.

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# Chapter 32

# Control by Physical Removal

## Introduction

Microorganisms can be physically removed from solid and liquid foods by several methods. In general, these methods can partially remove microorganisms from food, and by doing so, they reduce the microbial level and help other antimicrobial steps that follow to become more effective. They are generally used with raw foods before further processing.

## Physical Methods

### *Centrifugation*

Centrifugation is used in some liquid foods, such as milk, fruit juices, and syrups, to remove suspended undesirable particles (dust, leucocytes, and food particles). The process consists of exposing the food in thin layers to a high centrifugal force. The heavier particles move outward and are separated from the lighter liquid mass. Although this is not intended to remove microorganisms, spores, large bacterial rods, bacterial clumps and chains, yeasts, and molds can be removed because of their heavier mass. Under high force, as much as 90% of the microbial population can be removed. Following centrifugation, a food will have fewer thermoduric microorganisms (bacterial spores) that otherwise would have survived mild heat treatment (e.g., milk pasteurization) and increased microbial load in the pasteurized product.

### *Filtration*

Filtration[1,2] is used in some liquid foods, such as soft drinks, fruit juices, beer, wine, and water, to remove undesirable solids and microorganisms and to give a sparkling clear appearance. As heating is avoided or given only at minimum levels, the natural flavors of the products and heat-sensitive nutrients (e.g., vitamin C in citrus juices) are retained to give the products natural characteristics. The filtration process can also be used as a step in the production of concentrated juice with better flavor and higher levels of vitamins. Many types of filtration systems are available. In many filtration processes, coarse filters are used initially to remove large particles; this is



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followed by ultrafiltration. Ultrafiltration methods, depending on pore size of the filter materials (0.45–0.7 mm), are effective in removing yeasts, molds, and most bacterial cells and spores from liquid products.

Filtration of air is also used in some food-processing operations, such as spray drying of milk, to remove dust from the air used for drying. The process also removes some microorganisms with dust from the air, and this reduces the microbial loads in food.

## Trimming

Fruits and vegetables showing damage (with a greater chance of microbial contamination) and spoilage are generally trimmed.[3–5] In this manner, areas heavily contaminated with microorganisms are removed. Trimming the outside leaves in cabbage used for sauerkraut production also helps reduce microorganisms coming from soil. Trimming is also practiced for the same reason to remove visible mold growth from hard cheeses, fermented sausages, bread, and some low-pH products. However, if a mold strain is a mycotoxin producer, trimming does not ensure removal of toxins from the remaining food. Trimming is also used regularly to remove fecal stain marks, unusual growths, and abscesses or small, infected areas from carcasses of food animals and birds. Although this method helps remove highly contaminated areas, it does not ensure complete removal of the causative microorganisms. Thus, a beef carcass can have an area contaminated with feces along with enteric pathogens. Merely removing the visibly tainted area by trimming does not help remove pathogens from the surrounding areas that do not show the taint. This is an important concern in the production of safer foods.

## Washing

Washing equipment and work areas has been discussed under cleaning and sanitation in Chapter 31. Here, only washing foods is discussed.[6–8] Fruits and vegetables are washed regularly to reduce temperature (which helps reduce the metabolic rate of produce and microbial growth) and remove soil. Washing also helps remove the soil-laden microorganisms from food, especially from raw fruits and vegetables. It is also used for shell eggs to remove fecal materials and dirt. During the processing of chicken and turkey, the carcasses are exposed to water several times. During defeathering, they are exposed to hot water; following evisceration, they are given spray washings; and finally they are exposed to cold water in a chilling tank. Although these treatments are expected to reduce microbial load, they can spread contamination of undesirable microorganisms, particularly enteric pathogens. Thus, higher percentages of chicken have been demonstrated to be contaminated with salmonellae when coming out of the chill tank than before entering the tank. This aspect is discussed in Chapter 36. Carcasses of food animals, such as beef, pork, and lamb, are washed to remove hair, soil particles, and microorganisms. Instead of hand-washing, automated machine-washing at a high pressure is currently used to effectively remove undesirable materials and microorganisms from the carcass. In addition to high pressure, the effectiveness of hot water, steam, ozonated water, and water containing chlorine, acetic acid, propionic acid, lactic acid, tripolyphosphates, or bacteriocins (nisin and pediocin) of lactic acid bacteria has been studied for removing microorganisms, particularly enteric pathogens, such as *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*, separately or in combination. The results were not consistent. Some of these agents also have a bactericidal property. However, studies show that all the agents can reduce bacterial contamination to a certain level from the carcass surfaces, and a combination of two or more components may be better.[3,4] The European

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Food Safety Authority (EFSA) has established guidelines toward carcass decontamination that include application of (a) a physical method, such as hot water, steam, and steam vacuuming; (b) a chemical method, such as organic acids, chlorine, acidified sodium chlorite, polyphosphates; and (c) biological methods, such as bacteriophages and bacteriocins.[9] Combined physical and chemical treatments are also proposed and have proven to be more effective than a single treatment scheme.

The suitability of the combinations as well as their concentration and duration of application must be determined. One has to recognize that, with time, microorganisms can form a biofilm on the carcass surface (Chapter 7). The nature of the biofilm varies with microbial species and strains. Also, with time, the biofilm becomes more stable, and removing microorganisms by washing after the formation of a stable biofilm is relatively difficult. This aspect needs to be considered while developing effective methods of carcass washing. Removal of pathogens from carcass surfaces will be an important area to develop a suitable intervention strategy for ensuring the safety of meat and meat products (see Appendix C).

## Conclusion

Microorganisms that have gained access to food can be controlled by various means, one of which is to remove them physically. This can be achieved by the different methods discussed in this chapter. Some of them are used in liquid foods but have limited applications; others are used in solid foods. Chapter 33 discusses different methods of heating to kill microorganisms already present in food.

### QUESTIONS

1. List the methods used to remove microorganisms from foods and discuss one advantage and one disadvantage of each method.
2. Discuss two specific microbiological disadvantages, with examples, of trimming food for visible microbial growth or microbial contamination.
3. Discuss two specific microbiological disadvantages, with examples, of washing carcasses of animals and birds to reduce microorganisms.
4. List six different agents that have been tested and discuss their relative efficiency in removing microorganisms from beef carcasses.
5. A milk processor found that a batch of milk had very high levels of bacterial spores that could increase the SPC level following pasteurization. Discuss a physical removal procedure that he could use to remove the spores to attain the desired SPC level.

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# Chapter 33

# Control by Heat (Thermal Processing)

## Introduction

The desirable effect of heat (from fire) on the taste of foods of animal and plant origin, especially seeds, tubers, and roots, was probably accidentally discovered, following a natural forest fire, by our ancestors long before civilization. They also possibly recognized that heated foods did not spoil as rapidly as raw foods or cause health hazards. Since then, particularly following the invention of pottery and ovens, heat has been used to roast, boil, bake, and concentrate foods to improve taste and to enhance shelf life (and probably safety). However, it was not until approximately 1810 that extended shelf life of perishable foods was achieved by a specific method, appertization. Nicolas Appert, after whom the method was named, in France, reported that by filling a clean glass jar with a desirable food, heating the contents in boiling water for six hours or more, and hermetically sealing the container with a cork kept the food unspoiled and safe for a long time.[1] Some of his products (meat stew) prepared in 1824 were found nontoxic when opened in 1938 but were found to contain dormant spores. Heating of foods in cans was developed by Durand in England in 1810, and by 1820, the method was used in the United States. Methods to reduce the heating time to approximately 30 minutes were also developed by adding different types of salts to the water to raise the boiling point above 212°F (100°C). In 1870, the autoclave was invented, providing the possibility of developing pressure vessels that could be used to heat canned food at a much higher temperature for a relatively shorter period of time in order to retain the quality of foods as well as to enhance shelf life.

All these developments occurred without clearly knowing why a perishable food, following appertization or canning, did not spoil (or cause foodborne disease). The role of microorganisms in the spoilage of wine and milk was discovered by Louis Pasteur in approximately 1870. He also showed that mild heat treatment (pasteurization) killed these microorganisms, and in the absence of recontamination, the products stayed unspoiled. Subsequently, the microbial role in foodborne diseases was also recognized. Following these, studies were conducted to isolate the most heat-resistant microorganisms (spores) that could survive heating in canned foods and cause spoilage



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and foodborne diseases and to determine the temperature and time requirements for their destruction. Studies were also conducted to identify the time-temperature relationships for the destruction of less heat-resistant microorganisms (vegetative cells). From these results, mathematical expressions were developed to accurately predict time-temperature relationships to destroy microorganisms (also some enzymes and toxins) by heating foods at different temperatures and times.

## Objectives

The main objective (microbiological) of heating food is to destroy vegetative cells and spores of microorganisms that include molds, yeasts, bacteria, and viruses (including bacteriophages). Although very drastic heat treatment (sterilization) can be used to kill all the microorganisms present in a food, most foods are heated to destroy specific pathogens and some spoilage microorganisms, which are important in a food. This is necessary in order to retain the acceptance and nutritional qualities of a food. To control growth of surviving microorganisms in the food, other control methods are used following heat treatment.

Heating of foods also helps destroy undesirable enzymes (microbial and food) that would otherwise adversely affect the acceptance quality of food. Some microorganisms also produce heat-stable proteinases and lipases in food. Heating a food to a desired temperature for a specific time can help destroy or reduce the activity of these enzymes.[2] This is especially important in foods stored for a long time at room temperature.

Some microorganisms can release toxins in food; also, some foods can have natural toxins. If a toxin is heat sensitive, sufficient heating will destroy it, and consumption of such a food will not cause health hazards. It is also important to recognize that microbial (and natural) heat-stable toxins are not completely destroyed even after high heat treatment.

Heating (warming) of ready-to-eat foods before serving is also usually used to prevent growth of pathogenic and spoilage microorganisms. A temperature above 50°C (122°F), preferably 60°C (140°F), is important to control growth of many microorganisms in such foods during storage before serving.

Finally, heating of raw materials, such as milk, is done before adding starter culture bacteria for fermentation to kill undesired microorganisms (including bacteriophages) and to allow growth of the starter cultures without competition.

## Mechanism of Thermal Inactivation

Depending on the temperature and time of heating, microbial cells and spores can be heat shocked, sublethally injured, or dead. Heat-shocked cells acquire some resistance to subsequent heating, and sublethally injured cells and spores retain the ability to repair and multiply. These are discussed in Chapter 10.

Results of different studies have shown that following heat injury, bacterial cells show a loss of permeability and increased sensitivity to some compounds to which they are normally resistant. Sublethally injured cells seem to suffer injury in the cell membrane, cell wall, DNA (strand break), ribosomal RNA (degradation), and some important enzymes (denaturation). Death occurs from damages in some vital functional and structural components. Bacterial spores, following heating, were found to lose structural components from the spore coat, suffer damage to the structures that are destined to become membrane and wall, and develop an inability to use water for hydration

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during germination. Death results from the inability of a spore either to germinate or to outgrow (see Chapter 10).

Exposure of microbial cells to approximately 45°C–50°C (104°F–122°F) for a short time, which can occur while heating a large volume of a food, such as a large rare roast, may induce production of heat-shock proteins (stress proteins) by the cells. In the presence of these proteins, the microbial cells can develop relatively greater resistance to subsequent heating at higher temperatures (see Chapter 10). The implication of this phenomenon in the thermal destruction of microbial cells in low-heat-processed foods is important.[3,4]

## Influencing Factors

The effectiveness of heat in killing microbial cells and spores depends on many factors, some of which are related to the inherent nature of the foods and others to both the nature of the microorganisms and the nature of processing. An understanding of these factors is important to develop and adopt an effective heat-processing procedure for a food.[1]

### *Nature of Food*

Composition (the amount of carbohydrates, proteins, lipids, and solutes), $A_W$ (moisture), pH, and antimicrobial content (natural or added) greatly influence microbial destruction by heat in a food. In general, carbohydrates, proteins, lipids, and solutes provide protection to microorganisms against heat. Greater microbial resistance results in higher concentrations of these components. Microorganisms in liquid food and food containing small-sized particles suspended in a liquid are more susceptible to heat destruction than in a solid food or a food with large chunks in liquid. Microorganisms are more susceptible to heat damage in a food that has higher $A_W$ or lower pH. In low-pH foods, heating is more lethal to microorganisms in the presence of acetic, propionic, and lactic acids than phosphoric or citric acids at the same pH. In the presence of antimicrobials, not inactivated by heat, microorganisms are destroyed more rapidly, the rate differing with the nature of the antimicrobials.

### *Nature of Microorganisms*

Factors that influence microbial sensitivity to heat include inherent resistance of species and strains, stage of growth, previous exposure to heat, and initial load. In general, vegetative cells of molds, yeasts, and bacteria are more sensitive than spores. Cells of molds, yeasts, and many bacteria (except thermoduric and thermophilic), as well as viruses, are destroyed within 10 minutes at 65°C (149°F). Most thermoduric and thermophilic bacterial cells important in foods are destroyed in 5 to 10 minutes at 75°C–80°C (167°F–176°F). Yeast and most mold spores are destroyed at 65°C–70°C (149°F–158°F) in a few minutes, but spores of some molds can survive at as high as 90°C (194°F) for four to five hours. Bacterial spores vary greatly in their sensitivity to heat. Generally, heating at 80°C–85°C (176°F–185°F) for a few minutes does not kill them. Many are destroyed at 100°C (212°F) in 30 minutes, but there are bacterial species whose spores are not destroyed even after boiling (100°C) for 24 hours. All spores are destroyed at 121°C (249.8°F) in 15 minutes (sterilization temperature and time). Below this temperature (and time), spores of some bacterial species can survive; however, this depends on the initial number of spores and the nature of the suspending medium. Species and strains of bacterial cells and spores also differ in

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heat sensitivity. This is especially important if a food is heat treated on the basis of results obtained by using a heat-sensitive species or strain but contains heat-resistant variants.

Cells at the exponential stage of growth are more susceptible to heat than resting cells (stationary phase). Also, cells previously exposed to low heat become relatively resistant to subsequent heat treatment (a result of the synthesis of stress proteins).[4] Finally, the higher the initial microbial load in a food, the longer the time it takes at a given temperature to reduce the population to a predetermined level. This is because the rate of heat destruction of microorganisms follows first-order kinetics, which is discussed later. This suggests the importance of lower initial microbial loads (through sanitation and controlling growth) in a food before heat treatment.

### *Nature of Process*

Microbial destruction in food by heat is expressed in terms of its exposure to a specific temperature for a period of time, and these are inversely related: The higher the temperature, the shorter the period of time required to obtain the same amount of destruction when other factors are kept constant. As a food is heated by conduction (molecule-to-molecule energy transfer) and convection (movement of heated molecules), a liquid food is heated more rapidly than a solid food, and a container with high conduction (metal) is better. Also, food in a small container is heated more rapidly than in a large container. A product can have a cold point at the center (in a solid food in a can) or near the end (in a liquid food in a can), which may not attain the desired temperature within the given time. Finally, it needs to be emphasized that heating a food at a given temperature for a specific time means that every particle of that food should be heated to the specified temperature (say 71.6°C or 161°F) and stay at that temperature for the specified time (15 seconds used in milk pasteurization). This time is also called the holding time. The time during which milk is heated before the temperature reaches 161°F in this case is not considered (but considered in the commercial sterilization process, which uses a much higher temperature).

## Mathematical Expressions

When a population of microbial cell suspension is heated at a specific temperature, the cells die at a constant rate. This observation helps in expressing the microbial death rate resulting from heat as a function of time and temperature under a given condition. These expressions are helpful to design a heat-treatment method for a food.

### *Decimal Reduction Time (D Value)*

The $D$ value is the time in minutes during which the number of a specific microbial (cells or spores) population exposed to a specific temperature is reduced by 90% or 1 log. It is expressed as $D_T = t$ min, where $T$ is the temperature (°C or °F), and $t$ is the time (min) for 1 log reduction of the microbial strain used. Thus, it is a measure of heat sensitivity of microorganisms and varies with microbial species and strains, temperature used, and other variables, such as suspending media and age of the culture.

It can be determined by using the expression

$$D_T = \frac{t}{\log_{10} x - \log_{10} y},$$

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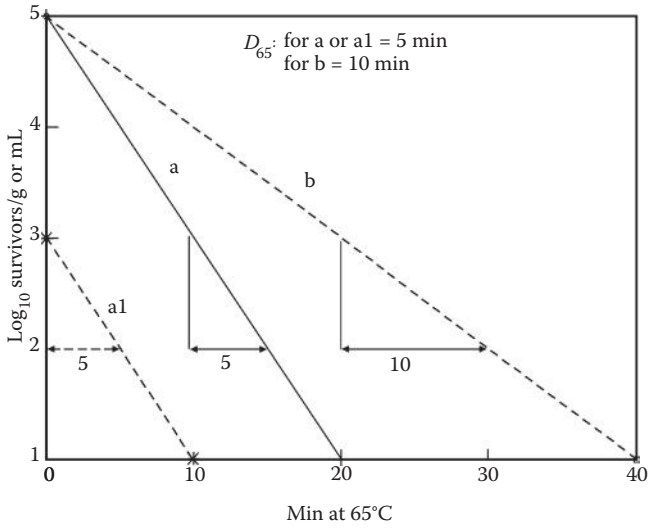



**Figure 33.1 Graphical representation of decimal reduction time (*D*). The graph also illustrates the number of *D* required with high and low populations of bacteria with the same heat sensitivity (a and a1) to obtain a desired survivor level (say $10^1$/g or /mL) and different *D* values for two bacterial species with different heat sensitivities at 65°C (a and b).**

where *x* and *y* represent, respectively, microbial numbers before and after exposure at temperature *T* for *t* min. It also can be determined by plotting $\log_{10}$ survivors against time of exposure (min) for a specific temperature (Figure 33.1). Ideally, it is a straight-line graph and is independent of the initial number of a microbial population. It can be extrapolated to $-\log_{10}$ values to obtain very low levels of microbial survivors, such as 1 cell or spore in 10 g, or 100 g, or 1000 g of a product and thus can be used to design heat-treatment parameters to obtain a desirable low level of a microbial population in a food. It is evident from the plot that to reach a desirable microbial level, a food with lower initial numbers will require less time (fewer *D*) than a food with higher initial numbers at a specific temperature. It can also be used to determine the relative sensitivity of two or more microbial species or strains with respect to a specific temperature (Figure 33.1).

The 12*D* concept is used in heat processing of high-pH foods (pH > 4.6, low-acid foods, such as corn, beans, and meat) to destroy the most heat-resistant spores of the pathogenic bacteria *Clostridium botulinum*. It means that the products are given heat treatment to reduce the population of *Clo. botulinum* spores by 12 log cycles. Hypothetically, if 1 billion ($10^9$) cans at one time, each containing $10^3$ spores of *Clo. botulinum*, are given proper heat treatment, only one can will contain one viable spore. This is an extreme processing condition used for a high degree of safety. The 12*D* value at D121.1°C is approximately 2.8 or approximately 3.0 minutes.[5]

## *Thermal Death Time (TDT), Z Value, and* F *Value*

TDT is the time in log that is necessary to completely destroy a specific number of microbial cells or spores in a population at a specific temperature. It indicates the relative sensitivity of a microorganism to different temperatures. A TDT curve can be constructed either by plotting log time of complete destruction against temperature or by plotting log *D* values against temperature (Figure 33.2; this is called a phantom TDT curve). The slope of the curve is the *Z* value, which

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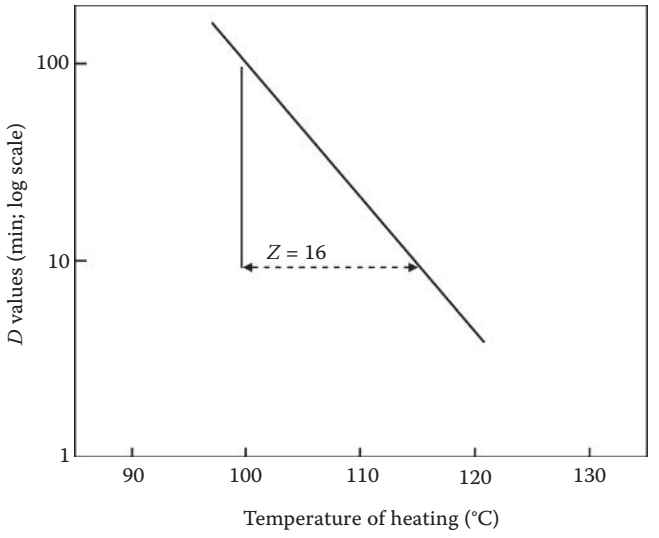



**Figure 33.2 Hypothetical thermal death time curve. *D*, decimal reduction time; *Z*, °C required for the thermal death time curve to transverse over a $\log_{10}$ cycle.**

indicates the temperature (°C or °F) required to change the *D* value (or TDT) to transverse by 1 log. A value of $Z = 10$ in °C implies that if the *D* value of bacterial spores at 100°C (212°F) is 50 minutes, at 110°C (230°F) it will be five minutes, and at 120°C (248°F) it will be 0.5 minutes. In developing heat-processing conditions of a food, *D* and *Z* values are used to obtain desirable destruction of microorganisms (cells and spores). In addition, the symbol *F* is used to express the time (minutes) necessary to completely destroy a specific number of microbial spores or cells at a reference temperature (121.1°C for spores and 60°C for cells). $F_{121.1°C}$ for *Clo. botulinum* Type A spores was found to be 0.23 minutes and was used to calculate the 12*D* value ($12 \times 0.23 = 2.78$ or 3 minutes) for heat processing of low-acid canned foods.

## Methods

On the basis of temperature and time heating the food to destroy microorganisms, methods can be broadly divided into low-heat processing and high-heat processing. Low-heat processing is used to destroy mainly the microorganisms relatively sensitive to heat and is not generally effective against thermoduric microorganisms. In contrast, high-heat processing is used to destroy thermodurics and, especially, the most heat-resistant spores of spoilage and pathogenic bacteria. Methods (e.g., baking, broiling, simmering, roasting, and frying) used in cooking foods and blanching used to destroy some natural enzymes in fresh vegetables and fruits are not discussed here. Although they destroy microorganisms, their main use is for food preparation and not destruction of microorganisms in foods.[6]

### *Low-Heat Processing or Pasteurization*

The temperatures used for low-heat processing or pasteurization is below 100°C (212°F). The objectives of pasteurization are to destroy all the vegetative cells of the pathogens and a large

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number (~90%) of associative (many of which can cause spoilage) microorganisms (yeasts, molds, bacteria, and viruses). In certain foods, pasteurization also destroys some natural enzymes (e.g., phosphatase in milk). The temperature and time are set to the lowest level to meet the microbiological objectives and to minimize thermal damage of the food, which otherwise could reduce the acceptance quality (such as heated flavor in milk) or pose processing difficulties (such as coagulation of liquid egg). Depending on the temperature used, thermoduric cells of spoilage bacteria and spores of pathogenic and spoilage bacteria survive the treatment. Thus, additional methods need to be used to control the growth of survivors (as well as post-pasteurization contaminants) of pasteurized products unless a product has a natural safety factor (e.g., low pH in some acid products). Refrigeration, modified atmosphere packaging, incorporation of preservatives, reduction of $A_W$, and other techniques are used, when possible in combination, to prevent or retard the problem of microbial growth in low-heat-processed products. Microbial heat-stable enzymes and toxins are not destroyed unless a food is heated for 30 minutes or longer at or above 90°C (194°F).

Pasteurization of milk has been used for a long time. Two methods, heating at 145°F (62.8°C) for 30 minutes or 161°F (71.7°C) for 15 seconds, are used to destroy the most heat resistant Q fever–causing pathogen, *Coxiella burnetii*. The methods are also designated, respectively, as low-temperature, long-time (LTLT) and high-temperature, short-time (HTST) methods. As indicated previously, regulation requires every particle of milk to be heated at 145°F for 30 minutes or at 161°F for 15 seconds (holding time). Foods that are not uniformly heated to the specified temperature and time can be involved in foodborne diseases. Immediately after the holding time, the milk is cooled to 40°F (4.5°C), packaged, and maintained at that temperature until consumed. Low-heat treatment is used for processing many products by using different times and temperatures, for example, ice cream mix, 180°F (82.3°C) for 25 seconds or 160°F (71.2°C) for 30 minutes; liquid whole egg, 140°F (60°C) for 3.5 minutes (intended to destroy *Salmonella*); fruit juices, 60°C–70°C (140°F–158°F) for 15 minutes or 80°C–85°C (176°F–185°F) for one minute; wine, 82°C–85°C (179.6°F–185°F) for one minute; pickles in acid (pH 3.7), 74°C (165.2°F) for 15 minutes; vinegar, 65.6°C–71.1°C for one minute or 60°C for 30 minutes (heating of all low-pH products is designed to destroy aciduric spoilage microorganisms); crab meat, 70°C (158°F) for 10 minutes; low-heat-processed meat products, 60°C–70°C (140°F–158°F) internal temperature (depending on the size, it can take two hours for the center of a product to reach the desired temperature; heating is not intended to kill *Clo. botulinum* Type A or nonproteolytic B spores). The time and temperature of pasteurization for different foods is also specified by regulatory agencies.

In addition to the use of hot water (e.g., for milk) or moist heat (e.g., for meat products) to heat process some foods, other products, such as dried egg whites and dried coconut, are pasteurized by dry heat. In such a condition, the products are exposed to 50°C–70°C (122°F–158°F) for five to seven days.

In the production of some fermented foods, the raw materials are heated to a high temperature to destroy vegetative cells of pathogens and spoilage microorganisms, which include thermoduric bacterial cells. Raw milk used for the production of buttermilk, acidophilus milk, and yogurt are given a 30- to 60-minute heat treatment at approximately 90°C (194°F) before adding starter-culture bacteria. Heating helps the starter culture bacteria grow preferentially, in addition to improving the gelling properties of the milk proteins at low pH.

### *High-Heat-Processed Foods*

This process involves heating foods at or above 100°C (212°F) for a desired period of time. The temperature and time of heating are selected on the basis of product characteristics and the specific

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microorganisms to be destroyed. Most products are given a commercially sterile treatment to destroy microorganisms growing in a product under normal storage conditions. Low-acid or high-pH (pH > 4.6) products are given 12*D* treatment to destroy *Clo. botulinum* Type A and B spores (the most resistant spores of a pathogen). However, the products can have viable spores of thermophilic spoilage bacteria (e.g., *Bacillus stearothermophilus*, *Bac. coagulans*, *Clo. thermosaccharolyticum*, and *Desulfotomaculum nigrificans*; see Chapter 20). As long as the products are stored at or below 30°C (86°F), these spores will not germinate. If the products are temperature abused to 40°C (104°F) and above even for a short time, the spores will germinate. Subsequent storage at or below 30°C will not prevent outgrowth and multiplication of these thermophiles to cause food spoilage. The time and temperature required for commercial sterility of a particular food are determined by actual pack inoculation studies. Generally, *Clo. sporogenes* PA 3679 is used to simulate *Clo. botulinum* because this is a nonpathogenic strain, but the spores have the same heat resistance as *Clo. botulinum* Type A or B (both proteolytic and nonproteolytic). For spoilage control studies, spores of *Bac. stearothermophilus* are used because spores of this species are the most heat resistant.

For high-acid or low-pH (pH ≤ 4.6) products, such as tomato products, fruit products, and acidified foods, a much lower heat treatment is used. Because *Clo. botulinum* spores cannot germinate or grow at this low pH, their presence is of little health hazard significance. The spore formers that can germinate and grow in low-pH products (e.g., *Bac. coagulans*) and the aciduric non-spore-forming bacteria (e.g., *Lactobacillus* and *Leuconostoc* spp.), yeasts, and molds that can grow at low pH are relatively heat sensitive. These products are generally heated to approximately 100°C (212°F) for a desired period of time. High-heat-treated products are either first packed in containers and then heated or heated first and then packed in sterile containers while still hot (hot pack).

Commercial sterility is also obtained by heating a food at very high temperatures for a short time. This process is designated as ultrahigh temperature (UHT) processing. Milk heated to approximately 150°C (302°F) for two to three seconds can be stored at room temperatures (≤30°C), and the products generally have a three-month shelf life. However, if microbial heat-stable proteinases or lipases are present in the raw milk, the product can show spoilage. In the UHT process, the milk is heated by injecting steam at high pressure for a rapid temperature increase. Following heat treatment in bulk, the milk is packed in small serving containers. Microbial heat-sensitive toxins will be destroyed, but heat-stable toxins may remain active even after heating for commercial sterility.

Under special circumstances, foods are heated to destroy all microorganisms (cells and spores) and to achieve sterility. Sterile foods are necessary for immunosuppressed individuals in order to avoid any complications from the microorganisms that are normally present in heated but nonsterile foods.

## *Microwave Heating*

Heating or cooking foods in the microwave at home is quite common in both developed and developing countries.[7,8] Frozen foods can be thawed and heated very rapidly, in a few minutes, depending on the size of the product. However, the method has not been well accepted as a source of rapidly generated high heat for commercial operations.

In a microwave oven, the waves change their polarity very quickly. Oppositely charged water molecules in a food rapidly move to align along the waves. The movement of the water molecules generates frictional heat, causing the temperature of the food to rise very rapidly. Depending on the exposure time and intensity of the wave, the temperature can be very high. Microwave

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treatment is quite lethal to microorganisms, and the destruction is caused by the high temperature. At present, microwave-heated foods cannot be considered safe from pathogens. Generally, when a food is heated in a microwave oven, it is not heated uniformly and some areas can remain cold. If a food harbors pathogens, there are chances that they will survive in the cold spots.[7]

## Conclusion

Heating has been used in food preparation and preservation long before civilization. Following the recognition of microbial roles in food spoilage and foodborne diseases, precise heating techniques have been developed to destroy vegetative cells of yeasts, molds, bacteria, viruses, and spores of bacteria. These include pasteurization, commercial sterilization, and UHT. Destruction of microorganisms by these methods is achieved by heat treatment of a food at a specific temperature for a specific time period. Microbial destruction at a high temperature results from structural and functional destabilization of cells and spores. At a lower heating temperature, the cells and spores can be sublethally injured and stressed. Because they are not dead, these two phenomena have important implications in developing heating methods to destroy microorganisms in food. Because extreme heat to kill all microorganisms cannot always be used, other methods are used mainly to prevent growth of microorganisms as well as to maintain acceptance qualities of food. One such method is low temperature and is discussed in Chapter 34.

### QUESTIONS

1. Discuss the ideological differences in the use of heating in food preservation before and after A.D. 1870.
2. List the microbiologically related objectives of heating a food.
3. Describe the mechanisms of sublethal and lethal heat injury in bacterial cells and spores. How can stress proteins alter this effect in food processed slowly at low heat?
4. List three important factors that one should consider in designing thermal preservation of a food. Discuss the implications of one of the factors.
5. Define $D$ value, $Z$ value, $F$ value, and 12$D$ value. Draw hypothetical plots to show how $D$ and $Z$ values can indicate relative heat resistance of two microbial species.
6. List the objectives of pasteurization of food. How do these objectives differ from those used in commercial sterilization of foods? Use a food system for each method of treatment to justify your explanations.
7. How does microwave heating differ from conventional heating to ensure food safety?
8. The SPC/mL of raw milk from two suppliers is as follows: (A) SPC/mL 95,000 (with thermodurics 15,000/mL) and (B) SPC/mL 65,000 (with thermodurics 25,000/mL). Briefly discuss $D$ value differences that a processor has to adopt to meet the legal bacterial counts for pasteurized Grade A milk. Can both be sold as Grade A pasteurized milk? Explain.

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# Chapter 34

# Control by Low Temperature

## Introduction

The effectiveness of low temperature, especially freezing, in food preservation was probably recognized by our ancestors at least in the last Ice Age, before 40,000 B.C. Natural freezing and thawing could also have been used to preserve food during the very early stages of civilization in approximately 10,000–12,000 B.C. In the colder regions of the world, foods (e.g., meat and fish) are still preserved in natural ice. Ice was used by the wealthy Romans to reduce the temperature of foods. Until approximately A.D. 1800, ice blocks from frozen lakes were cut, stored, and used to preserve raw foods (e.g., meats, milk, fish, and produce) by lowering the temperature. By 1840, with the help of ammonia-compressed refrigerator units, ice blocks were produced commercially and used to reduce the temperature of food for preservation. In 1880, refrigeration was used on ships and trains in Europe to transport meat and fish from other countries. Linde, in Germany, developed the first domestic refrigerator in approximately 1874 and started commercial production before 1890. The popularity of domestic refrigerators was initially slow in the United States; in 1930, 2%–3% of the households had the units. In the United States, methods to freeze fruits and vegetables were developed and commercially used around the 1930s. During this time, retail stores also started using cabinets to display frozen foods.

During World War II (1941–1944), consumer interest in refrigerated and frozen foods increased dramatically, which helped develop the technology necessary for processing, transporting, retailing, and home storage of refrigerated and frozen foods in the United States. The popularity of refrigerated and frozen foods has increased steadily since then. In the 1960s, 1970s, and early 1980s, frozen food consumption increased sharply, mainly because of the long shelf life. Since the mid-1980s, there has been increased interest in refrigerated and chilled foods, which consumers view as natural and healthy. The major drawback of many refrigerated foods is their relatively short shelf life, approximately one to two weeks. But in recent years, several technological improvements, such as oxygen-impermeable packaging materials, good vacuum-packaging equipment, innovative packaging systems, low-temperature refrigeration units (≤–1°C), and use of the hurdle concept to preserve food (Chapter 41) have helped increase the shelf life of many refrigerated foods to 60 days and, for some products, to more than 90 days. At present, of the total foods consumed, low-temperature preserved foods constitute more than 65%, and the trend



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looks to steadily increase in the future. To suit the taste of consumers, many new products are being developed that are low in fat (caloric); high in fiber, phosphates, and other additives; and have low amounts of or no preservatives. To achieve the long shelf life and to make these products safe, extra precautions are being introduced for microbiological control. This has helped many new or emerging pathogenic and spoilage bacteria, in the absence of competition from associated microorganisms, to become predominant (Chapter 29). New designs of processing equipment for high-production efficiency, centralized production of large volumes of products, transportation of products for long distances in regional storage facilities, retailing conditions, and consumers' handling of the products have facilitated these so-called new pathogenic and spoilage bacteria in gaining prominence (Chapter 21). Unless some effective intervention strategies are developed, new pathogenic and spoilage microorganisms will continue to surface in refrigerated foods stored for a long time.[1]

## Objectives

The main microbiological objective in low-temperature preservation of food is to prevent or reduce growth of microorganisms. Low temperatures also reduce or prevent catalytic activity of microbial enzymes, especially heat-stable proteinases and lipases. Germination of spores is also reduced. Low-temperature storage, especially freezing (and thawing), is also lethal to microbial cells, and under specific conditions, 90% or more of the population can die during low-temperature preservation. However, the death rate of microorganisms at low temperature, as compared with that of heat treatment, cannot be predicted (as $D$ and $Z$ values in heating). Also, spores are not killed at low temperatures. Thus, foods are not preserved at low temperature in order to kill microbial cells. Freezing is also used to preserve starter cultures for use in food bioprocessing. This has been discussed in Chapter 14.

## Mechanisms of Cold-Induced Inactivation

The metabolic activities, enzymatic reactions, and growth rates of microorganisms are maximized at the optimum growth temperature. As the temperature is lowered, microbial activities associated with growth slow down. Normally, the generation time, within a certain range, is doubled for every 10°C (50°F) reduction in temperature. Thus, a species dividing every 60 minutes in a food at 22°C (71.6°F) will take 120 minutes to divide if the temperature is reduced to 12°C (53.6°F). At a lower range, generation time can be even higher than double. For example, if the temperature is reduced from 12°C (53.6°F) to 2°C (35.6°F) and the species can grow at 2°C, the generation time for the species could be more than 240 minutes. The lag and exponential phases and the germination time (of spores) for some psychrotrophs (mesophilic types) become increasingly longer as the temperature is reduced to approximately 0°C or even to approximately –1°C (33.8°F). At this temperature, nongrowing cells of some mesophiles (nonpsychrotrophic) and thermophiles may be injured and killed, especially if they are stored for a long time (weeks) at 2°C or below and the foods have low $A_W$, low pH, or preservatives. The rate of catalytic activity of some enzymes also decreases as the temperature of an environment is reduced.

Water is present in a food as free water and bound (with the hydrated molecules) water. As the temperature in a food system drops to approximately –2°C (28.4°F), free water in the food starts freezing and forming ice crystals (pure water freezes at 0°C, but in a food with solutes, it

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freezes below 0°C). As the temperature drops further and more ice crystals form, the solutes get concentrated in the remaining water, which, in turn, depresses the freezing point of the water in the solution. The $A_W$ is also reduced. When the temperature is reduced to approximately –20°C (–4°F), almost all the free water freezes.

As the temperature of a food is reduced below –2°C (28.4°F), free water inside the microbial cells also undergoes similar changes. At a slow rate of freezing, as the water molecules in the food start freezing, water molecules from inside the microbial cells migrate outside, causing dehydration of cells and concentration of solutes and ions inside. When the temperature is reduced further (below –20°C) so that the water in the food has frozen, water inside the cell also freezes. However, before that, microbial cells are exposed to low pH (resulting from concentration of ions) and low $A_W$ (resulting from concentration of solutes) inside and outside the cells. This can cause denaturation and destabilization of the structural and functional macromolecules (enzymes) in the microbial cells, whose stability and functions depend on their three-dimensional structures, and can injure the cells. If the freezing is rapid, very small ice crystals form quickly, and the cells are not exposed to the solution effect. This is practiced in the freeze preservation of starter cultures and frozen stock cultures.

Microbial cells subjected to freezing and thawing suffer sublethal (repairable) as well as lethal injury. Studies show that different components of the cell wall (or outer membrane) and cell membrane (or inner membrane) are injured. DNA strand break, ribosomal RNA degradation, and activation and inactivation of some enzymes have also been reported in some studies. In sublethally injured cells, the structural and functional injuries are reversible. In lethally injured (or dead) cells, the damages are irreversible (Chapter 10).[2–4]

## Influencing Factors

The effectiveness of low temperatures in controlling microbial growth and microbial enzymatic activity in food depends on many factors. These factors can be arranged into three groups: those unique to low temperatures, those related to the food environment, and those inherent in microorganisms. These factors not only help prevent or reduce the growth of microorganisms, but can also greatly influence the extent of sublethal and lethal injury that microorganisms incur in food preserved at low temperatures. An understanding of the influence of these factors and interaction among them is important to design an efficient and predictable method to preserve a specific food at low temperature.

### *Nature of Process*

At temperatures above freezing of free water (≤–2°C), different types of bacteria, molds, and yeasts can grow in a food. But the lag and exponential phases become longer as the temperature is reduced. In the low range, even a difference in <1°C can be highly important. A *Pseudomonas fluorescens* strain was reported to have a generation time of approximately 6.7 hours at 0.5°C but 32.2 hours at 0°C. Thus, a reduction in 0.5°C increased the generation time by approximately 4.5-fold. This is much more than the theoretical estimate, which suggests that the generation time doubles for every 10°C (50°F) reduction. Spores of some spoilage *Bacillus* and *Clostridium* spp. can germinate even at refrigeration temperatures (4.5°C or 40°F). Cells of some mesophiles and thermophiles can be sublethally injured as well as die as the temperature drops below 4.5°C.

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As the temperature is reduced enough to cause a large portion of the water to freeze, the growth of most microorganisms stops except for some psychrophilic bacteria, yeasts, and molds. Although there are conflicting reports, slow growth probably can occur up to –10°C (14°F), especially by some molds. As the temperature drops further, to approximately –20°C (–4°F), and water in a food freezes completely, more cells will have sublethal and lethal injury. The rate of freezing and the lowest temperature of freezing dictate the extent of microbial damage from ice crystals. Damage and death are more extensive at a slower rate of freezing and at –20°C than at a rapid rate of freezing and at –78°C or –196°C (temperatures of solid $CO_2$ and liquid $N_2$, respectively). Death and sublethal injury are very high during initial storage (ca. seven days) but subsequently slow down.

Fluctuation of temperature of a food during low-temperature storage has great impact on growth, sublethal injury, and death of microorganisms. This quite readily happens to foods during storage, transport, retail display, and at home. A fluctuation of temperature of food from ≤4.4°C to 10°C–12°C not only stimulates rapid growth of psychrotrophic pathogenic and spoilage bacteria, but many mesophilic spoilage and pathogenic bacteria start to grow and their spores germinate at this range. Just from the spoilage aspect, six- to eight-hour temperature abuse (12°C) of a vacuum-packaged, refrigerated, low-heat-processed meat product can reduce its expected shelf life of eight weeks by approximately 7–10 days. A fluctuation in temperature of a frozen food increases microbial death and injury because of a repeated damaging solution effect and mechanical damage from larger ice crystals that form during repeated freezing and thawing. Dead microbial cells can also lyse, releasing intracellular enzymes, many of which (e.g., proteinases and lipases) can act on food components and reduce the acceptance quality of food (see Chapter 22).

The rate of cooling of a food is also very important for effective control of the growth of pathogenic and spoilage microorganisms. A slow rate of cooling of foods has been implicated as a major cause of foodborne diseases (Chapter 24). This can occur by trying to cool a large volume of hot or warm food in a big (deep) container in a refrigerator or overstuffing refrigerators with hot or warm foods. During thawing of a frozen food (such as an uncooked chicken), rapid thawing is desirable in order to control microbial growth, especially growth of pathogens. If the food is thawed slowly, the temperature on the food surface will soon increase, thereby allowing microbial growth, even when the inside is still frozen.

Refrigerated foods have limited shelf life, and, with time, microorganisms grow and spoil the products. In frozen foods, microorganisms (only cells, not spores) slowly die. However, even after long storage, some survive in frozen foods.

## *Nature of Food*

Composition, pH, $A_W$, and presence of microbial inhibitors (preservatives) in a food can greatly influence growth, sublethal injury, and viability of microorganisms during storage at low temperatures. A food with higher solid content (especially high proteins, carbohydrates, and lipids, but low ions), pH closer to 7.0, higher $A_W$, and the absence of microbial inhibitors facilitate growth and survival of microorganisms at refrigeration temperatures and inflict less injury and cause less death at frozen temperatures. Thus, the shelf life of refrigerated foods can be increased by using one or more of these factors, such as low pH, low $A_W$, incorporation of suitable microbial inhibitors, and, when possible, vacuum or modified air packaging (see Chapter 41).

In packaged frozen foods, ice may form in the packages (package ice), especially if the storage temperature fluctuates. During thawing, the ice melts and is absorbed by the food, resulting in an increase in the $A_W$ in a localized area (e.g., in a bread) and making it susceptible to microbial growth after thawing.

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### Nature of Microorganisms

Although some microorganisms can grow at as low as –10°C (14°F), many mesophilic and thermophilic bacterial cells can be sublethally injured and may die with time at low temperatures above freezing. At temperatures below –10°C, vegetative cells of microorganisms can sustain sublethal injury and die. In general, gram-negative or rod-shaped bacteria are more susceptible to the damaging effect of freezing than gram-positive or spherical-shaped bacteria. Also, cells from the early exponential phase of growth are more susceptible to freezing than those from the early stationary phase. Species and strains of microorganisms also differ greatly in sensitivity and resistance to freezing damage. Germination and outgrowth of spores of some *Clostridium* spp. can occur at as low as 2°C (35.6°F) and maybe at a slightly higher temperature for some *Bacillus* spp. spores. Spores do not lose their viability in frozen foods. Some microbial enzymes, either heat stable or released by the dead and lysed cells, can catalyze reactions at temperatures above –20°C (–4°F) but at a slow rate and can reduce the acceptance quality of a food.[4]

## Methods

Foods are stored at low temperatures in different ways to extend their shelf life. Many fresh fruits and vegetables are kept at temperatures between 10°C (50°F) and 20°C (68°F) or lower, mainly to reduce their metabolic rate. Microorganisms to which these products are susceptible, namely yeasts and molds (and some bacteria), can grow at this temperature. Maintaining a low relative humidity to prevent moisture buildup on the food surface is very important to reduce their growth. Highly perishable products are generally stored at a low temperature, below 7°C (44.6°F), often in combination with other preservation methods. The importance of rapid cooling of a food for microbiological safety has been mentioned before. The methods used for low-temperature preservation of foods and in food safety are briefly discussed.[5]

### Ice Chilling

This is used in retail stores where the foods are kept over ice; the surface in contact with the ice can reach between 0°C and 1°C (33.8°F).[6] Fresh fish, seafood, meats, cut fruits, vegetable salads (in bags), different types of ready-to-eat salads (prepared at the retail store), salad dressings (high pH, low caloric), sous vide, and some ethnic foods (e.g., tofu) are stored by this method. The trend is increasing.

Temperature fluctuation (resulting from the size of the container or melting of ice), duration of storage (fresh or several days), and cross-contamination (raw fish, shrimp, oysters, and ready-to-eat fish salads in an open container in the same display case at the retail store) can cause microbiological problems, especially from foodborne pathogens.

### Refrigeration

The temperature specification for refrigeration of foods has changed over time (Chapter 21). Previously, 7°C (44.6°F) was considered a desirable temperature. However, technological improvements have made it economical to have domestic refrigeration units at 4°C–5°C (39.2°F–41°F). For perishable products, ≤4.4°C (40°F) is considered a desirable refrigeration temperature. Commercial food processors may use as low as ~1°C (33.8°F) for refrigeration of perishable foods (such as fresh meat and

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fish). For optimum refrigeration in commercial facilities along with low temperatures, the relative humidity and proper spacing of the products are also controlled.

Raw and processed foods of plant and animal origin, as well as many prepared and ready-to-eat foods, are now preserved by refrigeration. Their volume is increasing rapidly because consumers prefer such foods. Some of these foods are expected to have a storage life of 60 days or more.

For refrigerated products expected to have a long shelf life, additional preservation methods are combined with the lowest possible temperature that can be used (close to –1°C). However, as the products are nonsterile, even a very low initial microbial population (e.g., ≤10 cells or spores per 10 g) capable of growing (or germinating) under the storage condition can multiply to reach hazard (for pathogen) or spoilage levels, thereby reducing the safety and stability of the product. Any fluctuation in temperature or other abuse (e.g., a leak in a vacuum or modified atmosphere package or oxygen permeation through the packaging materials) can greatly accelerate their growth. The processing and storage conditions may provide environments in which different types of spoilage and pathogenic microorganisms grow advantageously. This may increase spoilage and wastage of foods unless appropriate control measures are installed quickly.

### *Freezing*

The minimum temperature used in home freezers (in the refrigerator) is –20°C (–4°F), a temperature at which most of the free water in a food remains in a frozen state. Dry ice (–78°C) and liquid nitrogen (–196°C) can also be used for freezing; they are used for rapid freezing (instant freezing) and not for only freezing a food to that low temperature. Following freezing, the temperature of the food is maintained at approximately –20°C (–4°F) to –30°C (–22°F). Depending on the type, foods can be stored in a freezer for months or even more than a year. Many raw produce (vegetables, fruits), meat, fish, processed products, and cooked products (ready-to-eat) are preserved by freezing.

Microorganisms do not grow at –20°C (–4°F) in frozen foods. Instead, microbial cells die during frozen storage. However, the survivors can multiply in the unfrozen foods. Accidental thawing or slow thawing can facilitate growth of survivors (spoilage and pathogenic microorganisms); spores can also germinate and outgrow, depending on the temperature and time following thawing. Enzymes released by the dead microbial cells can reduce the acceptance quality of the food.

## Conclusion

Microbial growth in a food depends on the storage temperature and the microbial types. On the basis of these two factors and the specific need, foods are stored below room temperature, refrigerated, or frozen. Although microorganisms cannot grow in frozen foods, some can grow in refrigerated foods. In addition to preventing and retarding growth, microbial cells are also injured and killed at frozen and refrigerated temperatures. Because refrigeration maintains the acceptance quality of foods, lower temperatures (~ –1° to 5°C, i.e., 30.2°F–41°F) are used in combination with other factors (low $A_W$, pH, etc.) to extend the shelf life of foods for 60 days or more. Like low temperature, reduced $A_W$ is also used to control microbial growth in food and is discussed in Chapter 35.

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**QUESTIONS**

1. List the microbiological objectives of low-temperature preservation of food. How do these objectives differ from those for food preserved by heat?
2. Briefly discuss the mechanisms of microbial control by reducing the temperature of a food to 10°C, to –1°C, to –10°C, and to –20°C.
3. List the major factors that need to be considered for effective control of microorganisms in a food at low temperatures and briefly discuss the importance of each.
4. Discuss the microbial implications of the following in low-temperature preservation of foods: (a) fluctuation of storage (refrigerated and frozen) temperature, (b) slow cooling of a warm food, and (c) slow thawing of a frozen food.
5. Briefly discuss the microbiological problems of foods stored by chilling, refrigeration, and freezing.
6. List the psychrotrophic pathogens that can cause a food hazard in refrigerated ready-to-eat foods and suggest a method that can be used to overcome the problem (consult Chapter 41).

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# Chapter 35

# Control by Reduced Water Activity and Drying

## Introduction

The ability of dried seeds, grains, tubers, and fruits to resist spoilage was probably recognized by humans even before their discovery of agriculture. Subsequently, this simple method (drying) was practiced to preserve the large volume of foods produced during growing seasons to make them available during nongrowing seasons. Later, reduced $A_W$ was also extended to preserve other foods (e.g., meat, fish, and milk) not only by removing water by drying, but also by adding solutes (e.g., salt, honey, and starch) to bind water.

Since the beginning, reduced $A_W$[1] has been used throughout human civilization in many ways not only to preserve foods and stabilize the food supply, but also to develop different types of shelf-stable foods. Some of these include salted fish and meats; semidry and dry fermented sausages; dried fish, meat, vegetables, and fruits; evaporated and sweetened condensed milk; and dry milk, cheeses, bread and bakery products, flour, cereals, molasses, jams and jellies, chocolate, noodles, crackers, dried potatoes, dried eggs, and confectioneries. In more recent years, new technologies have helped produce foods with low $A_W$ by freeze-drying, puffed-drying, freeze-concentration, and osmotic-concentration methods. In addition, a better understanding of the relationship and influence of moisture and $A_W$ on microbial growth has been instrumental in producing many types of intermediate-moisture ready-to-eat foods. Efforts are also in progress to use low $A_W$ along with other microbial control parameters, such as low pH, vacuum packaging, and low heat, to produce ready-to-eat meat, fish, and dairy products that can be stored at ambient temperatures for relatively long periods of time. Because of their convenience, these foods are popular; the trend shows that the popularity will continue.

## Objectives

The main objective of reducing $A_W$ in food is to prevent or reduce the growth of vegetative cells and germination and outgrowth of spores of microorganisms. Prevention of toxin production by toxigenic molds and bacteria is also an important consideration. Microbial cells (not spores) also



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suffer reversible injury and death in foods with low $A_W$ although not in a predictable manner as in heat treatment. Finally, reduced $A_W$ is also used to retain viability of starter-culture bacteria for use in food bioprocessing, which is discussed in Chapter 14. In this chapter, preservation of food by controlling microbial growth at low $A_W$ is described.

## Mechanism of Action

Microorganisms need water for transport of nutrients, nutrient metabolism, and removal of cellular wastes. In a food, the total water (moisture) is present as free water and bound water; the latter remains bound to hydrophilic colloids and solutes (it can also remain as capillary water or in a frozen state as ice crystals) and is not available for biological functions. Thus, only the free water (which is related to $A_W$) is important for microbial growth. Microorganisms also retain a slightly lower $A_W$ inside the cells than in the external environment to maintain turgor pressure, and this is important for cell growth. If the free water in the environment is reduced either by removing water or by adding solutes and hydrophilic colloids, which cannot readily enter the cells, the free water from the cells flows outside in an effort to establish equilibrium. The loss of water causes an osmotic shock and plasmolysis, during which the cells do not grow. The water loss can be quite considerable even with a slight reduction in $A_W$. A 0.005 reduction in $A_W$ from 0.955 to 0.950 in the environment reduces the intracellular water content by 50% in *Staphylococcus aureus* and reduces the cell volume by 44% in *Salmonella enterica* ser. Typhimurium. Hence, even a slight reduction in $A_W$, necessary for minimal growth of a microbial species or strain, prevents its growth. Unless a microbial cell regains its intracellular turgor by reducing internal $A_W$, it will either remain dormant or die. This is often the case with microorganisms sensitive to slight $A_W$ reduction. However, some other microorganisms have developed very effective mechanisms such as transporting solutes inside or metabolizing solutes to overcome plasmolysis and regain turgor. Microorganisms that are relatively resistant to a great reduction in $A_W$ and grow at relatively lower $A_W$ have this capability.[2,3]

## Influencing Factors

### *Nature of Process*

Water activity and the total amount of water (% moisture) a food contains are different. The $A_W$ of a food indicates the amount or fraction of the total amount of water available for some chemical or biochemical reactions. In pure water, both values are the same, but in food, $A_W$ is always less than the total amount of water. Under a set of conditions, the relationship between the moisture content and the $A_W$ of a food can be determined from the sorption isotherm. However, instead of a single line, the sorption isotherm forms a loop (hysteresis loop), depending on whether it is determined during removal of water from (desorption) or during addition of water to (adsorption) a food (see Figure 6.1). At the same moisture level, the $A_W$ value obtained by desorption is lower than that obtained by adsorption. In controlling microbial growth by reducing $A_W$, this is quite important.

Solutes differ in their ability to reduce $A_W$. The amounts (% w/w) of NaCl, sucrose, glucose, and inverted sugar required to reduce $A_W$ at 25°C of pure water to 0.99 are 1.74, 15.45, 8.9, and 4.11 g; and to 0.92, they are 11.9, 54.34, 43.72, and 32.87 g, respectively. These solutes do not freely enter the microbial cells and thus have a greater inhibitory effect on microbial cells

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as compared with solutes that enter freely in cells (e.g., glycerol), which are required in higher amounts for similar inhibition.

Although microorganisms can be injured and killed at reduced $A_W$, a lower $A_W$ value is less detrimental. For a 90% reduction of salmonellae population in a product at 15°C (59°F), it took 27 days at an $A_W$ of 0.71 and 67 days at an $A_W$ of 0.34.

Studies on minimal $A_W$ values to support the growth of specific microorganisms have generated conflicting data. This could be a result of the inherent problems with different techniques used to measure $A_W$. However, with the modern electronic hygrometers, this problem is expected to be minimized.[1,3,4]

## Nature of Foods

Minimal $A_W$ values for growth of microorganisms, as well as influence of $A_W$ on viability loss, vary with the food characteristics and the food environment. In a homogeneous food, $A_W$ will remain unchanged provided other factors do not change. However, a heterogeneous food with ingredients or items of different $A_W$ (e.g., a sandwich or a meal with different items in the same package) will generate a gradient. This can lead to microbial growth in an item preserved by reduced $A_W$ alone and stored with an item of high $A_W$ containing a preservative. Also, condensation of water during storage with temperature fluctuation, followed by dripping of moisture in some areas of food, can alter a safe $A_W$ level in these areas to an unsafe state.

The minimum $A_W$ for growth of microorganisms in a food can be higher than that in a broth. Thus, *Sta. aureus* has a minimal $A_W$ for growth of 0.86 in a broth, but it does not grow in shrimp at an $A_W$ of 0.89. As the $A_W$ is reduced, anaerobic bacteria will require more of an anaerobic environment for growth. *Clostridium perfringens* grew in a broth with an $A_W$ of 0.995 at an O-R potential of +194 mV; when the $A_W$ was reduced to 0.975, an O-R potential of +66 mV was required for growth. As the incubation temperature is moved in either direction from optimum without changing the $A_W$, the microorganisms require a longer time to grow. In a broth of $A_W$ of 0.975, a *Clo. botulinum* E strain grew in six days at 30°C, in 19 days at 15°C, and in 42 days at 7.2°C. The minimum $A_W$ for growth of *Sta. aureus* was 0.865 at 30°C but changed to 0.878 at 25°C. Reduced $A_W$ and low pH interact favorably in inhibiting microbial growth. A *Clo. botulinum* B strain grew at an $A_W$ of 0.99 up to pH 5.3 and at an $A_W$ of 0.97 up to pH 6.0, but at an $A_W$ of 0.95, it failed to grow even at pH 7.0. Similarly, a spoilage strain of *Clo. butyricum* grew at an $A_W$ of 0.98 up to pH 3.8, but at an $A_W$ of 0.97, it failed to grow at pH 4.5 even after 30 days at 30°C. Many chemical preservatives enhance the inhibitory effect of lower $A_W$ on microbial growth. In the presence of low concentrations of sorbate, citrate, and phosphate, different microorganisms were found not to grow at the lowest $A_W$ in which they grew in the absence of these chemicals.

Food composition can influence microbial death rate even at the same $A_W$. At an $A_W$ of 0.33, *Escherichia coli* counts reduced by $\log_{10}$ 2.8 in ice cream powder and $\log_{10}$ 4.8 in dried potatoes but by more than $\log_{10}$ 6 in coffee. Under the same conditions, the death rate of *Enterococcus faecalis* was much less. Although the survivors remain dormant in a low-$A_W$ food, as soon as it is rehydrated, the microorganisms regain the ability to metabolize and multiply. Thus, a rehydrated food should be treated as a perishable food that, unless effective control methods are used, can be unsafe and spoiled.[3]

## Nature of Microorganisms

Microorganisms differ greatly in their minimal $A_W$ requirement for growth, sporulation, and germination (Table 35.1). In general, molds and yeasts can grow at lower $A_W$ values than bacteria;

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**Table 35.1 Minimum $A_W$ for Microbial Growth at Optimum Growth Temperature**

| *Microorganism* | $A_W$ |
|---|---|
| **Bacteria** | |
| *Bacillus cereus* | 0.95 |
| *Bacillus stearothermophilus* | 0.93 |
| *Clostridium botulinum* Type A | 0.95 |
| *Clostridium botulinum* Type B | 0.94 |
| *Clostridium botulinum* Type E | 0.97 |
| *Clostridium perfringens* | 0.95 |
| *Escherichia coli* | 0.95 |
| *Salmonella enterica* | 0.95 |
| *Vibrio parahaemolyticus* | 0.94 |
| *Staphylococcus aureus* | 0.86 |
| *Pseudomonas fluorescens* | 0.97 |
| *Lactobacillus viridescens* | 0.94 |
| **Yeast** | |
| *Saccharomyces cerevisiae* | 0.90 |
| *Saccharomyces rouxii* | 0.62 |
| *Debaryomyces hansenii* | 0.83 |
| ***Molds*** | |
| *Rhizopus nigricans* | 0.93 |
| *Penicillium chrysogenum* | 0.79 |
| *Penicillium patulum* | 0.81 |
| *Aspergillus flavus* | 0.78 |
| *Aspergillus niger* | 0.77 |
| *Alternaria citri* | 0.84 |

among pathogenic and spoilage bacteria, gram-negatives require a slightly higher $A_W$ than gram-positives for growth. *Sta. aureus*, however, can grow at an $A_W$ of 0.86. Sporulation by spore-forming bacteria occurs at $A_W$ values in which the species and strains grow; whereas germination may occur at slightly lower $A_W$ values. Toxin production may occur at the $A_W$ of growth (by *Clo. botulinum*) or at a slightly higher than minimum $A_W$ of growth (by *Sta. aureus* at 0.867 at 30°C

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and 0.887 at 25°C). The minimum $A_W$ for microbial growth can vary with the type of solutes in a food. *Clo. botulinum* Type E failed to grow below an $A_W$ of 0.97 when NaCl was used as a solute, but grew up to an $A_W$ of 0.94 when glycerol was the solute. *Pseudomonas fluorescens* similarly showed a minimum $A_W$ of 0.957 for growth with NaCl but 0.94 with glycerol. This is because glycerol enters freely inside the cell and thus does not cause osmotic stress as do nonpermeable NaCl, sucrose, and similar solutes. Germination of spores in glycerol occurs at lower $A_W$ because *Clo. botulinum* E spores, with glycerol, germinate at an $A_W$ of 0.89 but do not grow below an $A_W$ of 0.94. The growth rate of microorganisms also decreases as the $A_W$ value is lowered. The growth rate of *Sta. aureus* reduces to about the 10% level at an $A_W$ of 0.90 of its optimum growth rate at an $A_W$ of 0.99.

Among microbial groups, halophiles, osmophiles, and xerophiles grow better or grow preferentially at lower $A_W$ values. Halophiles, such as some vibrios, need NaCl in varying amounts for growth. Osmophiles and xerophiles, yeasts, and molds grow at an $A_W$ less than 0.85 as they do not have competition from bacteria.

Optimum growth of most microorganisms in foods occurs at an $A_W \geq 0.98$. At $A_W \geq 0.98$, gram-negatives, having a faster growth rate, predominate if other needs for optimum growth are fulfilled. As the $A_W$ drops to 0.97, gram-positive bacteria, such as Bacilli, Lactobacilli, Micrococci, and Clostridia, predominate. Below an $A_W$ of 0.93, gram-positive bacteria, such as Micrococci, Staphylococci, Enterococci, and Pediococci as well as yeasts and molds grow preferentially. As the $A_W$ drops below 0.86, osmophilic yeasts and xerophilic molds predominate. They can grow in foods with an $A_W$ up to 0.6; as $A_W$ drops below 0.6, microbial growth stops.[3,5,6]

## Methods

The water activity of foods can be reduced by using one or more of these three basic principles: (1) removing water by dehydration, (2) removing water by crystallization, or (3) adding solutes to bind water. Some of these methods and their effects on microorganisms are briefly described.[4,7]

### *Natural Dehydration*

Natural dehydration is a low-cost method in which water is removed by the heat of the sun. It is used to dry grains as well as to dry some fruits (raisins), vegetables, fish, meat, milk, and curd (from milk), especially in warmer countries. The process is slow; depending on the conditions used, spoilage and pathogenic bacteria as well as yeasts and molds (including toxigenic types) can grow during drying.

### *Mechanical Drying*

Mechanical drying is a controlled process, and drying is achieved in a few seconds to a few hours. Some of the methods used are tunnel drying (in which a food travels through a tunnel against a flow of hot air that removes the water), roller drying (in which a liquid is dried by applying a thin layer on the surface of a roller drum heated from inside), and spray drying (in which a liquid is sprayed in small droplets, which then come in contact with hot air that dries the droplets instantly). Vegetables, fruits, fruit juices, milk, coffee, tea, and meat jerky are some foods that are dried by mechanical means. Liquids may be partially concentrated before drying by evaporation, reverse osmosis, freeze-concentration, and addition of solutes.

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Depending on the temperature and time of exposure, some microbial cells can die during drying; whereas other cells can be sublethally injured. Also, during storage, depending on the storage conditions, microbial cells can die rapidly at the initial stage and then at a slow rate. Spores generally survive and remain viable during storage in a dried food.

## *Freeze Drying*

The acceptance quality of food is least affected by freeze-drying as compared with both natural and mechanical drying. However, freeze-drying is a relatively costly process. It can be used for both solid and liquid foods. The process initially involves freezing the food, preferably rapidly at a low temperature, and then exposing the frozen food to a relatively high vacuum environment. The water molecules are removed from the food by sublimation (from solid state to vapor state) without affecting its shape or size. The method has been used to produce freeze-dried vegetables, fruits, fruit juices, coffee, tea, and meat and fish products, some as specialty products.[8] Freeze-drying is a popular method of food preservation for space foods for astronauts, especially the side dishes, such as green beans, corns, scrambled eggs (Figure 35.1), tomatoes, and artichokes, etc.[9] These foods




**Figure 35.1 (See color insert.) Freeze-dried scrambled egg for astronauts. Water is added to rehydrate the food before consumption. (Courtesy of NASA, Johnson Space Center, Houston, TX.)**

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are rehydrated before consumption. Microbial cells are exposed to two stresses—freezing and drying—that reduce some viability as well as inducing sublethal injury. During storage, especially at a high storage temperature and in the presence of oxygen, cells die rapidly initially and then more slowly. Spores are not affected by the process.

### *Foam Drying*

The foam-drying method consists of whipping a product to produce stable foam to increase the surface area. The foam is then dried by warm air. Liquid products, such as egg whites, fruit purees, and tomato paste, are dried in this manner. The method itself has very little lethal effect on microbial cells and spores. However, a concentration method before foaming, the pH of the products, and low $A_W$ cause both lethal and reversible damages to microbial cells.

### *Smoking*

Many meat and fish products are exposed to low heat and smoke for cooking and depositing smoke on the surface at the same time. The heating process removes water from the products, thereby lowering their $A_W$. Many low-heat-processed meat products (dry and semidry sausages) and smoked fishes are produced this way. Heat kills many microorganisms. The growth of the survivors is controlled by low $A_W$ as well as the many types of antimicrobial substances present in the smoke.

### *Intermediate Moisture Foods*

Intermediate moisture foods (IMF) have $A_W$ values of approximately 0.70–0.90 (with moisture contents of ca. 10%–40%). They can be eaten without rehydration but are shelf stable for a relatively long period of time without refrigeration and are considered microbiologically safe. Some of the traditional IMFs include salami, liverwurst, semidry and dry sausages, dried fruits, jams and jellies, and honey. However, in recent years, many other products have been developed, such as pop tarts, Slim Jims, ready-to-spread frosting, breakfast squares, soft candies, fruit rolls, food sticks, and soft granola bars. The low $A_W$ value and relatively high moisture is obtained by adding water-binding solutes and hydrophilic colloids. Microorganisms can survive in the products, but because of low $A_W$, bacteria cannot grow. However, yeasts and molds can grow in some. To inhibit their growth, specific preservatives, such as sorbate and propionate, are added or irradiated.[10,11]

## Conclusion

The $A_W$ of a food is directly proportional to the level of free water it contains. As microbial growth is adversely affected even with a slight reduction in $A_W$, it has been used, where possible, to retard and prevent growth mainly of spoilage bacteria in food. It is achieved either by removing or binding the free water. At lower $A_W$, microbial cells can lose viability and become injured with time. However, this alone cannot be used to ensure safety of food from pathogens. Although, as a single method, it has limited application in food preservation, along with other methods, such as low temperature or low pH, it can be used to extend the shelf life of food very effectively. The effectiveness of low pH, either alone or with other methods, in controlling microorganisms in food is discussed in Chapter 36.

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**QUESTIONS**

1. List the microbiological objectives of reducing $A_W$ in foods.
2. Discuss the mechanisms by which low $A_W$ produces an antimicrobial effect.
3. Define sorption isotherm. Describe the hysteresis loop and discuss how there can be problems in selecting the correct $A_W$ in a food from the hysteresis loop.
4. Discuss the influences of different solutes in lowering the $A_W$ value of a food and controlling microbial growth. (Use NaCl, sucrose, and glycerol as examples.)
5. Briefly describe the interaction of $A_W$ with other intrinsic and extrinsic factors in controlling microorganisms in a food.
6. List how different microorganisms are controlled in a food as the $A_W$ value drops from 0.99 to 0.60. Discuss the minimal $A_W$ for growth, toxin production, sporulation, and germination by a bacterial species (*Clo. botulinum*).
7. Briefly explain the microbiological concerns of naturally dried foods, freeze-dried foods, smoked foods, rehydrated foods, and IMFs.
8. Briefly discuss how in the rural areas in many developing countries lower $A_W$ along with lower pH and a suitable preservative can be used to extend the shelf life of some perishable foods (use one example) at room temperature (see Chapter 40).

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# *Chapter 36*

# Control by Low pH and Organic Acids

## Introduction

During the early stages of human history when food was scarce, our ancestors probably recognized that some foods from plant sources, especially fruits, resisted spoilage. Later, they observed that the fermented foods and beverages prepared from fruits, vegetables, milk, fish, and meat were much more shelf stable than the raw materials from which they were produced. That probably provided an incentive to develop large varieties of fermented foods, especially in tropical areas where, unless preserved, many foods spoil rapidly.[1]

When the microbial involvement in food spoilage and foodborne diseases was recognized, methods to control their growth as well as to kill them in food were studied. It was observed that over a restricted pH range, many microorganisms present in food can grow, but at lower pH ranges many of them die. Once this was recognized, many organic acids were used as food additives. In addition to their effectiveness as food preservatives, they are also used to improve the acceptance qualities of foods. The amounts and types of organic acids that can be added to foods are governed by regulatory agencies.

Organic acids can be present in foods in three ways. They can be present naturally, such as citric acid in citrus fruits, benzoic acid in cranberries, and sorbic acid in rowan berries. Some, such as acetic, lactic, and propionic acids, are produced in different fermented foods by desirable food-grade starter-culture bacteria as fermentation byproducts. Many acids are also added to foods and beverages to reduce the pH. Among the organic acids used in food as preservatives are acetic, propionic, lactic, citric, sorbic, and benzoic acids; their salts; and some derivatives of benzoic acid (e.g., paraben). The influence of these acids in reducing food pH and producing antimicrobial effects are briefly discussed in this chapter.



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## Objectives

The major antimicrobial objective of using weak organic acids is to reduce the pH of food to control microbial growth. As the pH drops below 5.0, some bacteria become injured and die. However, the death rate in low pH is not predictable as in the case of heat. Thus, it could not be used with the objective of destroying a predictable percentage of a microbial population in the normal pH range of foods.[1,2]

## Mechanisms of Antimicrobial Action

The antimicrobial action of a weak organic acid is produced by the combined actions of the undissociated molecules and the dissociated ions.[2,3] Microorganisms that are important in food tend to maintain an internal cytoplasmic pH of approximately 6.5–7.0 in acidophiles and 7.5–8.0 in neutrophiles. The internal pH (pHi) is tightly regulated and drops by approximately 0.1 unit for each 1.0 unit change in the environmental pH (pHo). For nutrient transport and energy synthesis, the microorganisms also maintain a transmembrane pH gradient (ca. 0.5–1.0 unit with alkaline pHi) and a proton gradient (ca. 200 mV); together, they form the proton motive force (PMF).

When a weak organic acid is added to the environment (in a food), depending on the pH of the food, the pK of the acid, and the temperature, some of the molecules dissociate, whereas others remain undissociated (Table 36.1). At the pH of most foods (pH 5–8), except for paraben, the organic acid molecules remain generally dissociated; as a result, $[H^+]$ in the environment increases, which interferes with the transmembrane proton gradient of microbial cells. To overcome this, the cells transport protons through the proton pump, which causes depletion in energy and a decrease in pHi. The structures on the cell surface, outer membrane or cell wall, inner membrane or cytoplasmic membrane, and periplasmic space are also exposed to $[H^+]$. This

**Table 36.1 Infuence of pH on the Amount (%) of Dissociated Ions of Weak Organic Acids**

| | | *% Dissociated at pH* | | |
|---|---|---|---|---|
| *Acid* | *pK* | *4* | *5* | *6* |
| Acetic | 4.8 | 15.5 | 65.1 | 94.9 |
| Propionic | 4.9 | 12.4 | 58.3 | 93.3 |
| Lactic | 3.8 | 60.8 | 93.9 | 99.3 |
| Citric | 3.1 | 81.1 | 99.6 | >99.1 |
| Sorbic | 4.8 | 18.0 | 70.0 | 95.9 |
| Benzoic | 4.2 | 40.7 | 87.2 | 98.6 |
| Paraben[a] | 8.5 | <0.1 | 0.1 | 0.3 |

[a] Paraben: esters of benzoic acids.

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can adversely affect the ionic bonds of the macromolecules and thus interfere with their three-dimensional structures and some related functions. At pH < 5.0, the undissociated molecules of some acid can be considerably high. Being lipophilic (except citric), they enter freely through the membrane as a function of the concentration gradient. Because the pHi is much higher than the pK of the acid, the molecules dissociate, releasing protons and anions. Some anions (e.g., acetate and lactate) are metabolized by several microorganisms as a carbon source. If they are not metabolized, the anions are removed from the cell interior. However, the $[H^+]$ will reduce the internal pH and adversely affect the proton gradient. To overcome this problem, the cells pump out the excess protons by expending energy. At lower pHo (pH 4.5 or below), this represents an expense of a large amount of energy that cells may not be able to generate. As a result, the internal pH drops, adversely affecting the pH gradient. The low pH can also act on the cellular components (such as proteins) and adversely affect their structural (by interfering with the ionic bonds) and functional integrity.[2–4]

These changes can interfere with the nutrient transport and energy generation and, in turn, interfere with microbial growth. In addition, low pH can reversibly and irreversibly damage cellular macromolecules, which can subsequently inflict sublethal as well as lethal injury to cells. Low pH can alter the ionic environment of the spore coat by replacing its ions (e.g., $Ca^{2+}$) with $H^+$ and make the spores unstable toward other environmental stresses, such as heat and low $A_W$.

## Influencing Factors

### *Nature of Acids*

The weak organic acids used in food vary in antimicrobial effectiveness because of their differences in pK.[4] An acid with higher pK has proportionately higher amounts of undissociated molecules at a food pH and is more antimicrobial. Limited studies have revealed that, in general, under similar conditions, the antimicrobial effectiveness of four acids follows the order acetic > propionic > lactic > citric. Similarly, at lower pH and higher concentrations, an acid is more antimicrobial. Solubility of the acids in water is also important for the desirable effect. Acetate, propionate, lactate, and citrate are very soluble in water, whereas benzoate (50% g), sorbate (0.16% g), and paraben (0.02%–0.16% g) are poorly soluble in water and, thus, at the same concentration, have different effectiveness. In many studies, the antimicrobial effectiveness of these acids against microorganisms is studied on a percentage basis (g in 100 mL). However, they vary in molecular weight; thus, at the same concentration, they have different numbers of molecules and produce different concentrations of undissociated molecules as well as dissociated ions. For a comparison, it is better to use the acids on a molar concentration basis (see Chapter 17).

Organic acids also differ in their lipophilic properties, which, in turn, regulate their ease in entering the cells. Acetic and propionic acids are more lipophilic than lactic acid and have more antimicrobial effectiveness than lactic acid. Citrate is transported through the membrane by a specific transport mechanism (citrate permease) and is less effective than lipophilic acids. Many microorganisms can metabolize the anions of weak acids, such as acetate, lactate, and citrate. Use of salts of these acids may decrease the antimicrobial effect at higher pH. Some acids show synergistic effects when used in suitable combinations (e.g., acetic and lactic acids; propionic and sorbic acids) or with another preservative (e.g., benzoic acid with nisin; propionic, acetic, or lactic acid with nisin or pediocin AcH; propionate or benzoate with $CO_2$).[1,4,5]

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### Nature of Foods

The normal pH of foods varies greatly from a very acid range (pH 3.0; citrus juice) to an alkaline range (pH 9.0; egg albumen). The initial pH can strongly influence the antimicrobial effect of an acid. An acid is more inhibitory in a food at a lower pH than in one at a higher pH. The buffering action of the food components also reduces the effectiveness of low pH. Nutrients can also facilitate repair of sublethal acid injury of microorganisms.

### Nature of Microorganisms

Microorganisms important in food vary greatly in the lower limit of pH that allows growth (Table 36.2). In general, gram-negative bacteria are more sensitive to low pH than are gram-positive bacteria, and yeasts and molds are the least sensitive. Fermentative bacteria are more resistant to lower pH than are respiring bacteria, probably because they are able to resist changes in external pH as well as withstand slightly lower internal pH. The ability of yeasts and molds to withstand low pH is also a result of these factors.[5] The antimicrobial property of an organic acid is enhanced by heat, low $A_W$, the presence of some other preservatives, and low storage temperatures.

The inhibitory effect of weak acids is reduced at a higher microbial load. Also, in a mixed microbial population, the metabolism of an acid (such as lactate) by one resistant species can reduce its effective concentration against another sensitive species, allowing the latter to grow. Some microorganisms important in food, such as some *Salmonella* serovars and *Esc. coli* O157:H7, seem to have genetic determinants that enable them to grow at higher acid concentrations (or lower pH) than other strains of the same species. The acid tolerance seems to be related to overproduction of a group of proteins (stress proteins) by these strains (see Chapter 10).

Finally, microorganisms differ in their sensitivity to different organic acids. Yeasts and molds are particularly sensitive to propionic and sorbic acids, and bacteria are more sensitive to acetic acid. Bacterial spores at lower pH become susceptible to heat treatment and do not germinate and outgrow at minimum $A_W$ of growth. Also, the inhibitory effect of $NO_2$ against spores is more pronounced at the lower pH range of growth.

## Acids Used

### Acetic Acid

Acetic acid is used usually as vinegar (5%–10% acetic acid) or as salts of sodium and calcium at 25% or higher levels in pickles, salad dressings, and sauces. It is more effective against bacteria than yeasts and molds. Bacteria that grow better above pH 6.0 are more inhibited. The inhibitory concentrations of undissociated acid are 0.02% against *Salmonella*, 0.01% against *Staphylococcus aureus*, 0.02% against *Bacillus cereus*, 0.1% against *Aspergillus* spp., and 0.5% against *Saccharomyces* spp. The inhibitory action of acetic acid is produced through neutralizing the electrochemical gradient of the cell membrane as well as denaturing proteins inside the cells. Besides its use in food, acetic acid has been recommended for use (1%–2%) in carcass wash to reduce bacterial loads.[1,2]

### Propionic Acid

Propionic acid is used as salts of calcium and sodium at 1000–2000 ppm (0.1%–0.2%) in bread, bakery products, cheeses, jams and jellies, and tomato puree. It is effective against molds and

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**Table 36.2 Minimum pH at which Growth Occurs**

| *Microorganism* | *Minimum Growth pH* |
|---|---|
| **Gram-Negative Bacteria** | |
| *Escherichia coli* | 4.4 |
| *Pseudomonas* spp. | 5.6 |
| *Salmonella* serovars | 4.5 |
| *Vibrio* spp. | 4.8 |
| *Serratia* spp. | 4.4 |
| **Gram-Positive Bacteria** | |
| *Bacillus cereus* | 4.9 |
| *Bacillus stearothermophilus* | 5.2 |
| *Clostridium botulinum* | 4.6 |
| *Clostridium perfringens* | 5.0 |
| *Enterococcus faecalis* | 4.4 |
| *Lactobacillus* spp. | 3.8 |
| *Staphylococcus aureus* | 4.0 |
| *Listeria monocytogenes* | 4.6 |
| ***Yeasts*** | |
| *Candida* spp. | 1.5–2.3 |
| *Saccharomyces* spp. | 2.1–2.4 |
| *Hansenula* spp. | 2.1 |
| *Rhodotorula* spp. | 1.5 |
| ***Molds*** | |
| *Aspergillus* spp. | 1.6 |
| *Penicillium* spp. | 1.6–1.9 |
| *Fusarium* spp. | 1.8 |

bacteria but almost ineffective against yeasts at concentrations used in foods. The inhibitory concentration of undissociated acid is 0.05% against molds and bacteria. The antimicrobial action is produced through the acidification of cytoplasm as well as destabilization of membrane proton gradients.[2]

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## Lactic Acid

Lactic acid is used as acid or sodium and potassium salts up to 2% in carbonated drinks, salad dressings, pickled vegetables, low-heat-processed meat products, and sauces. It is less effective than acetic, propionic, benzoic, or sorbic acids, but more effective than citric acid. It is more effective against bacteria but quite ineffective against yeasts and molds. It produces an inhibitory effect mainly by neutralizing the membrane proton gradient. The sodium salt of lactic acid may also reduce $A_W$. L(+)-lactic acid is preferred over D(–)-lactic acid as a food preservative. It has also been recommended at a 1%–2% level to wash carcasses of food animals to reduce microbial load.[2,6]

## Citric Acid

Citric acid is used at 1% (or more) in nonalcoholic drinks, jams and jellies, baking products, cheeses, canned vegetables, and sauces. It is less effective than lactic acid against bacteria as well as yeasts and molds. Its antibacterial effect is probably by a mechanism different than that for lipophilic acids. The antibacterial effect is partially a result of its ability to chelate divalent cations. However, many foods can have sufficient divalent cations to neutralize this effect.

## Sorbic Acid

Sorbic acid is an unsaturated acid and used either as acid or as salts of sodium, potassium, or calcium. It is used in nonalcoholic drinks, some alcoholic drinks, processed fruits and vegetables, dairy desserts, confectioneries, mayonnaise, salad dressings, spreads, and mustards. The concentrations used vary from 500 to 2000 ppm (0.05%–0.2%). It is more effective against molds and yeasts than against bacteria. Among bacteria, catalase-negative species (e.g., lactic acid bacteria) are more resistant than catalase-positive species (e.g., aerobes, *Sta. aureus*, and *Bacillus* spp.). Also, aerobic bacteria are more sensitive to it than anaerobic bacteria. The inhibitory concentrations of undissociated acid are as follows: ≤0.01% (100 ppm) for *Pseudomonas* spp., *Sta. aureus*, *Esc. coli*, and *Serratia* spp.; ~0.1% for *Lactobacillus* spp. and *Salmonella* serovars; and ≤0.02% for most yeasts and molds, but ~1.0% for *Clostridium* spp.

The antimicrobial effect of sorbate is produced through its inhibitory action on the functions of some enzymes, some from the citric acid cycle. It also interferes with synthesis of the cell wall, protein, RNA, and DNA. Also, like other organic acids, it interferes with the membrane potential and inhibits spore germination.[2,7]

## Benzoic Acid

Benzoic acid is used as an acid or sodium salt at 500–2000 ppm (0.05%–0.2%) in many low-pH products, such as nonalcoholic and alcoholic beverages, pickles, confectioneries, mayonnaise and salad dressings, mustards, and cottage cheese. It is more effective against yeasts and molds than bacteria. The inhibitory effect is produced by both the undissociated and dissociated acids. The inhibitory concentrations of undissociated acid are 0.01%–0.02% against bacteria and 0.05%–0.1% against yeasts and molds. The inhibitory action is produced in several ways. It inhibits the functions of many enzymes necessary for oxidative phosphorylation. Like other acids, it also destroys the membrane potential. In addition, it inhibits functions of membrane proteins.[2,8,9]

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### *Parabens (Esters of p-Hydroxybenzoic Acid)*

Parabens are used as methyl, ethyl, butyl, or propyl parabens. They are broad-spectrum antimicrobial agents and used in food and cosmetic and pharmaceutical products. Because of high pK values, they are effective at high pH and against bacteria, yeasts, and molds. They are used at 100–1000 ppm (0.01%–0.1%) in nonalcoholic and alcoholic beverages, fruit fillings, jams and jellies, pickles, confectioneries, salad dressings, spreads, and mustards. The undissociated inhibitory concentrations are 0.05%–0.1% against yeasts, molds, and bacteria. Propyl and butyl parabens are more inhibitory than the others.

Parabens produce antimicrobial action by acting on several targets of microbial cells. They may inhibit the functions of several enzymes. They dissolve membrane lipids and interfere with membrane functions, including transport of nutrients. They also interfere with the synthesis of proteins, RNA, and DNA. In addition, they destroy the membrane potential as other weak organic acids do. However, toxic side effects of parabens, such as reproductive toxicity resulting from an estrogenic effect, asthma, allergic response, and dermatitis have raised serious concerns in recent years.[10,11]

## Conclusion

A pH lower than the minimum growth pH prevents microbial growth by affecting energy production, enzymatic activity, transportation of nutrients, and others. In addition to controlling growth, microbial cells are sublethally injured or killed by low pH. Many food-grade organic acids along with other methods are used to control microbial growth in food. Low pH is also used to prevent germination of bacterial spores in food. Although many Gram-negative pathogenic bacteria are very sensitive to low pH, it cannot be used to eliminate these pathogens during storage of food. The fact that some strains can become acid resistant has created concern about such a practice in the processing and preservation of some low-pH foods. In addition to low pH and the methods discussed before, low O-R potential is used to control microbial growth in food and is discussed in Chapter 37.

### QUESTIONS

1. Discuss the mechanisms of antimicrobial action of weak organic acids and low pH. Explain the differences in sensitivity to low pH among gram-negative and gram-positive bacteria, bacterial spores, yeasts, and molds.
2. Define the pK of an organic acid and, using acetic and lactic acids as examples, describe their differences in antibacterial effect at pH 5 and 6.
3. Briefly discuss the influence of the following factors on antimicrobial effectiveness of low pH: acids, foods, and microorganisms.
4. List the specific acids to be used to inhibit growth of gram-positive bacteria, gram-negative bacteria, yeasts, and molds.
5. Discuss the antimicrobial properties of acetic acid, propionic acid, lactic acid, benzoic acid, and parabens.
6. Some low-pH foods, such as apple juice, orange juice, and fermented sausage, have recently been implicated in foodborne disease outbreaks by strains of *Esc. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*. Explain the possible reasons and suggest methods that could be used to reduce this problem.

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# Chapter 37

# Control by Modified Atmosphere (or Reducing O-R Potential)

## Introduction

Humans probably recognized, in the early ages of agriculture and animal husbandry, that many foods were susceptible to quality loss when stored in air. This probably led to the preservation of foods in approximately 6000 B.C. by initially excluding air in pits and later, with the invention of pottery and baskets, in large vessels and containers. Grains, semidry products, some fermented products, concentrated syrups and molasses, and similar products are still stored in large, closed, airtight vessels in many parts of the world to prevent insect infestation and the growth of molds and yeasts. However, this method of changing the gaseous environment to preserve more perishable products, such as fresh meat, fish, fruits and vegetables, and other processed products, originated in the early 20th century. During the 1920s and 1930s, studies revealed that by using $CO_2$ in higher concentrations (4%–100%), the growth of molds on fresh meats could be greatly reduced, and ripening of fruits and vegetables could be prolonged. In the 1960s, refrigerated beef carcasses were transported by ship from New Zealand and Australia to other countries in controlled $CO_2$-rich environments.

In the 1960s, the availability of suitable plastic materials for packaging and the necessary technology helped develop methods to preserve foods in different-sized packages in an altered atmosphere. This was achieved either by vacuum packaging or by gas flushing the package with one gas or a mixture of gases. During storage, the gaseous environment could change because of the metabolic activities of the food products and microorganisms and also as a result of the permeability of gases from the air through the packaging materials.[1,2]

In recent years, modification of the storage environment to preserve foods has become one of the methods used most often. The desire of consumers for fresh foods, foods that have been given fewer processing treatments to maintain nutritive value, and without undesirable preservatives, as well as the availability of the necessary technology, have helped in the economical production of



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many convenient and ready-to-eat foods that are preserved by altering the environment. As the demand for such food is expected to grow in the future, the use of an altered atmosphere to preserve these foods will increase.[1–6]

Four terminologies are used to alter the atmosphere in foods in order to preserve and increase their acceptance quality. To avoid confusion, these terms are defined here:

1. *Controlled atmosphere packaging (CAP).* In this method, the atmosphere in a storage facility is altered and the levels of the gases are continually monitored and adjusted as required. This is expensive and used for long-term storage of fruits and vegetables to maintain their freshness.
2. *Modified atmosphere packaging (MAP).* This method, unlike CAP, does not require a high degree of control of the gaseous environment during the entire storage period. In this method, a food is enclosed in a high gas-barrier packaging material; the air is removed from the package, which is then flushed with a particular gas or combination of gases; and the package is hermetically sealed.
3. *Active packaging (AP).* The active components in the packaging system interact with the content or the environment to extend the shelf life and quality of the product. This includes moisture control, $O_2$ generators, $CO_2$ controllers, $O_2$-permeable films, $O_2$ scavengers, flavor enhancement, or odor removal, etc.
4. *Vacuum packaging (VP).* This method involves the removal of air from the package and then sealing the package hermetically.

MAP and VP have been described as two separate methods by some but as one method (e.g., MAP) by others. In this chapter, MAP has been used both to describe vacuum packaging and gas-flush packaging. Advantages and disadvantages of MAP are summarized in Table 37.1.

**Table 37.1 Advantages and Disadvantages of Modified Atmospheric Packaging (MAP)**

| *Advantages* | *Disadvantages* |
|---|---|
| • Maintains product quality and extends shelf life of products that traditionally have short shelf life, such as raw meats and fresh fruits and vegetables<br>• Prevents the use of chemical preservatives thus products are perceived as "natural" and "healthy"<br>• Reduced distribution costs as fewer deliveries over longer distances possible<br>• Allows transportation to remote destinations | • Different gas formulations are required for each product type<br>• Requires specialized and expensive equipment<br>• Requires temperature control<br>• Product safety needs to be established for different MAP systems<br>• Increased package volume adversely affects transport costs and retail space<br>• Beneficial effects are lost for the unused product once the package is opened for consumption or damaged |

*Source:* McMillin, K.W., *Meat Sci.*, *80*, 43–65, 2008; Rodriguez-Aguilera, R. and Oliveira, J., *Food Eng. Rev.*, *1*, 66–83, 2009.

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## Objectives

The objectives of MAP are to control or reduce the growth of undesirable microorganisms in food. The technique also helps retard enzymatic and respiratory activities of fresh foods. The growth of aerobes (molds, yeasts, and aerobic bacteria) is prevented in products that are either vacuum packaged or flushed with 100% $CO_2$, 100% $N_2$, or a mixture of $CO_2$ and $N_2$. However, under these conditions, anaerobic and facultative anaerobic bacteria can grow unless other techniques are used to control their growth.

## Mechanism of Action

The antimicrobial action in MAP foods can be produced by changes in the redox potential (Eh) and $CO_2$ concentrations based on the methods used. Aerobes and anaerobes, depending on the microbial species and reducing or oxidizing state of food, have different Eh requirements for growth, whereas facultative anaerobes grow over a wide Eh range. Vacuum packaging and gas flushing, especially with $CO_2$ or $N_2$ or their mixture and no $O_2$, discourage growth of aerobes but encourage growth of facultative anaerobes and anaerobes. However, even under these conditions of packaging, tissue oxygen and dissolved and trapped oxygen can allow the initial growth of aerobes to produce $CO_2$ even by the proteolytic microbes. In addition, natural reducing components in foods, such as the –SH group in protein-rich foods, and ascorbic acid and reducing sugars in fruits and vegetable products, can alter the Eh of a food and encourage growth of anaerobes and facultative anaerobes. Thus, it is not possible to control microbial growth just by changing the Eh. Other methods, in addition to modification of the environment, are necessary for effective preservation of foods. However, by controlling the growth of aerobic bacteria, many of which have short generation times, the shelf life of a product is greatly extended.

When $CO_2$ is used in high concentrations (20%–100%), alone or in combination with $N_2$ or $O_2$, or both, the shelf life of MAP foods is also extended. Several mechanisms by which $CO_2$ increases the lag and exponential phases of microorganisms have been proposed, such as rapid cellular penetration of $CO_2$ and alteration in cell permeability, solubilization of $CO_2$ to carbonic acid ($H_2CO_3$) in the cell with the reduction of the pH inside the cells, and interference of $CO_2$ with several enzymatic and biochemical pathways, which, in turn, slow the microbial growth rate. The inhibitory effect of $CO_2$ on microbial growth occurs at the 10% level and increases with the increase in concentration. Too high a concentration can inhibit growth of facultative spoilage bacteria and stimulate growth of *Clostridium botulinum*.[1,5]

## Influencing Factors

### *Nature of Process*

These factors include efficiency of the vacuum, permeability of the packaging materials to $O_2$, and the composition of gas (in the gas-flushing method) used.[6] A high-efficiency vacuum can effectively control the growth of aerobes by removing $O_2$ from the products, except probably trapped and dissolved $O_2$. Similarly, packaging films that prevent or considerably reduce permeation of $O_2$ during storage effectively control the growth of aerobes. Minute leaks in the film can adversely affect the protective effect of vacuum packaging or gas-flush packaging. The development of polymeric film has helped greatly reduce the $O_2$ permeability. In gas flushing, $CO_2$ and $N_2$, and in the case of fresh

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meat along with some $O_2$, are used in a mixture. $N_2$ is used as inert filler, and $O_2$ is used to give the red oxymyoglobin color to the meat. $CO_2$ is used for its antimicrobial effect. As low as 20% $CO_2$ has been found to control the growth of aerobes, such as *Pseudomonas*, *Acinetobacter*, and *Moraxella*; however, in general, $CO_2$ at 40%–60% gives better results. In some cases, 100% $CO_2$ is used.

### Nature of Foods

The amounts of oxygen (dissolved and trapped), metabolizable carbohydrates, and other nutrients and the reducing components present influence the growth of microbial types in a MAP food. In the presence of $O_2$, aerobes, such as *Pseudomonas*, utilize glucose and lactate. Growth of facultative anaerobes, however, discourages the growth of aerobes but can stimulate the growth of anaerobes. Reducing components also encourage the growth of anaerobes. The presence of specific inhibitors, either present in a food or produced by associated bacteria (such as bacteriocins by some lactic acid bacteria), low $A_W$, and low pH also influence the microbial ability to grow or not grow under specific packaging conditions.

### Nature of Microorganisms

Aerobes can have limited initial growth, depending on the oxygen present in a vacuum-packaged or gas-flushed packaged product. However, their growth will stop as soon as the facultative anaerobes start growing. These include lactic acid bacteria, *Brochothrix thermosphacta*, some *Enterobacteriaceae*, and *Corynebacteriaceae*. In low-pH products, a favorable growth of lactic acid bacteria can reduce or prevent growth of other bacteria because the former can produce acids and other antibacterial substances (e.g., bacteriocins). Also, the composition of the gas mixture influences the predominant types; with 100% $CO_2$, lactic acid bacteria, especially *Leuconostoc* and *Lactobacillus* spp., predominate. $CO_2$ at approximately 20% concentration controls growth of *Pseudomonas* spp., but a $CO_2$ concentration of more than 60% is required to control growth of *Enterobacteriaceae*. As the Eh of a product starts reducing, the anaerobes, especially *Clostridium* spp., start growing. The presence of reducing agents in the food also favors the growth of anaerobes. The possible growth of *Clo. botulinum* in MAP refrigerated foods, especially Type E and nonproteolytic Type B, is a major concern. Non-spore-forming psychrotrophic pathogens, especially the facultative anaerobes (*Listeria monocytogenes* and *Yersinia enterocolitica*), can also multiply in MAP foods. In addition, some mesophilic facultative anaerobic pathogens with growth capabilities at 10°C–12°C (some *Salmonella* and *Escherichia coli* O157:H7 strains and *Staphylococcus aureus*) can also grow if the MAP foods are temperature abused during storage. Suitable preservatives, low pH, or low $A_W$, or their combination, should be used as additional hurdles for their control.[4,6]

## Methods

### Vacuum Packaging

Vacuum packaging is predominantly used as retail packages in many fresh and ready-to-eat meat products, including beef, pork, lamb, chicken, and turkey. Packaged this way, red meat, because of a change in color to purple (reduced myoglobin), is not very popular with consumers. The refrigerated storage life of these products varies greatly: approximately three to four weeks in fresh meat and as long as eight weeks or more in processed meats. If the products have low $A_W$, or low pH, or both, they are produced under sanitary conditions.

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### Gas Flushing

The gas-flushing method is used in both bulk and retail packages to increase the shelf life of many refrigerated foods in European countries. In the United States, it is used in products, such as fresh pasta, bakery products, cooked poultry products, cooked egg products, fresh and cooked fish and seafood, sandwiches, raw meats, and some vegetables. The gases usually used are a mixture of $CO_2$ and $N_2$, with some $O_2$ for packaging red meats. Generally, the composition of gas mixtures must be tailored for each product. In raw meats, a composition of 75% $CO_2$, 15% $N_2$, and 10% $O_2$ was found to effectively prevent growth of *Pseudomonas fragi* for a limited period. Products flushed with $CO_2$ alone were dominated by lactic acid bacteria, principally *Leuconostoc* spp. and *Lactobacillus* spp. When $N_2$ and $CO_2$ were used together, along with lactic acid bacteria, *Bro. thermosphacta*, some coryneforms, and *Enterobacteriaceae* were also found. In the presence of some $O_2$, lactic acid bacteria, *Bro. thermosphacta*, *Enterobacteriaceae*, and *Pseudomonas* spp. were isolated. The product storage life, depending on a product, can be four weeks or more for fresh products and eight weeks or more for processed products.[2,4]

## Conclusion

The use of a modified atmosphere to reduce the O-R potential of the environment has been a very widely used method to control growth mainly of aerobic microorganisms in food. However, facultative anaerobes and anaerobes can gain an advantage because of the absence of competition from aerobes. They can also be controlled in an O-R potential–reduced food by combining other methods, especially some chemical preservatives, which are discussed in Chapter 38.

### QUESTIONS

1. Describe the methods used for the alteration of atmosphere to preserve foods.
2. Discuss the mechanisms of antimicrobial action in the MAP method used in foods.
3. Explain the different factors that can inhibit or influence microbial growth in a MAP food.
4. List the predominant microorganisms that can grow during the storage of vacuum-packaged and gas-flushed packaged foods.
5. What is the major microbiological concern of MAP foods? How can this be solved?
6. *Lis. monocytogenes* has been implicated in foodborne disease outbreaks from the consumption of vacuum-packaged, refrigerated, low-heat-processed meat products. These products are also spoiled (gas fermentation) by *Leuconostoc* spp. Briefly, discuss how these heat-sensitive bacterial species can be implicated as a health hazard and spoilage in the properly heated, packaged products. Also suggest a methodology to reduce the problems.

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# *Chapter 38*

# Control by Antimicrobial Preservatives and Bacteriophages

## Introduction

Many chemical compounds, either present naturally, formed during processing, or legally added as ingredients, can kill microorganisms or control their growth in foods. They are, as a group, designated as antimicrobial inhibitors or preservatives.[1–3] Some of the naturally occurring preservatives can be present in sufficient amounts in foods to produce antimicrobial action, such as lysozyme in egg whites and organic acids in citrus fruits. Similarly, some of the antimicrobials can be formed in enough quantities during food processing to control undesirable microbial growth, such as lactic acid in yogurt fermentation. However, others can be naturally present or formed in small quantities and essentially do not produce antimicrobial action in foods, for example, lysozyme in milk (13 mg/100 mL) and diacetyl in some fermented dairy products. Among the many food additives, some are specifically used to preserve foods against microorganisms, such as nitrite ($NO_2$) in cured meat to control spore germination, especially of *Clostridium botulinum*, whereas others are added principally to improve the functional properties of a food, such as butylhydroxyanisol (BHA) used as an antioxidant, although it has antimicrobial properties. Use of some of the preservatives started as far back as 6000 B.C. Examples include salting, which had nitrate ($NO_3$) as a contaminant, of fish and meat; burning sulfur to generate sulfur dioxide gas ($SO_2$) to sanitize the environment and equipment used for baking and brewing; smoking fish and meats; and adding spices, herbs, acids, and alcohol to foods (from fermentation).

Following the discovery of the microbial role in food spoilage and in foodborne diseases, many chemicals have been introduced since the late 1900s to control microorganisms in foods. The safety of the chemicals on humans was not tested before adding them to foods, and some of the chemicals used were later found to be harmful to humans. To protect U.S. consumers, in 1958, the Food Additive Amendment was passed (see Appendix C). According to this amendment, a food processor wanting to use a food preservative and other additives must prove their safety, by the procedures recommended,



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before their incorporation. This law, however, granted an exemption to substances used for a long time, found to be safe, and considered as generally regarded as safe (GRAS) substances.

In recent years, the possible effect of different preservatives and other additives on human health from long-term use through different foods has been questioned. Many of them are of nonfood origin or added at a level not normally present in foods, and some are synthetic. The effect of interactions of the different preservatives in the body, consumed through different foods, especially in children and debilitated and elderly people; the possible cumulative effect from the consumption over many years; and their interactions with each other and with other chemicals (e.g., medications) have not been determined. Many health-conscious consumers are interested in foods that do not contain any preservatives, especially those not normally found in foods or present in a much lower concentration than that added to foods. This has resulted in the search for preservatives that are either naturally present in the food of plant and animal origin or produced by safe food-grade microorganisms used to produce fermented foods. They are also designated as biopreservatives.[2] Some of these have been used, not always as preservatives, for a long time in foods and have been found to be safe (such as lactic, acetic, and propionic acids), whereas others have not been used directly as preservatives. Although many of them have been unknowingly consumed through foods without any adverse effects (such as bacteriocins of many lactic acid bacteria), they probably have to be tested for safety and regulatory approval before use in foods.

## Objectives

Antimicrobial chemicals are used in food in relatively small doses either to kill undesirable microorganisms or to prevent or retard their growth. They differ greatly in their abilities to act against different microorganisms. Some are effective against many microorganisms (broad spectrum), whereas others are effective against either molds and yeasts or only bacteria (narrow spectrum). Similarly, some compounds are effective against either gram-positive or gram-negative bacteria or bacterial spores or viruses. Those capable of killing microorganisms are designated as germicides (kill all types), fungicides, bactericides, sporicides, and viricides, depending on their specificity of killing actions against specific groups. Those that inhibit or retard microbial growth are classified as fungistatic or bacteriostatic. However, under the conditions in which most antimicrobials are used in foods, they cannot completely kill all the microorganisms or prevent their growth for a long time during storage. They can also cause injury.

## Influencing Factors

Several factors need to be considered in evaluating the suitability of an antimicrobial agent as a food preservative,[1,3,4] based on their antimicrobial properties, suitability for application in a food, and ability to meet regulatory requirements. Regarding antimicrobial properties, a compound that kills (-cidal) instead of controlling growth (-static) is preferred. Similarly, a compound with a broader antimicrobial spectrum is more suitable for application in foods so that it is effective against many types of microorganisms important in foods (namely, molds, yeasts, bacteria, and viruses) as compared with one that has a narrow spectrum. Also, a compound effective not only against vegetative cells but also against spores is preferred. Finally, it should not allow development of resistant strains. Most compounds do not meet all these requirements. Many times, more than one compound is used in combination to increase the inhibitory spectrum. In addition, food environments may restrict growth of many types

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of microorganisms. Under such circumstances, a preservative that can effectively control the growth of the microorganisms of concern can be used alone.

To be suitable for application in a food, a compound should not only have the desired antimicrobial property but also not affect the normal quality of a food (texture, flavor, or color). It should not interact with food constituents and become inactive. It should have a high antimicrobial property at the pH, $A_W$, Eh, and storage temperature of the food. It should be stable during the storage life of the food. Finally, it should be economical and readily available.

The regulatory requirements include the expected effectiveness of an antimicrobial agent in a food system. It should be effective in small concentrations and should not hide any fault of a food (e.g., conceal poor quality and spoilage). Most importantly, it should be safe for human consumption. Finally, when required, it should be listed on the label, indicating its purpose in the food.

## Examples of Antimicrobial Preservatives

Foods can have antimicrobial compounds in three ways: present naturally, formed during processing, or added as ingredients. Antimicrobial agents may be of animal, plant, or microbial origin or from other food-grade chemicals. Those added have to be GRAS-listed and approved by regulatory agencies. Some of these are added specifically as antimicrobial preservatives, whereas others, although they have antimicrobial properties, are added for different reasons. Those in the latter group are also called indirect antimicrobials. In this chapter, the antimicrobial effectiveness, mode of action, and uses in foods of some inorganic and organic antimicrobials added to foods are briefly discussed. Table 38.1 and 38.2 list some antimicrobial preservatives (direct or indirect) used in foods.[5,6]

**Table 38.1 Some Antimicrobial Chemical Preservatives[a] Used in Foods**

| | | | |
|---|---|---|---|
| Acetaldehyde | $CO_2$ and CO | Lauric acid | Propylene oxide |
| Acetic acid | Dehydroacetate | Lysozyme | Propyl gallate |
| Ascorbic acid | Diacetate | Malic acid | Smoke |
| Bacteriocins | Diacetyl | Methyl bromide | Sodium chloride |
| Benomyl | Diethyl bicarbonate | Monolaurin | Sorbic acid |
| Benzoic acid | Diphenyl | Natamycin | Spices |
| Betapropiolactum | Ethyl alcohol | Nitrite and nitrate | Succinic acid |
| BHA, BHT, and TBHQ | Ethyl formate | Parabens | Sucrose |
| Boric acid | EDTA | Peracetate | Sulfites and $SO_2$ |
| Caprylic acid | Ethylene oxide | Polyphosphates | Tetracyclines |
| Chitosan | $H_2O_2$ | Propionic acid | Thiabendazole |
| Citric acid | Lactic acid | Propylene glycol | Tylosin |

[a] Not all are permitted in the United States. The preservatives that are allowed by US FDA are listed in Table 38.2.

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**Table 38.2 FDA-Approved Antimicrobial Chemical Preservatives for Use in Foods**

| *Compound* | *Target Microbes* | *Foods Allowed* |
|---|---|---|
| Acetic acid, acetates, dehydroacetic acid | Yeasts, bacteria | Backed foods, condiments, confections, dairy products, fats and oils, meats, sauces |
| Benzoic acid, benzoates | Yeast, molds | Beverages, fruit products, margarine |
| Dimethyl dicarbonate | Yeasts | Beverages |
| Lactic acid, lactates | Bacteria | Meats, fermented foods |
| Lactoferrin | Bacteria | Meats |
| Lysozyme | *Clostridium botulinum*, other bacteria | Cheese, casings for frankfurters, cooked meat and poultry products |
| Natamycin | Molds | Cheese |
| Nisin | *Clostridium botulinum*, other bacteria | Cheese, casings for frankfurters, cooked meat and poultry products |
| Nitrite, nitrate | *Clostridium botulinum* | Cured meats |
| Parabens | Yeasts and molds | Beverages, backed goods, syrups, dry sausage |
| Propionic acid, propionates | Molds | Bakery products, dairy products |
| Sorbic acid, sorbates | Yeasts, molds and bacteria | Most foods, beverages, wines |
| Sulfites | Yeasts and molds | Fruits, fruit products, potato products, wines |

*Sources:* Adapted from Anonymous, *Comp. Rev. Food Sci. Food Safety*, *5*, 71–137, 2006; Weiss, J. et al., *Global Issues in Food Science and Technology*, B.-C. Gustavo, M. Alan, L. David, S. Walter, B. Ken, and Paul, C., Eds., Academic Press, San Diego, pp. 425–479, 2009.

## Food-Grade Chemicals

### *Nitrates and Nitrites*

Sodium and potassium nitrites ($NaNO_2$ and $KNO_2$) or sodium and potassium nitrates ($NaNO_3$ and $KNO_3$) are used as curing agents in the meat.[7] Curing agents that contain nitrite, together with NaCl, sugar, spices, ascorbate, and erythorbate, are permitted for use in heat-processed meat, poultry, and fish products to control growth and toxin production by *Clo. botulinum*. Nitrate and nitrite are also used in several European countries in some cheeses to prevent gas blowing by *Clo. butyricum* and *Clo. tyrobutyricum*. The mechanisms of antibacterial action of nitrite are not properly understood, but the inhibitory effect is probably produced in several ways, such as

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reactions with some enzymes in vegetative cells and germinating spores, restriction of the bacterial use of iron, and interference with membrane permeability, thereby limiting transport. In addition to clostridial species, nitrite is inhibitory, to some extent, to *Staphylococcus aureus*, *Escherichia*, *Pseudomonas*, and *Enterobacter* spp. at 200 ppm; *Lactobacillus* and *Salmonella* serovars seem to be resistant to this concentration of $NO_2$.

The antibacterial effect of $NO_2$ is enhanced at lower pH (pH 5.0–6.0), in the presence of reducing agents (e.g., ascorbate, erythorbate, and cysteine), and with sorbate. The current regulatory limit in the United States is 156 ppm of $NO_2$, but this varies widely in other countries. This amount can also be reduced by supplementing $NO_2$ with other reducing agents as well as sorbates. The $NO_2$ effect is also enhanced by reducing $A_W$ and at low Eh. In cured meat products, $NO_2$ reacts with myoglobin to form a stable pink color of nitrosyl hemochrome during heating. In bacon, nitrite can lead to the formation of carcinogenic compounds, such as nitrosoamines. Because of this, there is a trend to reduce $NO_2$ or to use other preservatives to control *Clo. botulinum* in low-heat-processed meat products.

## *Sulfur Dioxide ($SO_2$) and Sulfites ($SO_3$)*

Sulfur dioxide, sodium sulfite ($Na_2SO_3$), sodium bisulfide ($NaHSO_3$), and sodium metabisulfite ($Na_2S_2O_5$) are used to control microorganisms (and insects) in soft fruits, fruit juices, lemon juices, beverages, wines, sausages, pickles, and fresh shrimp.

Currently, these additives are not permitted in the United States in meat, as they destroy vitamin $B_1$. They are more effective against molds and yeasts than bacteria; among bacteria, the aerobic gram-negative rods are the most susceptible. The antimicrobial action is produced by the undissociated sulfurous acid that rapidly enters the cell and reacts with the thiol groups in structural proteins, enzymes, and cofactors, as well as with other cellular components. At low pH (≤4.5) and low $A_W$, the fungicidal effect is more pronounced. In bacteria, they are effective at high pH (≥5.0) but are probably bacteriostatic at lower concentrations and bactericidal at higher concentrations. The concentrations used in foods vary greatly in different countries. In the United States, 200–300 ppm is generally permitted for antimicrobial uses.

Sulfur dioxide and sulfites are also used as antioxidants in fresh and dried fruits and vegetables (salads) to prevent browning. However, people with respiratory problems can be mildly to severely allergic to sulfites. The products need to be labeled to show the presence of sulfites.

## *$H_2O_2$*

Hydrogen peroxide is also produced during microbial fermentation and is used as a food preservative (see Chapter 17). A solution of $H_2O_2$ (0.05%–0.1%) is recommended as an antimicrobial agent in raw milk to be used in cheese processing (to control growth of psychrotrophic gram-negatives that produce heat-stable enzymes), liquid egg to facilitate destruction of *Salmonella* by low-heat pasteurization, packaging material used in aseptic packaging of foods, and food processing equipment. In raw milk and liquid egg, catalase is used before pasteurization to hydrolyze $H_2O_2$ to water and oxygen. $H_2O_2$ is a strong oxidizing agent, and the germicidal action is associated with this property (see Chapter 17).

Recently, $H_2O_2$ has been used to produce modified plant fiber flour from straws for use in low-calorie foods; for bleaching and color improvement of grains, chocolate, instant tea, fish, sausage casings, and many others; and to reduce sulfite in wines. In the future, the use of $H_2O_2$ in foods is expected to increase.

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### Epoxides (Ethylene Oxide, Propylene Oxide)

Ethylene oxide and propylene oxide are used as fumigants to destroy microorganisms (and insects) in grains, cocoa powder, gums, nuts, dried fruits, spices, and packaging materials. They are germicidal and effective against cells, spores, and viruses. Ethylene oxide is more effective. Epoxides are alkylating agents and react with various groups (e.g., –SH, $–NH_2$, and –OH) in cellular macromolecules, particularly structural proteins and enzymes, adversely affecting their functions. They can react with some food components, such as chlorides, and form toxic compounds that can remain as residue in treated foods. They can be toxic at high concentrations (as residue), particularly to people who are sensitive to them.

### Organic Acids

Acetic, propionic, lactic, citric, benzoic, and sorbic acids are used as food preservatives and are discussed in Chapters 17 and 36. Organic acids are produced during microbial fermentation; however, production of many of them at the industrial scale requires further optimization.[8]

### Parabens

Methyl and propyl esters of *p*-hydroxybenzoic acids are discussed in Chapter 36.

### Diacetyl

Diacetyl is discussed in Chapter 17.

### $CO_2$

$CO_2$ is discussed in Chapter 37.

### Butylated Hydroxyanisol (BHA), Butylated Hydroxytoluene (BHT), and t-Butyl Hydroquinone (TBHQ)

BHA, BHT, and TBHQ are primarily used at 200 ppm or less as antioxidants to delay oxidation of unsaturated lipids. Additionally, they have antimicrobial properties and thus can be regarded as indirect antimicrobials. In concentrations of 50–400 ppm, BHA inhibits growth of many gram-positive and gram-negative bacteria; however, some species may be resistant to it. They also effectively prevent growth and toxin production by molds and growth of yeasts, but BHA seems to be more effective. The antimicrobial action is most likely produced by their adverse effect on the cell membrane and enzymes. Their antimicrobial effectiveness increases in the presence of sorbate but decreases in foods with high lipids and at low temperature.

### Monolaurin (Glycerol Monolaurate)

Monolaurin, the ester of lauric acid and glycerol, is one of the more effective bactericidal agents among the different derivatives of lauric acid tested in foods. Its effectiveness in deboned meat, chicken sausages, minced fish, and other foods has been observed against undesirable bacteria, particularly the anaerobes. It also enhances the thermal inactivation of spores of *Bacillus* spp. The

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antimicrobial property of this lipophilic compound is enhanced with lactate, sorbate, ascorbate, and nisin but may be reduced by starchy and proteinaceous compounds. In combination with monolaurin, the fungistatic activity of several antifungal compounds is enhanced. The antimicrobial activity of monolaurin is produced through its ability to destabilize the functions of the membrane. At lower concentrations, it is bacteriostatic by interfering with the uptake of nutrients. It can be used up to 500 ppm without affecting the taste of the food.

### *Ethylene-Diamine-Tetraacetate (EDTA)*

The sodium and calcium salts of EDTA at 100 ppm are approved for use in foods to chelate trace metals in order to prevent their adverse effect on food quality. At a low dose (5000 ppm), EDTA appears to have no toxic effect and mostly passes through the GI tract unabsorbed. By itself, EDTA may not have much antimicrobial effect, but because of its ability to chelate divalent cations, it can destabilize the barrier functions of the outer membrane of gram-negative bacteria and, to some extent, the cell wall of gram-positive bacteria. In this way, it enhances antibacterial action of other chemicals, especially those that are membrane acting, such as surface-active compounds, antioxidants, lysozymes, and bacteriocins. EDTA is also inhibitory for germination and outgrowth of spores of *Clo. botulinum*. In the presence of divalent cations in the food environment (e.g., dairy products), the effectiveness of EDTA is greatly reduced.

## Antimicrobials of Microbial Origin[1–4]

### *Bacteriocins of Lactic Acid Bacteria*

Bacteriocins of food-grade lactic acid bacteria, with special reference to nisin, pediocin AcH, and reuterine and their application as food preservatives, are discussed in Chapter 17.

### *Natamycin*

Natamycin, a microlid produced by *Streptomyces natalensis*, is a broad-spectrum antifungal agent. It is amphiphilic; thus it has a lower solubility in water than bacteriocins. Its use as a dip or spray to prevent growth of molds and formation of mycotoxins on the surface of some cheeses, sausages, and in raw peanuts has been approved by the Expert Committee of the World Health Organization (WHO). It is customarily used at 500 ppm, which leaves detectable but safe levels of the antibiotic on the product surface. It is also effective against fungal growth on fruits such as strawberries, cranberries, and raspberries. Natamycin binds to ergosterol on the fungal cell membrane and increases membrane permeability, leakage of essential ions and cell lysis. Because bacteria do not have sterol on their membrane, natamycin is ineffective against them.

### *Tylosin*

Tylosin, a microlid produced by *Streptomyces*, inhibits protein synthesis, is a bactericidal antibiotic that is more effective against gram-positive than gram-negative bacteria and also inhibits outgrowth of germinated endospores. Because of its high heat resistance, it has been studied at a low concentration (1 ppm) to determine its effectiveness in controlling the growth of spore formers in low-acid canned products.

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### Polylysine

Polylysine is produced by *Streptomyces actinomycetes* and is inhibitory to gram-positive and gram-negative bacteria including *Lis. monocytogenes*, *Salmonella*, *Esc. coli* O157:H7 and fungi. It is thermostable (100°C for 100 minutes or 120°C for 20 minutes), active in a wide range of pH, and water soluble and does not affect food flavor. It is widely used in Japan as a food preservative.

## Antimicrobials of Animal Origin[1,3]

### Chitosan

Chitosan, a polycationic polymer [poly (β-1,4)-2-amino-2-deoxy-D-glucopyranose], is obtained by alkaline hydrolysis of chitin from the shells of Crustaceans, such as shellfish and arthropods. It is inhibitory against bacteria, yeasts, and molds and causes destabilization of the cell wall and cell membrane functions. It is safe for human consumption. It has many applications in foods, including direct application into product formulations to improve shelf-life and also into packaging film to prevent microbial growth on the surface of products. It has low solubility in water; thus, it is dissolved in organic acids (acetic or lactic acid) for use during food processing. Acidic conditions may hydrolyze chitosan, so modified water-soluble stable derivatives have been developed. Chitosan coating on eggs prevented *Salmonella* Enteritidis, *Esc. coli*, and *Lis. monocytogenes* growth. Chitosan-based edible packaging films prepared in combination with essential oils and nisin protected the meat surface from growth of *Lis. monocytogenes* and *Esc. coli* O157:H7.

### Lysozyme

The enzyme lysozyme (a muramidase) is present in large quantities in some foods, such as egg whites and shellfish (oysters and clams), as well as in small amounts in milk and some plant tissues. It is also abundant in human saliva and other body fluids. It hydrolyzes the peptidoglycan (β-1,4 linkage between N-acetylmuramic acid and N-acetyl-glucosamine) layer present in the cell wall of gram-positive and gram-negative bacteria. In gram-negative bacteria the cell wall is located in the middle layer and lysozyme is effective only after the barrier function of the outer membrane (OM) is destabilized by chemical (e.g., EDTA) and physical (e.g., freezing or heating) stresses. The antimicrobial effect is manifested by the lysis of cells. Lysozyme is most effective at pH 6.0–7.0 and at concentrations of approximately 0.01%–0.1%. It can be used directly to control gram-positive bacteria and with EDTA and other similar compounds to control gram-negative bacteria. It has been used in wine (sake, a Japanese beverage) to prevent growth of undesirable lactic acid bacteria and in tuna and salmon products to control *Lis. monocytogenes*.

### Lactoferrin

Lactoferrin, an iron-binding glycoprotein is naturally present in milk and also in human and animal tissues and body fluids. It a has broad-spectrum antimicrobial effect against bacterial and viral pathogens. Besides, in humans and animals, it regulates immune function, intestinal proliferation, and facilitates iron adsorption. Its antimicrobial action depends on binding to the surface of bacteria, such as the outer membrane of gram-negative bacteria leading to damaged OM. Lactoferrin has a strong affinity for iron, rendering it unavailable for bacterial metabolism and thus preventing bacterial growth. It is now approved by USDA-FSIS for use on a variety of meat products.

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### Lactoperoxidase

Lactoperoxidase is an enzyme (glycoprotein) and is naturally present in raw milk, colostrum, saliva, and tears. The antimicrobial activity of lactoperoxidase is mediated by oxidizing thiocyanate ($SCN^-$) present in milk in the presence of hydrogen peroxide ($H_2O_2$) to form hypothiocyanate ($OSCN^-$) and hypothiocyanous acid (HOSCN). Both $OSCN^-$ and HOSCN have antimicrobial activity against pathogens, including *Staphylococcus aureus*, *Lis. monocytogenes*, *Esc. coli* O157:H7, *Salmonella*, and *Yersinia enterocolitica* in food products such as beef, vegetable juices, milk, and liquid whole egg.

### Ovotransferrin

Ovotransferrin, also known as conalbumin, is a glycoprotein present in egg whites, which also possesses antimicrobial activity. It is a metalloprotease and has strong affinity for iron, hence depriving bacteria from using iron. It is also effective against a broad range of spoilage and pathogenic bacteria. Because egg albumin is allergy-causing in certain consumers, its widespread use as a food preservative may be limited.

### Protamine

Protamine is a cationic peptide derived from Salmon. It has broad-spectrum activity against gram-positive and gram-negative bacteria and fungi. The antimicrobial activity is exerted by a strong electrostatic affinity of protamine to the negatively charged surface of bacteria during growth. It was found to be highly inhibitory to *Lis. monocytogenes*, *Clo. botulinum*, *Brochothrix thermosphacta*, and *Esc. coli*.

### Pleurocidin

Pleurocidin is a polypeptide present in mucosal and myeloid tissues of vertebrates and invertebrates. It has been isolated from the skin of flounder (fish) and has broad activity against gram-positive and gram-negative bacteria including *Lis. monocytogenes* and *Esc. coli* O157:H7. It is heat stable, salt tolerant, and nontoxic.

### Defensins

Defensins are cationic antimicrobial peptides, produced by epithelial cells and immune cells, and are responsible for natural immunity in mammals, insects, and birds. They have a broad range of activity against bacteria, viruses, and fungi. Honeybee defensins exist as two peptides: defensins 1 and 2. Defensin 1 is synthesized in the salivary glands and is responsible for social immunity, and defensin 2 is synthesized by hemolymph and is responsible for individual immunity.[4]

## Antimicrobials of Plant Origin

### Herbs and Spices[4,9,10]

Herbs and spices of plant origin are added to foods to enhance flavors and fragrances, and they possess antimicrobial activity. Antimicrobial activity is primarily associated with plant essential oils (EO), which are complex mixtures comprised of up to 45 different constituents, and the active components are divided into four major groups based on their structure: (i) terpenes,

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(ii) terpenoides, (iii) phenylpropenes, (iv) and others. Essential oils are generally more inhibitory against gram-positive than gram-negative bacteria. It is postulated that the lipopolysaccharide (LPS) of the outer membrane of gram-negative bacteria creates a barrier for active ingredients to interact with the bacteria. The antimicrobial action of phenolic compounds in essential oils is concentration-dependent. At low concentrations, phenols affect enzyme activity associated with energy production; at high concentrations, they cause protein denaturation. Essential oils alter cell membrane permeability, causing loss of macromolecules from the interior, and also interfere with membrane function, such as electron transport, nutrient uptake, protein, and nucleic acid synthesis, and enzyme activity.

The antimicrobial activity of EO is extracted from leaves (sage, rosemary, basil, oregano, thyme, and marjoram), flowers or buds (cloves), bulbs (onion, garlic), seeds (nutmeg, fennel, parsley, grape seed, etc.), fruits (pepper and cardamom), and barks and skins (cinnamon, lemon). Examples of some of the active components include cinnamaldehyde in cinnamon; eugenol (2-methoxy-4-allyl phenol) in

**Table 38.3 Antimicrobial Spectrum of Active Ingredients in Spice and Plant Extracts**

| *Active Components of Essential Oils* | *Common Name of Spices or Plants* | *Antimicrobial Spectrum* |
|---|---|---|
| Thymol, eugenol | Basil, Bishop's weed, thyme, savory | *Erwinia, Escherichia, Klebsiella, Listeria, Proteus, Pseudomonas, Salmonella, Shigella Staphylococcus* |
| Cinnamaldehyde, coumarin | Cinnamon | *Bacillus, Campylobacter, Enterobacter, Escherichia, Listeria, Pseudomonas, Salmonella, Staphylococcus* |
| Linalool, decanol | Coriander | *Candida, Listeria* |
| Eugenol | Cloves | *Campylobacter, Enterobacter, Escherichia, Listeria, Salmonella, Staphylococcus* |
| Cumin aldehyde, terpinen | Cumin | *Bacillus* |
| Allicin | Garlic | *Candida, Escherichia, Listeria, Salmonella, Staphylococcus* |
| Citronellol, limonene, linalool | Kaffir lime | *Aspergillus* |
| Geranial | Lemongrass | *Listeria, Staphylococcus* |
| Limonene, *p*-cymene | Lime | *Aspergillus, Escherichia, Salmonella* |
| Menthol | Menthol | *Escherichia, Micrococcus, Salmonella, Staphylococcus* |
| Carvacrol | Oregano | *Candida, Escherichia, Pseudomonas, Staphylococcus* |
| Carnosic acid, carnosol, rosmadial, rosmaric acid | Rosemary | *Candida, Saccharomyces, Bacillus, Escherichia, Staphylococcus* |

*Source:* Adapted from Hyldgaard, M. et al., *Front. Microbiol.*, *3*, 12, 2012.

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cloves, allspice, and cinnamon; linalool in coriander; allicin in garlic; limonene from lime; and thymol in oregano and thyme (see Table 38.3 for more compounds and their antimicrobial spectrum). Their bacteriostatic and fungistatic properties depend on the active agent. Because of the small amounts used as spices in foods, they probably do not produce any antimicrobial effects. However, the antimicrobial components can be used in higher concentrations as oleoresins or essential oils.

The antimicrobial properties of garlic, onion, and ginger, as well as cabbage, Brussels sprouts, carrots, and others have generated interest for their possible use as natural preservatives. There is an increased interest in using "natural" or "green" antimicrobial compounds as food preservatives, such as those from food plants and spices, and further studies are underway to determine their delivery and efficacy in foods and beverages.

### *Wood Smoke*

Many processed-meat products and fishes are processed with smoke generated by burning hardwood, such as hickory, oak, maple, walnut, and mahogany. As an alternative, liquid smoke, obtained as a distillate of hardwood smoke, is also used with the ingredients of the products. The main reason for smoking meat, fish, and cheese is to impart desirable flavor, texture, and color to the products. The other benefit is the long shelf life of smoked products, especially those exposed to smoke during heating. The smoke contains several different types of chemicals that deposit on the food surface, many of which have antibacterial properties. The most important antibacterial agents are formaldehyde, phenols, and cresols. Depending on the temperature and time of heating, degree of surface drying ($A_W$), and the concentrations, smoking can be both bacteriostatic and bactericidal to bacterial cells. Although smoke has a slight antifungal action, it does not have any adverse effects on the survival or germination of bacterial spores. Liquid smoke, under similar conditions, is less antimicrobial than wood smoke. Smoke also contains some chemicals that are carcinogenic, such as benzopyrene and dibenzanthracene. One of the recommendations to reduce colon cancer is to minimize the consumption of foods treated with smoke.

## Bacteriophages[1,11,12]

Bacteriophages are viruses that infect bacteria (see Chapter 14). They are highly specific for a bacterium, multiply inside and either lyse the bacterial cell or are maintained as a prophage. They are now used to control foodborne bacterial pathogens and spoilage bacteria in live animals, meat, dairy products, seafoods, and fresh produce. They are the most abundant form of life ($10^{31}$ phages) on the planet. These viruses are very small (20–200 nm) and are categorized based on their shape: tailed, polyhedral, filamentous, and pleomorphic. A majority of viruses are tailed (96%) virus. A typical tailed bacteriophage possesses a head, tail, and tail fibers (Figure 38.1). The head contains the genome, consisting of double-stranded or single-stranded DNA or RNA and protected by capsid protein. The tail is a hollow tube through which nucleic acids (DNA or RNA) are transferred to the host during infection. Bacteriophages may transfer virulence genes from one bacterial pathogen to others. The tail fiber interacts with the host (bacterium) and provides specificity. Bacteriophages do not infect humans or animals; thus they are considered suitable, especially the lytic bacteriophages, for treatment of bacterial infection, to prevent pathogen colonization in meat animals, and as "biocontrol" for use in the food industry. Historically, bacteriophages had been used as therapeutic agents to control varieties of bacterial diseases in humans. First, therapeutic use was reported in 1919 by Felix d'Herell, who used phages to control bacillary dysentery and

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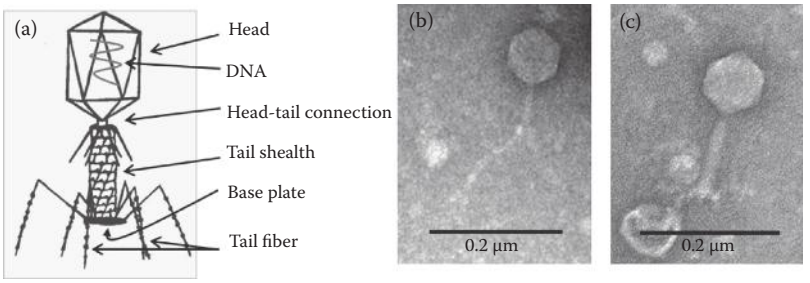



**Figure 38.1 (See color insert.) (a) Schematics of typical tailed bacteriophage, T4 and transmission electron microscopic (TEM) picture of *Escherichia coli*—specific (b) siphovirus (thin tail), and (c) myovirus (thick tail). (TEM photographs were provided by Dr. Paul Ebner and Yanying Pan, Purdue University.)**

cholera in India. Currently, it is being used in countries in Eastern Europe, especially in Russia and Poland. Bacteriophages are now considered attractive therapeutic agents because of the emergence of antibiotic-resistant bacterial strains.

## Bacteriophages to Control Pathogens in Food[11–13]

As discussed in Chapter 14, the bacteriophage life cycle is of two types: (i) the virulent or lytic and (ii) the temperate or lysogenic. The infection steps are comprised of initial attachment, penetration (injection of nucleic acid), the latent period, maturation, and lysis or lysogeny (Figure 14.3). During attachment, bacteriophage tail fibers interact with a specific receptor on the bacterial surface, and binding is irreversible. In the penetration step, viral nucleic acid is injected into the host cell while the remainder of the viral body stays behind. In the latent period, integration of phase DNA into the host genome, replication of the phage genome, viral protein transcription, and translation occur. In the maturation phase, new phage components are assembled, and DNA is packaged into heads, and virions are made. In the lysis phase, the bacteriophage makes two lytic enzymes: holin to form a pore in the cytoplasmic membrane and endolysin to degrade peptidoglycan (cell wall), and all matured viruses are released from bacteria after lysis of the cells. In the laboratory, bacteriophages are counted as plaque-forming units (PFU) based on the number of plaques they form on a lawn of bacterial cells on an agar plate (Figure 38.2). The entire cycle may last for one to two hours. In the lysogenic life cycle, viral DNA remains incorporated into the host genome and maintained as prophage (temperate phage) without producing any virions or lysis of bacteria.

Bacteriophage application in food safety is divided into two broad areas: (i) preharvest control of pathogens in meat-producing animals, and (ii) post-harvest control in raw or processed foods. For preharvest controls, bacteriophages are either applied on hides or feathers or introduced orally or intramuscularly to prevent pathogen colonization in cattle, swine, and poultry. The most common pathogens that were targeted for phage therapy include *Listeria*, *Salmonella*, *Escherichia*, and *Campylobacter*. For post-harvest control of pathogens, phages are applied to raw or processed meat (hotdogs, deli meats); smoked salmon; cheeses; fresh produce, such as sprouts, lettuce, melon, apples, cantaloupes, etc. The foodborne pathogens that were a target for post-harvest

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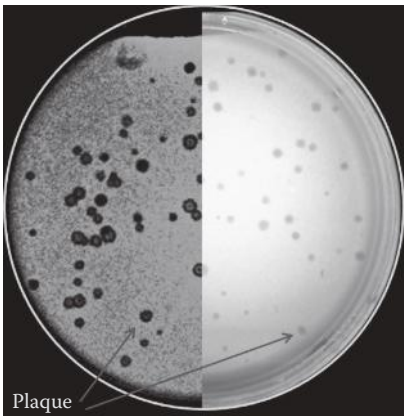



**Figure 38.2 (See color insert.) Plaque-forming unit (PFU) assay to determine bacteriophage-mediated lysis of host bacteria. Plaque showing lysis of bioluminescent *Pseudomonas fluorescence* lawn by ΦS1 lytic phage. Left half of the plate is taken with a photon-counting camera showing bioluminescent *Pseudomonas* lawns and right half taken with natural light showing clear lysis of cells. (Picture courtesy of Dr. Bruce Applegate and Philip Myer, Purdue University.)**

control include *Lis. monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Cronobacter sakazaki*, and *Staphylococcus aureus*.

To select bacteriophages for food application, several criteria must be fulfilled: (i) they must be strongly lytic, (ii) the host range should cover all epidemiologically significant strains, (iii) the safety of phages must be thoroughly checked to ensure that they do not carry any genes for virulence, and (iv) they must be stable in the food environment in which they are intended for use. In general, bacteriophages are stable at pH range 5–9 and are inactivated at 60°C for 30 minutes; thus they are considered favorable attributes for food application. Several bacteriophages are approved by the FDA for application in the food industry. In 2006, a commercial bacteriophage mixture of six (LMP-102) has been approved for controlling *Lis. monocytogenes* in ready-to-eat meat and poultry products. Another commercial anti-*Listeria* phage product, LISTEX$^{TM}$P100 received GRAS status to control *Lis. monocytogenes*. Likewise, bacteriophages to control *Escherichia coli* O157:H7 and *Salmonella* in meat, cattle hides, and poultry feathers have received regulatory approval by USDA-FSIS.

The advantages of bacteriophage use in food safety include (i) they infect only live bacterial cells, (ii) interaction is highly host-specific (i.e., an *E. coli*–specific phage will not infect *Salmonella* or other bacteria), (iii) the interaction with host bacteria is irreversible, (iv) there is no apparent ill effect on humans or animals, and (v) the phages can be inactivated by thermal processing. The disadvantages are (i) the bacteriophages do not work on bacterial toxins or any other microbes; (ii) because a majority of foods are solid, diffusion/distribution of bacteriophages throughout the food matrix when a target pathogen is present may be limited; (iii) high concentrations of phases ($1 \times 10^8$ PFU/mL) need to be applied to foods so that bacteriophages have the chance to come in contact with fewer bacterial pathogens present in processed or minimally processed foods; (iv) bacteriophages require live bacteria as a host, and if most target host cells are killed, desirable bacteriophage titers may not be maintained in the product for prolonged duration; and (v) a narrow host spectrum sometimes makes bacteriophages undesirable as antimicrobials. To overcome this problem, mixtures of bacteriophages (bacteriophage cocktails) are used.

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## Conclusion

Many chemical compounds, approved by regulatory agencies, have been used to control growth of pathogenic and spoilage microorganisms in food. In addition, some of them can kill microbial cells and prevent germination of bacterial spores at concentrations used. Many of these compounds are more effective in combination with other methods. However, food environments can greatly influence their effectiveness. They are, like the methods discussed before (except heat), not used to eliminate pathogens from a contaminated food. However, irradiation (besides heat) is used specifically to kill microbes present in food and is discussed in Chapter 39. In recent years, herbs and spices are gaining considerable interest as food preservatives because they are considered "green" natural antimicrobials. Bacteriophages are also a natural enemy of bacterial pathogens and have received regulatory approval to control many foodborne pathogens not only for final processed products but also to control pathogens in meat animals during preharvest.

### QUESTIONS

1. Discuss the uses of antimicrobial chemicals before and after A.D. 1865, after 1958, and since the 1980s.
2. Read the labels of 10 different foods from at least five food categories and identify in them the chemicals having antimicrobial action (direct or indirect).
3. Define the terms bacteriostatic, germicidal, bactericidal, fungicidal, sporicidal, viricidal, and sporostatic. Give one example of a preservative for each.
4. List five characteristics one should consider in selecting a suitable chemical preservative for a specific food. What is a GRAS-listed preservative?
5. Discuss the antimicrobial properties and any health concern of the following chemical preservatives, and list two foods (for each) in which they are used: $NO_2$, sulfites, bacteriocins, parabens, benzoic acid, lysozyme, natamycin, and lactic acid.
6. Indicate the mechanism of antimicrobial actions of a bacteriocin, lysozyme, and EDTA. From the information, indicate how, if used together, they can produce a synergistic antimicrobial effect.
7. Briefly discuss the life cycle of a lytic bacteriophage.
8. What are the advantages and disadvantages of using bacteriophage as food preservatives?

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# Chapter 39

# Control by Irradiation

## Introduction

### *Irradiation (Radiation) and Radioactivity*

In the electromagnetic spectrum, energy exists as waves, and the intensity of the energy increases as the waves get shorter. On either side of visible rays (~400–800 nm) are invisible long waves (>800 nm; IR and radio waves for radio, TV, microwave, and radar) and invisible short waves (<300 nm; UV rays, X-rays, β-rays, γ-rays, and cosmic rays). Exposure to long waves, visible light waves, and UV rays does not cause any change in the atomic structures. In contrast, exposure to X-rays, β-rays, and γ-rays can remove electrons from the outer shell of an atom and thus form an ion pair (negatively charged and positively charged). Ion formation or ionization does not make an atom radioactive. To induce radioactivity, the nucleus of an atom has to be disrupted by much higher energies, such as by neutrons; X-rays, β-rays, and γ-rays do not have that much energy.

For application in food preservation, X-rays, β-rays, and γ-rays were studied for their ability to penetrate inside foods and kill microorganisms, their efficiency, and their effect on food quality. β-rays (actually electrons, similar to cathode rays) have very little penetration power; they cannot penetrate inside metal cans and are thought to be ineffective in food preservation. X-rays, although they have good penetration power, cannot be effectively focused on foods and, because of this low efficiency, are not considered favorably for application in food. In contrast, γ-rays (photons) have high penetration power (~40 cm thick) and may be considered effective and economical for use in foods. Cobalt-60 ($^{60}$Co) and cesium-137 ($^{137}$Cs) are considered to be good sources of γ-rays. $^{60}$Co, an artificially induced radioactive isotope, is produced for use in nuclear medicine. When its energy level becomes so low that it cannot be further used in medicine, it is used to irradiate foods. Because of the easy availability of $^{60}$Co over $^{137}$Cs, it is preferred for food irradiation. Thus, foods irradiated with $^{60}$Co do not become radioactive. Instead, the atoms (and molecules) in a food and in microbial cells form ion pairs and other components that inhibit the multiplication of microorganisms (and food cells, such as the inhibition of sprouting in potatoes).[1–3]



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### Use of Irradiation in Food

The ability of X-rays, γ-rays (E-beam), and β-rays to kill microorganisms was recognized soon after their discovery in the late 19th and early 20th centuries. Some were even tested to determine their effectiveness in killing microorganisms in food in those early years. However, until the late 1940s and early 1950s, their use in food preservation was not actively studied. One reason was the unavailability of a large economical supply of radioisotopes, the technology of which was developed during World War II. Early studies showed that different foods can be irradiated to extend shelf life without making them radioactive, but the process affected the flavor qualities of the foods. Later, the problems were overcome by reducing exposure time and lowering product temperature. At that time, it was thought that irradiation of food would give storage stability for indefinite periods of time. The U.S. Army started a research program to determine whether storage-stable, acceptable, safe army rations could be produced by irradiation. Although foods do not become radioactive at low doses of irradiation, it was found that food accumulated radiolytic products. Extensive studies showed that they are present in very low concentrations (~3 ppm). Most of them are not unique to irradiated foods because they are present naturally in different foods and are also produced in heated foods (thermolytic products). Well-designed feeding studies, with animals as well as humans and for a fairly long period, showed that irradiated foods did not cause any toxic effect or genetic defects. A WHO expert committee on the wholesomeness of irradiated food, after extensive review of more than 200 well-designed studies conducted worldwide, has recommended irradiation of food up to a certain dose level (10 kGy). This level is currently approved in more than 30 countries for use in meat, fish, vegetables, fruits, and grains. Many countries are regularly selling irradiated foods, especially some vegetables, fruits, and grains. However, consumer resistance has been quite high. This is mainly because of their lack of information and understanding that irradiated foods are not radioactive and radiolytic products are not unique to only irradiated foods. This is probably the only food preservation method that has been studied for safety for a long time (more than 40 years) before its recommendation and whose use in many countries has been found to be safe by the world body of expert scientists. The other reason is political. A small but vocal group of lobbyists are waging a very successful campaign against the acceptance of irradiated food by large numbers of consumers.[4–6] As a result, food industries are in a wait-and-watch situation.

There is no controversy among experts that food irradiation is an economical and effective food preservation method. As the world population increases and food production decreases, effective preservation methods must be used to feed the hungry mouths, otherwise social unrest may start. Politics and lobbying will not stop hunger, but food preservation methods (such as irradiation) will.

Globally, in 2005, 405,000 tons of food, such as spices, dry vegetables, grains, fruits, meat and fish, sprouts, garlic, mushrooms, honey, potatoes, and other food items, were disinfected by irradiation. While commercial food irradiation is increasing significantly in Asian countries, the trend is opposite in the European Union.[2,5]

## Objectives

A food is irradiated because of the destructive power of ionization on the microorganisms a food harbors. Depending on the method used, it can either completely or partially destroy molds, yeasts, bacterial cells and spores, and viruses. In addition, irradiation can destroy worms, insects,

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and larvae in food. It also prevents the sprouting of some foods, such as potatoes and onions. However, irradiation cannot destroy toxins or undesirable enzymes in a food; in this respect, it differs from heat treatment (heat also does not destroy heat-stable toxins and enzymes). Irradiation is a cold sterilization process inasmuch as the temperature of a food does not increase during treatment, and thus irradiated foods do not show some of the damaging effects of heat on food quality. However, irradiation can cause oxidation of lipids and denaturation of food proteins, especially when used at higher doses.[1,7,8]

## Mechanisms of Antimicrobial Action

When an object (food or microorganism) is exposed to high-energy γ-rays ($10^{-1}$ to $10^{-2}$ nm), the energy is absorbed by thousands of atoms and molecules in a fraction of a second, which strips electrons from them. This produces negative and positive ion pairs. The released electrons can be highly energized and thus can remove electrons from other atoms and convert them into ions. This energization and ionization can adversely affect the normal characteristics of biological systems.

Ionizing radiation produces both direct and indirect effects on microorganisms. The direct effect is produced from the removal of electrons from the DNA, thereby damaging them and from the ionization of water molecules present in the cell. The hydrogen and hydroxyl radicals formed in this process are highly reactive and cause oxidation, reduction, and the breakdown of C–C bonds of other molecules, including DNA. Studies have shown that a hydroxyl radical can break both single and double strands in DNA at the sugar-phosphate backbone. In addition, the radicals can change the bases, such as thymine, to dihydroxydihydrothymine. The consequence of these damages is the inability of microorganisms to replicate DNA and reproduce, resulting in death.

Indirectly, ionizing radiation causes damage in the membrane and other structures, causing sublethal injury. Some microorganisms can repair the damage to the DNA strands (especially single-strand breaks) and in the bases and are designated as radiation-resistant microorganisms.

Microbial death by ionizing radiation, as in heat treatment, occurs at a predictable rate, which, like heat, is dependent on dose (strength and exposure time), microbial species, and environmental factors. Because of this, the *D* value (minutes to reduce cell viability by 1 log of a species in a population at a given exposure) can be derived. This, in turn, can be used to determine the time necessary to reduce the population to a desirable level under a specific condition of treatment.

When microorganisms are exposed to UV radiation (~260 nm), the energy is absorbed by the nucleotide bases in the DNA. The bases can react with each other to form dimers (e.g., thymine dimers) and cause breaks in the DNA strand. Microbial death and injury are associated mainly with DNA damage.[1]

## Influencing Factors

### *Nature of Process*

Among the several methods available (X-rays, β-rays, and γ-rays), γ-rays have a higher potential for effective and economical use in food preservation. $^{60}$Co is predominantly used in food irradiation because it is more readily and economically available. It has a half-life of ~5.3 years. It continuously

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emits γ-rays and thus can be lost even when it is not used. $^{137}Cs$ can also be used in foods, but it is relatively difficult to obtain and is also required in larger amounts. The antimicrobial efficiency of ionizing radiation increases as the dose is increased. The antimicrobial efficiency, however, decreases in the absence of oxygen (because of reduced oxidizing reactions) and at low $A_W$ (because of reduced free-radical formation with less water). Freezing also reduces the efficiency because of reduced availability of reactive water molecules.

### Nature of Foods

γ-rays (E-beam) have a penetration capability of approximately 40 cm and can penetrate through paper, plastic, and cans. Thus, foods can be exposed to γ-radiation in packages, cans, baskets, and bags. Frozen, dry, or anaerobically packaged foods need higher doses of treatment to obtain the desirable antimicrobial effect. In contrast, treatments, such as curing, high hydrostatic pressure, high temperature, and low pH, enhance the antimicrobial effect of radiation in food.[9] Food compositions (thickness and particle size) also determine the efficiency of irradiation in reducing microbial numbers.

### Nature of Microorganisms

Microorganisms vary greatly in their sensitivity to ionizing (and UV) radiation. Because of size differences, molds are more sensitive than yeasts, which are more sensitive than bacterial cells; bacterial cells are more sensitive than viruses (including phages). Among bacteria, gram-negative cells are more sensitive than gram-positive bacteria, and rods are more sensitive than cocci. Species and strains of bacterial cells vary greatly in their sensitivity to irradiation. Some strains, designated as radiation-resistant, have effective metabolic systems to repair the cellular damages (especially single- and double-strand breaks of DNA and base damage). These include some bacterial strains that are important in foods, such as *Salmonella* Typhimurium, *Escherichia coli*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Spores are quite resistant to irradiation, probably because their water content is very low. Among the spore formers, spores of *Clostridium botulinum* Type A and *Bacillus pumilus* are probably the most resistant to irradiation. Generally, *Bacillus* spores (aerobes) are less resistant than *Clostridium* spores (anaerobes).

The rate of death of microorganisms by irradiation follows first-order kinetics (i.e., straight line patterns) as the thermal destruction curve and can thus be expressed as a *D* value (time to destroy 90% of viable microorganisms). Accordingly, it can be influenced by a higher initial population, relative numbers of resistant cells in the population, number of spores present, and age and condition of growth of a strain. Toxins of microorganisms are not destroyed by ionizing radiation at the dose levels recommended in foods. Although irradiation can cause mutations in some microbial cells in a population, a possible increase in neither pathogenicity nor an induction of a gene that transcribes for a toxin is expected to occur as observed from the many studies conducted in these areas.

## Methods

### Doses

A radiation dose was originally designated as a *rad*, and 1 rad was defined as the quantity of ionizing radiation that results in the absorption of 100 ergs of energy per gram of an irradiated material. The current unit is gray (Gy), and 1 Gy is equivalent to 100 rad. When 1 kg of food absorbs the

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energy of 1 joule (1 joule = $10^7$ ergs), it has received a dose of 1 Gy. According to the international health and safety authorities, foods irradiated up to 10,000 Gy (10 kGy) are considered safe.

The relative sensitivity of microorganisms to an irradiation dose is a function of their size and water content. Approximate lethal dose levels for insects and different microorganisms have been suggested as follows: insects, ≤1 kGy; molds, yeasts, bacterial cells, 0.5–10 kGy; bacterial spores, 10–50 kGy; and viruses, 10–200 kGy. Thus, at the recommended level of 10 kGy, *Clo. botulinum* spores are not destroyed in foods (they need ca. 30–60 kGy) although cells of pathogenic (and spoilage) bacteria are destroyed. The products thus treated should have other barriers (low pH, low $A_W$, temperature ≤ 4°C) to control germination and growth of spore formers. But some spore formers (such as *Clo. laramie*) can germinate and multiply below 4°C. At present, a low dose level (<1 kGy) is used to control insects in fruits and grains, parasites in meat and fish, and sprouting in vegetables. Medium doses (1–10 kGy) are used to control foodborne pathogens and spoilage microorganisms to extend the safety and stability of refrigerated foods. Higher doses (>10 kGy) to destroy spores are not used in foods except in spices and vegetable seasonings that are used in very small quantities.

Irradiated foods, such as post-heat contamination in heated foods, can be contaminated later with pathogenic and spoilage microorganisms from various sources unless proper precautions (such as packaging) are used. Also, a food can be reradiated to inactivate microorganisms.

## Specific Terms

### Radurization

Radiation pasteurization is mainly intended to destroy spoilage bacteria in high-pH–high-$A_W$ foods, especially Gram-negative psychrotrophs in meat and fish, and yeasts and molds in low-pH–low-$A_W$ foods. The treatment is generally milder (~1 kGy). The products should be packaged and chilled to prevent growth of pathogens, which were previously thought to be mesophiles. However, with the recognition of psychrotrophic pathogens and the importance of psychrotrophic gram-positive spoilage bacteria, this treatment may not be effective.

### Radicidation

This is the radiation of foods to destroy vegetative foodborne pathogens. The dose level used is approximately 2.5–5.0 kGy. Although it is effective against pathogenic vegetative bacterial cells and molds, spores of the pathogens are not destroyed. Also, some radiation-resistant strains of pathogens can survive, such as some *Salmonella enterica* serovar Typhimurium strains. Irradiated products thus need to be stored at ≤4°C, especially to prevent germination and outgrowth of spores of *Clo. botulinum*.

### Radappertization

This method involves radiation of food at a high dose (~30 kGy) to destroy *Clo. botulinum* spores in order to get a safety similar to that by 12*D* heat treatment. However, this is not recommended for use in food.

## Current Recommendations

Irradiated fresh fruits, vegetables, spices and herbs, meat, and fish have been approved in 37 countries and are used in nations such as France, Belgium, and the Netherlands. In the United States,

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irradiated foods have been used by the army and in the space program for some time. Before 1985, irradiation was permitted by regulatory agencies for spices, wheat, wheat flour (to destroy insects), and potatoes (to prevent sprouting). Subsequently, permission for irradiation was approved in 1985 for pork (against *Trichinnella*), in 1986 for fresh foods (fruits and vegetables to destroy insects and larvae), and in 1992 for poultry and poultry parts (to destroy pathogens, specifically *Salmonella*). Currently, radiation of seafood (to destroy pathogens and spoilage bacteria), lettuce, and spinach are approved by the FDA (see Table 39.1). In addition, in the United States, radiation sterilization of beef steaks in the space program and refrigerated shelf-stable food for the military are being studied.

Currently, irradiated fruits (strawberry, mango, and papaya) and poultry are being marketed in limited amounts, and the consumer response has been favorable. Irradiated foods will have a special logo (Figure 39.1) along with the words "Treated with Radiation" or "Treated by Irradiation" for consumer information.

**Table 39.1 Approval of Food Irradiation in the United States**

| *Date* | *Products* | *Dose (kGy)* | *Purpose* |
|---|---|---|---|
| 1964, 1965 | Potatoes | 0.05–0.15 | Inhibit sprouting |
| 1983 | Spices and dry seasonings | <30 | Disinfestation and decontamination |
| 1985 | Pork | 0.3–1.0 | Control of *Trichinella spiralis* |
| 1985, 1986 | Dehydrated enzymes | <10 | Control insects and microbes |
| 1986 | Fruits and vegetables | <1 | Delay maturation and disinfection |
| 1986 | Herbs, spices, and seasonings | <30 | Control of microorganisms |
| 1990 | Poultry, fresh and frozen | <3.0 | Control of microorganisms |
| 1995 | Meat, frozen and packaged | >44 | Sterilization of products only for National Aeronautics and Space Administration (NASA) use |
| 1997, 1999 | Red meat, chilled | <4.5 | Control of microorganisms |
| | Red meat, frozen | <7.5 | |
| 2000 | Shell eggs | <3.0 | Control of *Salmonella enterica* ser. Enteritidis |
| 2000 | Sprouts | <8.0 | Control of pathogens in seeds |
| 2005 | Fresh or frozen mollusks and other shellfish | <5.5 | Control of *Vibrio* species and other foodborne pathogens |
| 2008 | Iceberg lettuce and spinach | <4.0 | Control of foodborne pathogens and extension of shelf life |

*Source:* Adapted from Ahn, D.U. et al., *Poult. Sci.*, *92*, 534–545, 2013.

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**Figure 39.1 (See color insert.) Logo for irradiated foods. The Codex Alimentarius, an international committee on food safety, has developed this logo to put in green color on the package of irradiated foods. Irradiated foods from the United States are labeled with this logo, along with the words "Treated with Radiation" or "Treated by Irradiation."**

### *UV Radiation*

Microorganisms are especially susceptible to UV light between 200 and 280 nm. Because of low penetration power, it has been used to inactivate microorganisms on the surface of foods (meat, fish, and bread) as well as in air and on walls, shelves, and equipment in the food handling and processing area. In addition, liquids, such as water and syrups, in thin layers, have been treated with UV.

## Conclusion

Irradiation, like heat, kills microbial cells and destroys their spores at a predictable rate that is basically dependent on dose level, exposure time, and microbial type. Its effectiveness on microbial control in food has been well studied since the beginning of the 20th century. Although irradiated foods have been found to be safe, many consumers view them as unsafe. As a result, irradiation is used in a limited way in food. It is expected that with proper education of consumers on the safety of irradiated food, consumers' demand will increase and irradiation will be used to destroy many foodborne pathogens in raw and processed foods. In recent years, several other methods that can be used to kill microorganisms effectively have been studied and are discussed in Chapter 40.

**QUESTIONS**

1. Briefly discuss why irradiated foods are not commercially successful in many countries.
2. Explain the mechanisms of antimicrobial actions of irradiation by γ-ray and UV light.
3. List the factors that can reduce or increase the antimicrobial effectiveness of irradiation in food.

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4. What recommended levels of γ-irradiation are approved in different foods, and what are their objectives? What is the status of handling spore problems in irradiated foods?
5. Briefly explain the importance of the following in relation to food irradiation: (a) irradiated products, (b) radioactive products, (c) radiolytic products, (d) thermolytic products, (e) free radicals, (f) radarization, (g) radicidation, and (h) radappertization.
6. In a beef-processing operation, fresh beef in 30- to 50-lb sizes was vacuum packaged and effectively irradiated to destroy pathogenic bacteria. In a retail operation, the packages were then opened and cut into retail size (1–5 lb). Some of these were found to contain pathogens. Discuss how this contamination could have occurred and suggest a method to overcome this problem.

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# Chapter 40

# Control by Novel Processing Technologies

## Introduction

Many of the conventional food preservation methods used at present and discussed here have several disadvantages. High-heat treatment given to foods for safety and long shelf life results in loss of heat-sensitive nutrients (e.g., thiamine, riboflavin, folic acid, and vitamin C); denatures proteins and causes changes in texture, color, and flavor; and induces formation of new compounds through covalent bonds (e.g., lysinoalanine). Low-heat processing, such as pasteurization, minimizes the disadvantages of high-heat processing of foods, but the foods have limited shelf life even with refrigerated storage. Drying and freezing also reduce nutritional and acceptance qualities of food, especially when stored for a long time, and moreover, not all food can be stored by drying or freezing. Irradiated foods have not been well accepted by consumers. Many of the chemical preservatives used are of nonfood origin and have limited efficiency.

Since the 1980s, health-conscious consumers, especially in the developed countries, have been concerned about the possible adverse effects that "harshly produced" and "harshly preserved" foods might have on their health and on the health of future generations. There is a concern that the cumulative effects and interactions of different types of food preservatives on the human body during one's lifetime are not properly understood. The revelation about the harmful effects of some of the additives that were once allowed to be incorporated in foods has shattered consumer confidence. The philosophy of the consumers has changed from "How long will I live?" to "How well will I live?" which, in turn, has shifted the desire of these consumers toward nutritious, natural, and minimally processed foods that have not been subjected to harsh processing or harsh preservation techniques. Because of changes in socioeconomic patterns and lifestyles, many consumers are also interested in foods that have a long shelf life and take very little time to prepare.[1]

The suitability of several thermal and nonthermal processing and preservation methods are being studied to produce such foods. The principal advantages, disadvantages, and the current status of some of these methods are briefly described here. Major emphasis is given to their antimicrobial properties.[2–4]



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## Summary of Processing Methods

Some of the novel technologies currently being studied to process foods, to control microorganisms, as well as to produce new types of foods and their current status in the application of food are briefly described. This includes processing under thermal and nonthermal conditions, and the details are summarized in a recent document compiled by National Advisory Committee on Microbiological Criteria for Foods (NACMCF).[5]

### *Microwave and Radio-Frequency Processing*[5–7]

These methods involve exposing a food to electromagnetic waves at microwave and radio frequencies. Generally, microwaves at 915 MHz and 2450 MHz and radio frequencies at 13.6, 27.1, and 40.7 MHz are used in food applications, but very little study has been conducted with radio frequency. Both techniques generate internal heat by dielectric and ionic mechanisms; water molecules, because of their dipolar nature, are associated with dielectric heating. Heating of food is very rapid but not uniform as compared with that in conventional heating. Destruction of microorganisms (cells and spores) is accomplished by the heat mainly through denaturation of proteins and nucleic acids. Microwave ovens are widely used now to cook and reheat foods. This method has the potential of producing commercially pasteurized and sterilized foods. However, because of the possible presence of cold spots in solid and semisolid foods, the method cannot be used to assure safety and is thus currently not regulated by the FDA (see Chapter 33). Many studies are now being conducted to improve the uniform heating of the microwave oven.

### *Ohmic and Inductive Heating*[5,8]

In ohmic heating, electric currents are passed to heat food through an electrode that is in contact with the food. In contrast, in inductive heating, electric coils placed near a food generate an oscillating electromagnetic field that sends electric currents to heat the food. Of the two, ohmic heating has been studied well for its potential use in the production of commercially pasteurized and sterilized food products. The destruction of microbial cells and spores is achieved primarily by destabilizing proteins, nucleic acids, membrane, and other vital functional components by heat. Food materials are heated rapidly and uniformly from the generation of internal energy. This method also produces cold spots in foods containing high fats and solids. Techniques are being studied to minimize their occurrence.

### *Infrared Heating*[5,9]

In infrared (IR) heating, also known as IR radiation, is an electromagnetic wave (wavelength 0.78 to 10,000 μm), which generates thermal energy that is absorbed by food materials to improve product shelf life. The heating rate is fast and has a very high thermal efficiency and also provides uniform heating, reduced quality losses, and significant energy saving. IR in combination with microwave heating is also becoming an effective method for food processing.

### *Pulsed Electric Fields*[10]

Use of pulsed electric fields (PEF), a nonthermal method, involves application of high-intensity electric pulses between two electrodes placed in a food for a very short time to inactivate

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microorganisms. Various aspects of this method, including applications in food, have been studied. A short review of the method is presented in the section on pulsed electric fields in this chapter. PEF has also been used effectively together with high hydrostatic pressure (HHP) technology[11] or ultraviolet (UV) radiation as a hurdle approach for microbial inactivation.[12]

### *High-Pressure Processing*[13,14]

High-pressure processing (HPP), also known as high hydrostatic pressure (HHP), is a nonthermal method involving pressurization of a packaged food in a water-filled, closed chamber for a short duration to inactivate microorganisms. A liquid food can be pressurized directly. In addition to an antimicrobial effect, it has many other food applications. The method has generated a great interest, and several foods have already been commercially produced. A review of the current status of the method has been presented in the section on hydrostatic pressure processing in this chapter.

### *Pulsed Light Technology*[5,15]

This is a nonthermal method in which microorganisms on the surface of food (usually in a transparent package) are inactivated by high-energy light pulses. Light pulses in the wavelengths of 170–2600 nm are used for a very short duration (≤0.01 seconds). Processing units have been developed in which electric energy can be stored in a capacitor over a long period, which then can be released in a shorter time to produce an antimicrobial effect. The antimicrobial effect is produced through the action on nucleic acids (especially in the UV range), proteins, membranes, and other cellular components. The antimicrobial effectiveness of the process has been studied on the food contact surface, the packaging materials, and the surface of various foods, including cheeses, processed meat products, bakery products, and fishery products. Following treatment, the products retained acceptance qualities during extended shelf life.

### *Oscillating Magnetic Fields*[5,16]

In this nonthermal method, a food, sealed in a plastic bag, is exposed to oscillating magnetic fields (OMF) (<500 kHz) for a duration to receive 1–100 pulses over a short period (≤100 ms). Limited studies showed that microbial inactivation is two to three log cycles, but the results were inconsistent. Some studies have been conducted using milk, yogurt, orange juice, and dough. The antimicrobial effect is produced from the damages in the DNA during exposure to the magnetic field.

### *Ultrasound*[17,18]

Lysis of microbial cells by sonication forms the basis of the use of ultrasound to inactivate microorganisms in foods. The method consists of generating high-frequency sound waves in the test materials. The antimicrobial effect of ultrasound is attributed to intracellular cavitation that disrupts the cellular structures and functional components. Limited studies revealed that the antimicrobial effect of ultrasound in food is rather low. However, it can be enhanced by combining ultrasound with heat above 50°C (122°F). In addition to the antimicrobial effect, ultrasound has been studied as a means to determine texture, viscosity, thickness, and composition of different foods.

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### High-Voltage Arc Discharge[5,16]

In this method, stored high-voltage electrical energy is rapidly discharged in the form of an arc through the gap of electrodes placed in a liquid food. The process not only raises the temperature of the liquid to some extent but also produces electrohydraulic shock. Antimicrobial effect is produced mainly because of membrane damage by the electrohydraulic shock. The major disadvantages of the method are the generation of electrolytic products and disintegration of food particles. Improved techniques are now being developed to overcome the problems.

### Pulsed X-Rays[5,19]

Irradiation of food by γ-rays (E-beam) using $^{60}Co$ (and $^{137}Cs$) has been discussed in Chapter 39. One of the disadvantages of this method is that the radionuclides emit radiation in all directions and all the time; it is thus uneconomical and also hazardous. In contrast, X-rays can be generated only when they are needed and in the desired linear direction. It was first recognized in 1895 and soon after that it became a routine diagnostic tool in medicine. A pulsed X-ray, which delivers high-energy pulses in a very short time, is being studied to inactivate microorganisms in foods only recently. The equipment can produce high energy with a high penetrating power and the least variation in dose uniformity. Limited studies have revealed that when used properly, the method can destroy five to six log cycles of microbial cells and spores at 20°C (68°F). The microbial inactivation occurs, like in γ-radiation, from the damages in the DNA. X-ray irradiation has been shown to enhance the safety and shelf life of many heat-sensitive products, such as lettuce, spinach, tomatoes, and raw almonds.

## Plasma Technology[20–22]

Moreau et al. defined "plasma" as a neutral ionized gas generated by excitation of gas by electric current,[22] which consists of atoms, ions, free-radicals and excited and nonexcited molecules, photons and electrons. The free-radicals and ionic species are highly toxic to microbial vegetative cells or endospores, including gram-positive (*Bacillus*, *Clostridium*, *Staphylococcus*, *Listeria*), gram-negative (*Escherichia*, *Salmonella*, *Vibrio*) bacteria, yeast (*Saccharomyces*) and mold (*Aspergillus*). There are two types of plasma, thermal and nonthermal, based on the conditions used for their generation. High-temperature plasma is used mostly for industrial processes, such as the automative industry and in microelectronics, and low-temperature plasma is used for food preservation and disinfection of food contact surfaces, and the latter one is receiving increasing interest as a novel food processing technology. The chemical composition of low-temperature plasmas of gas mixtures of nitrogen, oxygen, and carbon dioxide consisted of $O^-$, $O_2^-$, $N^+$, $N_2^+$, $O^+$, $O_2^+$, $NO^+$, and $CO_2^+$, which are highly toxic to microbial cells or endospores.

## Pulsed Electric Field[5,10,16]

The antimicrobial effect of high electric field pulses is not a result of the electric heat or electrolytic products, but rather the ability to cause damage to the cell membrane. When microbial cells in a suspension are exposed to pulses of high-voltage electric fields, a potential difference occurs between the outside and inside of the membrane. When the external electric field strength

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is moderately higher, so that the transmembrane potential does not exceed the critical value by 1V, pore formation occurs in the membrane; the process is reversible. (This principle is used in electroporation of cells to introduce foreign DNA.) However, if a much higher external electric field strength is applied so that membrane potential exceeds the critical value, the pore formation becomes irreversible, causing the destruction of membrane functions and cell death. For destruction of microbial cells, an electric field strength of approximately 15–25 kV/cm for 2–20 milliseconds (ms) is necessary. Destruction of bacterial and fungal spores requires a higher voltage and a longer period of time.

The lethal effect of PEF against microorganisms has generated interest for its use in nonthermal pasteurization and commercial sterilization of foods. During the process, there is little increase in the temperature of the suspension. However, to obtain greater microbial destruction, the temperature of the suspension can be increased to 60°C or higher. Also, by increasing the number of pulses, greater microbial destruction can be achieved.

In the PEF processing of food, a high-voltage pulse (20–80 kV/cm) is discharged between two electrodes in the food in a short time from a series of capacitors that act as storage. PEF can be applied as an exponentially decaying or square wave; the square wave appears to be more uniform and lethal. Pulses can be given once or more than once to enhance lethality. PEF treatment in the presence of other antimicrobial compounds (such as bacteriocins), at higher temperature (≥50°C), and at lower pH is more lethal to microbes. A process called Elsterile has been developed in Germany for microbial destruction by PEF in liquid food. The liquid food, in a treatment chamber that has two carbon electrodes, is subjected to high-voltage electric pulses. A 4-log reduction was obtained for *Lactobacillus brevis* in milk by treating with 20 pulses at 20 kV/cm for 20 ms. A similar reduction was also observed by treating *Saccharomyces cerevisiae* in orange juice with five pulses at 4.7 kV/cm for 20 ms. The increased reduction of the yeast cells, as compared with bacterial cells, was thought to be a result of the low pH of orange juice and larger cell size of yeasts.

In the United States, the antimicrobial efficiency of PEF has been studied in several fluid foods, such as dairy products, fruit juices, and liquid egg products. Apple juice treated with 10 pulses of PEF at 50 kV/cm for 2 ms each at 45°C (113°F) retained its acceptance qualities and vitamins for 28 days at 4°C (compared with 21 days of fresh juice). Aerobic plate counts (APCs/mL) in orange juice were reduced by three to four log cycles by applying 32 kV/cm at ambient temperature, and the product had a 90-day shelf life at 4°C. The population of *Listeria innocua* was reduced by 2.5 log cycles in skim milk by PEF treatment at 50 kV/cm. PEF treatments in the presence of nisin reduced the *Listeria* population by 3.4 log. *Escherichia coli* and *Listeria monocytogenes* populations in milk were reduced respectively by 3 log and 2.5 log at ambient temperature. In liquid egg treated at 36 kV/cm at ambient temperature, APCs reduced by 2.7 log and the product had a longer shelf life than untreated products. Inactivation of microbial cells by PEF in particulate food has been studied. However, to achieve a comparable reduction (as with liquid foods), a much higher voltage was necessary.

Limited studies showed that, at ambient temperature, PEF treatment had very little lethal effect on spores. However, in a combination of higher temperatures (~70°C–80°C or 158°F–176°F), low pH, and the presence of EDTA and lysozyme, some spore inactivation can be achieved. A combination of 80°C (176°F) heat shock and lysozyme, followed by PEF at 60°C (140°F) reduced spores of a *Bacillus subtilis* strain by three to four log cycles.

These studies showed that PEF, as a nonthermal processing technique, can be used in liquid food to reduce mainly vegetative cells of spoilage and pathogenic microorganisms to extend its shelf life and ensure safety.

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## Hydrostatic Pressure Processing

### History

Microbial cells exposed to high hydrostatic pressure (100–800 MPa or more) in a water-filled (or a suitable liquid-filled), closed chamber die rapidly. Bob Hite in 1890 first studied the effectiveness of destroying microorganisms by high hydrostatic pressure to preserve foods. But it took almost 90 years to apply high hydrostatic pressure to food preservation and production of several commercial foods in Japan. The technique, since then, has generated worldwide interest and has been referred to as ultrahigh hydrostatic pressure processing (UHP), high hydrostatic pressure processing (HHP), or simply hydrostatic pressure processing (HPP). Early studies were conducted with high-acid and low-protein fruit products, such as fruit juices, jams, and jellies, where the objective was to destroy aciduric spoilage microorganisms, mainly yeasts, molds, and lactic acid bacteria as well as the vegetative cells of some potent pathogenic bacteria. Some acid-sensitive vegetative bacteria and spores of spoilage and pathogenic bacteria were not considered because the cells do not multiply and spores do not germinate and outgrow in a low-pH (<4.6) environment. The current interest in HPP has included both low-pH (<4.6; high acid) and high-pH (≥4.6; low acid) foods and foods with high protein contents. At present, destruction of different foodborne spoilage and pathogenic bacteria, bacterial spores, yeasts, and molds is being studied. It will also be important to study the destruction of foodborne pathogenic enteric viruses, protozoa, and other parasites, as well as bacteriophages of lactic acid bacteria. As HPP alone cannot destroy all the important microorganisms in different types of foods, parameters are being developed for specific food groups based on similarities in their intrinsic characteristics. The effectiveness of other parameters, such as pressurization time, pressurization temperature, the presence of one or more antimicrobials during pressurization, pH, and $A_W$ are also being studied. Like in heat processing, studies on pressure processing are directed toward pressure pasteurization to destroy the vegetative cells of foodborne spoilage and pathogenic microorganisms and pressure sterilization to destroy foodborne bacterial spores. Whereas the pasteurization process can be nonthermal, the sterilization process is thermal. (Currently, the unit megapascal [MPa] is used to indicate pressure processing. The relationship between several units is as follows: 1 atm = 1 bar = 14.5 psi = 750 torr = 100 kilopascal [kPa] = 0.1 MPa.)[5,13,14,23–25]

### Methods, Mechanisms of Microbial Inactivation, and Advantages[5,13,26]

Hydrostatic pressure processing involves exposing a packaged solid or liquid food in water or water containing a little oil or an unpackaged liquid food (e.g., orange juice) in a closed steel chamber and then applying pressure by pumping more of the same liquid into the closed chamber (Figure 40.1). The pressure is transmitted instantaneously and uniformly throughout the closed chamber (as opposed to heat transmission). In general, the come-up and come-down times of pressure are very short (one to five minutes). The pressurization causes the volume of the pressurized material to be compressed (10%–15% between the range of 300–700 MPa) and a rise in temperature resulting from adiabatic heating (~3°C/100 MPa). These changes are temporary, and during depressurization, they return to their original states; however, both influence microbial inactivation, causing the phase transition of lipid bilayers and high-pressure disruption of ionic bonds, hydrophobic interactions, and hydrogen bonds of the molecules (without affecting the covalent bonds). This causes the macromolecules to unfold. When the pressure is released, the molecules refold but mostly in a manner different from the original configuration, changing the original characteristics. Volume compression (which brings the molecules closer) and adiabatic heating (resulting from temperature-related

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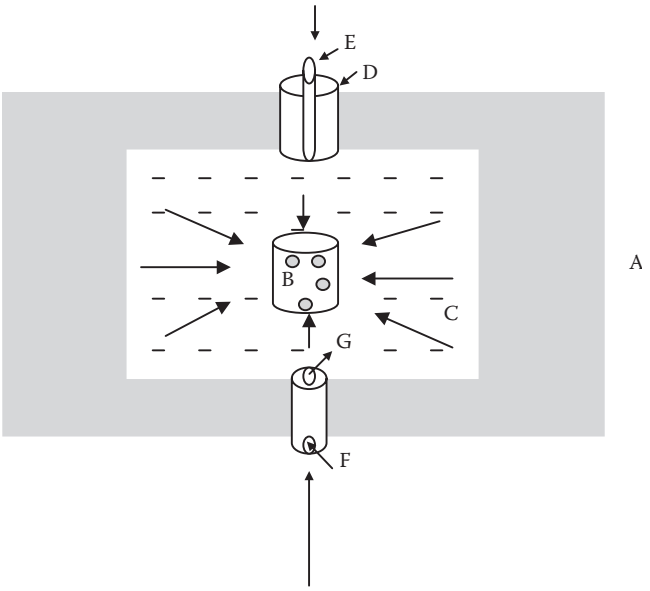



**Figure 40.1 Schematic diagram of hydrostatic pressure vessel (A). The packaged food (B) is put in the liquid from the top inside the chamber (C), and the top is closed by the movable cylinder (D) that has an opening valve (E) to remove air and excess liquid before pressurization. Once the chamber is closed, excess liquid is pumped in through the tube (F) that has a valve (G) that closes when the desired pressure is reached. The pressure is maintained constantly during pressurization time at the end of which the inlet valve (G) opens and the excess liquid is removed by gravity to bring the pressure inside the chamber to atmospheric pressure. Then the top cylinder is moved, and the package is removed from the chamber. The liquid in the chamber can be heated to the desired temperature by installing a heating coil around the vessel (A). The temperature of the liquid inside the chamber can be monitored by placing a thermocouple in the top movable cylinder (D). The process is clean, energy-efficient, and requires relatively less space to operate compared with other food-processing methods.**

actions) augment these molecular changes. Among the molecules, most changes occur in the secondary and tertiary structures of proteins as well as association of proteins with other molecules. In microbial cells, many vital structural and functional components are adversely affected, causing viability loss as well as reversible or sublethal injury. Although damage to the cell membrane is viewed as the major cause of cell death, damages in cell wall, DNA, and RNA; inactivation as well as activation of some enzymes; and lysis of cells have been reported. These changes are enhanced by increasing the pressure, pressurization temperature, and pressurization time. Bacterial spores at a very high pressure (≥700 MPa) are probably inactivated by the inactivation of enzymes associated with the germination process. At a lower pressure range (~200–300 MPa), spores are induced to germination by a mechanism similar to heat-shock germination. Once germinated, the outgrowing spores are susceptible to pressurization like the vegetative cells.

Hydrostatic pressure processing of food requires a very short time, less energy, and less space, and is safer than thermal processing. It does not act on covalent bonds of molecules; thus, vitamins, color pigments, and other small molecules are not adversely affected. Although it is basically a batch process, because of the short time requirements, it can be made continuous by installing

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three or more units in which while one is being filled, the second one is pressurized or depressurized and the third one is emptied. With the availability of units that can handle larger volumes, this becomes a commercial reality. The process has three parameters: pressure, temperature of pressurization, and time of pressurization. By adding suitable antimicrobials during pressurization, it can be made a four-dimensional unique process.

## *Destruction of Microbial Cells*[5,13,14,26]

### *Bacterial Cells*

There have been many studies on the pressure-induced viability loss and sublethal injury of foodborne bacterial cells, with more studies being reported for the pathogens. Some of these include pathogens, such as *Salmonella* serovars, *Campylobacter*, *Lis. monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, and *Esc. coli* O157:H7, and spoilage bacteria, such as *Pseudomonas* spp., *Serratia liquefaciens*, *Leuconostoc mesenteroides*, and *Lactobacillus sake*. The antibacterial effect of hydrostatic pressure can be summarized as follows:

- Gram-negative bacteria are relatively more susceptible than gram-positive bacteria, and rods are more sensitive than cocci.
- Species and strains of a species differ in sensitivity.
- A strain can be sensitive to thermal treatment but resistant to pressure treatment and vice versa.
- Pressure resistance increases with higher consistency, lower $A_W$, higher pH, and higher lipid content of the suspending media.
- Viability loss increases directly with the increase in pressure, temperature, and time with time having the least effect.
- Pressurization also induces sublethal injury in viable cells.
- Suitable antimicrobial agents (physical and chemical) can be included during pressurization to enhance viability loss.

Bacterial cell death in a given set of pressurization parameters occurs in a predictable way. Like in thermal treatment, the death rate follows first-order kinetics, especially at a pressure above 300 MPa and a pressurization temperature of 45°C (113°F) and above. *D* values of eight different foodborne bacterial cell suspensions in peptone solution following pressurization at 345 MPa and 50°C (122°F) were found to be <0.6 minutes. Thus, by a suitable combination of pressure and temperature, a 5- to 6-log reduction can be achieved within five minutes; it is increased further by 1–3 log by incorporating an antimicrobial agent (e.g., a bacteriocin) during pressurization.

### *Bacterial Endospores*

Bacterial spores are fairly resistant to pressures below 700 MPa at ambient temperatures. However, pressures above 700 MPa at temperatures above 70°C (158°F) can be detrimental to them. The information available from reported studies can be generalized as follows:

- Spore inactivation is directly proportional to the levels of pressurization (at ≥700 MPa), pressurization temperature (at ≥70°C), and pressurization time (at ≥30 minutes).
- Spores of species and strains of species vary greatly in pressure resistance.

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- In general, *Bacillus* spores are less resistant than *Clostridium* spores to pressure treatment.
- Dormant and superdormant spores are more pressure resistant.
- High concentrations of nonionic solutes and ionic solutes, low $A_W$, and high pH increase resistance.
- At a low-pressure range (~300 MPa), some spores germinate and outgrow and can be killed by one or more physical and chemical antimicrobial agents.

Although in low-pH foods inactivation of spores is not necessary, it is necessary to destroy (or prevent germination and growth) foodborne *Bacillus* and *Clostridium* spores in high-pH foods. Most results indicate that, even at 800 MPa and a pressurization temperature of 60°C–90°C (140°F–194°F), a 5-log cycle inactivation is difficult to attain in 10 minutes. Similarly, inducing germination of spores at a lower pressure range and then killing the germinated spores with a second cycle of pressurization does not ensure a predictable reduction of spores to achieve commercially sterile foods. It might be necessary to destroy some spores by a combination of parameters applicable for a food type and then prevent growth of the surviving spores with a suitable antimicrobial agent. This method is used in low-heat-processed meat and other food products. A recent study has reported that a combination of high pressures and high temperatures and a second cycle of repressurization can be used to produce commercially sterile food. This is discussed later.

### *Molds, Yeasts, Viruses, and Parasites*

Initial studies were conducted to develop pressurization parameters at ambient temperatures to destroy yeasts and molds because of their ability to grow in low pH, low $A_W$, high osmotic environments, and refrigeration temperatures in fruit juices, jams, and jellies. In general, a 4- to 5-log cycle of most foodborne yeasts and molds (including their spores) can be achieved by pressurizing at 300–350 MPa for 10 minutes at ambient temperatures. Although a few pressure-resistant species and strains of these microbes have been found, treatment at 400 MPa at 40°C (104°F) or a second cycle of pressurization eliminates them.

Almost no report is currently available on the pressure-induced destruction of foodborne protozoa and parasites in food systems. Similarly, the effect of pressure on the inactivation of foodborne pathogenic human viruses and bacteriophages of lactic acid bacteria (and prions) is not known. The current opinion is that the protozoa, parasites, and viruses will be destroyed by pressure at 300–400 MPa at ambient temperature. However, it is necessary to validate this by research.

## *Application in Food Processing*[5,13,14,26,27]

Hydrostatic pressure is being studied for many applications in food processing, including killing microorganisms. Applications can be divided into three groups: novel product development, improving processing techniques, and antimicrobial applications, as listed in Table 40.1. Antimicrobial applications to preserve foods are briefly discussed here. Books and reviews may be consulted for information on other applications.

Antimicrobial applications have been studied to produce mostly pressure-pasteurization products although limited studies are now being conducted to produce commercially sterile products. Pressure pasteurization is considered nonthermal because the foods are pressurized (≤700 MPa) at a lower temperature range (15°C to <70°C) to mainly kill vegetative cells of microorganisms, whereas pressure sterilization involves pressurization (≥700 MPa) at high temperatures (≥90°C or 194°F) to

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**Table 40.1 Some Potential Applications of High Hydrostatic Pressure in Food**

| |
|---|
| *1. Antimicrobial Applications* |
| • To destroy microbial cells at lower pressure range |
| • To destroy bacterial spores at higher pressure range |
| • To germinate bacterial spores at lower pressure range |
| • To prevent microbial growth in unfrozen food |
| *2. Novel Product Development* |
| • To produce fruit and vegetable products with natural taste |
| • To improve color of fruit products and egg yolks |
| • To facilitate gelation of proteins without heat |
| *3. Improved Food Processing* |
| • To tenderize meat |
| • To enhance cheese ripening |
| • To open valves of shellfish |
| • To inactivate undesirable food enzymes |
| • To thaw frozen food rapidly |
| • To store food unfrozen at –20°C |
| • To inactivate allergens and toxins |

destroy bacterial spores. Many types of food are currently being studied for pressure pasteurization, and a few are now commercially produced. A brief list of these is included in Table 40.2.

Some of the results from studies with processed meat products or alfalfa seeds are presented here.[28,29] In each of these studies, a five-strain mixture of pressure-resistant *Lis. monocytogenes*, *Salmonella* serovars, and *Esc. coli* O157:H7 was inoculated either at a high (~$10^7$/g) or a low (~$10^3$/g) level to frankfurters in packages, some of which were then inoculated with a mixture of two bacteriocins, pediocin and nisin. The bags were then pressurized at 345 MPa for five minutes at 50°C (122°F). Packages with high-level inoculation were enumerated for the survivors immediately after pressurization. Packages with low-level inoculation were stored at 4°C, and at selected intervals, multiple packages were tested for the survivors both by enumeration and by isolation

**Table 40.2 Current Status of Food Product Development**

| |
|---|
| • Fruit products: juices, jellies, jams, cut fruits, fruit yogurt |
| • Vegetable products: avocado dip, salsa |
| • Meat products: roast beef, salami, sausage, frankfurters, foie gras |
| • Seafood products: smoked salmon cream, oysters, surimi |
| • Miscellaneous: Spanish rice, spaghetti, rice wine |

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**Table 40.3 Viability Loss Determination of *Lis. monocytogenes* Strains by Pressurization in Inoculated Frankfurters (High-Level Inoculation)**

| *Treatment*[a] | *Log CFU/g* | *Log Viability Loss/g* |
|---|---|---|
| Control | 7.6 | – |
| PN | 6.1 | 1.5 |
| HP | 3.4 | 4.2 |
| HP + PN | ND[b] | 7.6 |

[a] Cell suspension of a five-strain mixture of pressure-resistant *Lis. monocytogenes* was inoculated in hotdog (sterile) packages (50 g/package at 7.6 log CFU/g). Some of the packages were inoculated with a mixture of pediocin AcH and nisin (PN; 1:1; 3000 AU/g). Packages were pressurized at 345 MPa for five minutes at 50°C. Enumeration was done by surface plating on prepoured plates of tryptic soy agar. Each result is the average of four packages. Control, without any treatment; PN, with pediocin and nisin only; HP, only pressurized; HP + PN, pressurized in the presence of PN.

[b] No CFU was detected in 0.4 g sample/package.

techniques. Results of high-level inoculation studies showed that pressurization in the presence of a bacteriocin mixture is more lethal than pressurization alone (Table 40.3). The results of low-level inoculation studies revealed that by the enumeration technique alone, a package might indicate an apparent absence of the pathogen because the technique cannot detect a very low level of survivors (theoretically ~1 CFU/package). However, even from very few survivors in the package, a psychrotrophic pathogen can multiply during refrigerated storage to reach a detectable level (Table 40.4). In contrast, the isolation technique can be effectively used to detect any survivor in a package. Thus, for a highly potent pathogen for which a zero tolerance is desired, the processing parameters

**Table 40.4 Viability Determination of *Listeria monocytogenes* Strains in Inoculated Frankfurters (Low-Level Inoculation)**

| | *Enumeration (log/g) After Storage at 4°C* | | | *Isolation (+/–) After Storage at 4°C* | | |
|---|---|---|---|---|---|---|
| *Treatment*[a] | *1 day* | *7 days* | *14 days* | *1 day* | *7 days* | *14 days* |
| Control (2) | 3.2 | 5.6 | 6.3 | 2/2 | 2/2 | 2/2 |
| BP (3) | ND[b] | 0.5 | 3.6 | 3/3 | 3/3 | 3/3 |
| HP (6) | ND | 0.4 | 0.7 | 1/6 | 5/6 | 6/6 |
| HP + BP (6) | ND | ND | ND | 0/6 | 0/6 | 0/6 |

[a] See footnote of Table 40.3 for explanations. Number in parentheses indicates number of samples tested. Enumeration was done by plating on tryptic soy agar and isolation was done on moxalactam agar. Each package was opened and mixed with a selective enrichment broth. A portion was used for enumeration and the remaining materials in the package were incubated at 37°C for one to two days. A loopful of material was then streaked on a moxalactam agar plate, which, after incubation for 48 hours, was examined for black characteristic colonies for *Listeria* survivors.

[b] No CFU was detected in 0.4 g sample/package.

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should be evaluated by the isolation technique. The results also showed that pressurization in the presence of a suitable antimicrobial (such as a bacteriocin mixture) is more effective in reducing foodborne bacteria.

## Conclusion

To meet consumer demand, several thermal and nonthermal technologies are being studied for the processing of foods. Among these, Plasma technology, PEF and HPP, especially the HPP technology, offer a great potential. Both technologies are nonthermal methods and suitable for the production of pasteurized food. HPP, however, in combination with high heat, can be used to produce commercially sterile foods that have a better acceptance quality than that of foods produced by the comparable thermal process. HPP can also be used to produce novel foods and novel processing techniques. Other parameters can be used with many of these technologies to provide an added advantage. This concept is discussed in Chapter 41 as the hurdle concept.

### QUESTIONS

1. List two thermal and four nonthermal processes among the currently studied technologies for application in food processing and preservation. What advantages do they have over the conventional methods now being used?
2. Briefly describe the principles of the antimicrobial actions of the four nonthermal processes, other than PEF and HPP, listed in this chapter.
3. Briefly discuss the mechanisms of antimicrobial action of PEF technology and how this is being used to improve preservation of foods.
4. Discuss why HPP treatment does not adversely affect the food quality.
5. Describe six other applications of HPP in food processing besides preservation and discuss the advantages of the methods in these applications.
6. Discuss the mechanisms of antimicrobial action of HPP against the vegetative cells of microorganisms.
7. Discuss the problems associated with the sublethal injury and resistance of foodborne pathogens in pressure-processed foods.
8. Discuss the mechanisms by which foodborne bacterial spores can be destroyed at low and high hydrostatic pressure.
9. Define pressure pasteurization and pressure sterilization of food and discuss their differences from pasteurization and commercial sterilization of food by heat treatment.
10. List eight foods from four food groups that have been studied for microbial control in HPP.

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# Chapter 41

# Control by a Combination of Methods (Hurdle Concept)

## Introduction

Many of the factors used to kill microorganisms or control microbial growth in foods are also able to facilitate microbial growth and survival. These factors, which include temperature, $A_w$, pH (acidity), Eh (redox potential), and some preservatives, have a range in which microbial growth varies from optimal to minimal. Beyond the range, depending on the factor, microorganisms either do not grow or die. When only one method is used to preserve food, the condition beyond the growth range of microorganisms is generally used. Examples of such methods are high-temperature treatment to produce a sterile food (preferably in a container to prevent post-heat contamination) or drying a food below an $A_w$ of 0.6. Although, microbiologically, these products can have a very long shelf life and be safe, their acceptance and nutritional qualities are generally lower, and they might not be preferred by many consumers. This is particularly true for consumers interested in foods that are fresh, natural, healthy, and convenient and not harshly processed or harshly preserved. There is a general interest in producing foods that are low in salt, sugar, and fat and that can be stored for a desirable period of time by refrigeration or chilling or even at room temperature and be eaten readily or by microwave heating. There is also an interest in producing such fresh-like foods for the U.S. Army and astronauts.

## Mechanisms of Antimicrobial Action

Many foods invariably contain different types of spoilage bacteria, and there is a good chance that some can have pathogens (e.g., *Staphylococcus aureus* cells, *Listeria monocytogenes*, and pathogenic clostridial spores). For safety and stability, the growth of pathogens and spoilage microorganisms must be controlled during the storage life expected for these products. To achieve this goal, two or more antimicrobial agents are employed together at a level at which the individual agents may facilitate some growth of the concerned microorganism but not when used in combination.[1–3] As



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each of these agents or methods is used "gently" (at a lower level) as opposed to "harshly," they normally do not adversely affect the acceptance and nutritional qualities of foods but do retain their desirable safety and stability. The mechanisms by which the combination of factors, or the hurdle concept, works can be explained with the following example (Figure 41.1A).

Two target microorganisms, a and b, can grow when preservation methods X, Y, or Z are used as individual hurdles. However, if X and Y are combined, the growth of a is arrested, and when X, Y, and Z are used in combination, both microorganisms fail to grow.

The concept can also be explained with another example (Figure 41.1B). Suppose a food has several types of psychrotrophic spoilage bacteria that can grow rapidly at 30°C (86°F) or at pH 6.0 or at an $A_w$ of 0.99 and spoil the food rather quickly. If any one of the factors is reduced (e.g., if the temperature is reduced to 5°C, keeping the other two unaltered), the growth rate will be slightly reduced, but the product can still be spoiled before the desired shelf life time (say 60 days). However, if all three parameters are reduced slightly, which will allow the target microorganisms to grow if used individually, such as 5°C, pH 5.0, and $A_w$ 0.93, their growth will either stop (C2) or occur at a very slow rate (C1), or they might even show some death during storage (C3). With this combination, the product may remain stable during its expected shelf life. Each of the three parameters used in this example can be ineffective individually, but when used together, their effect becomes additive, which the target organisms fail to accommodate for during growth. If more such parameters are added, the growth of microorganisms in foods can be reduced drastically for a considerable period of time.

The different parameters or factors that are used in combination include intrinsic factors (e.g., $A_w$, pH, Eh, and natural inhibitors), processing factors (e.g., heating, drying, fermentation, and preservatives), and extrinsic factors (e.g., temperature and an aerobic or anaerobic environment). Competitive flora (e.g., lactic acid bacteria) and nonthermal processing methods (Chapter 40) can be added to them. Food preservation by combining several of these factors has been used for a long time. An example is the preservation of jams and jellies, for which high heat, low pH (of

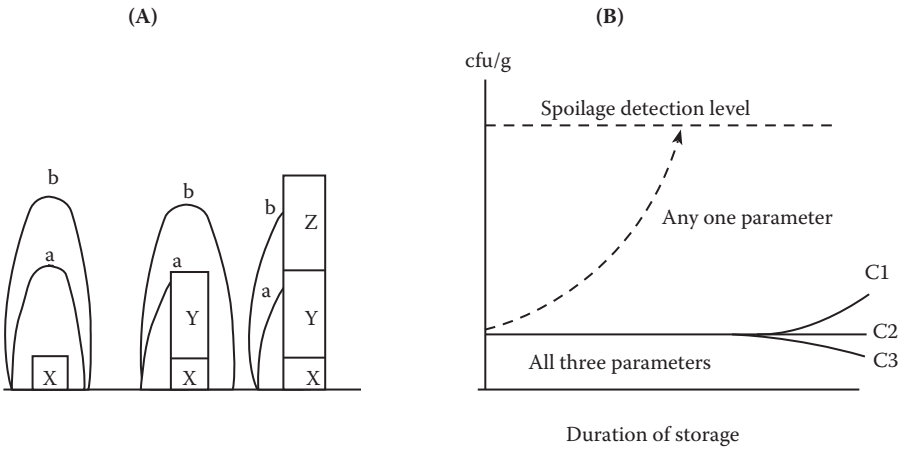



**Figure 41.1 (A) Schematic representation showing how the combination of several preservation techniques (X, Y, Z) is more effective in controlling different microbial groups (a, b). (B) An effective combination of several parameters can reduce the growth rate (C1), prevent growth (C2), or even kill (C3) microorganisms, which might not be achieved by using any one parameter.**

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fruits), low $A_w$ (sugar in fruits and added), and anaerobic packaging are used to reduce microbial numbers as well as the growth of survivors. However, the greatest challenge will be the long-term preservation of meat, dairy, and fish products at refrigerated temperatures or even at ambient temperatures, when refrigeration is not always available, such as during a military operation, a work relief during a natural disaster, or in the rural areas of many developing countries. To achieve this goal, it will be necessary to study the effectiveness of combining different factors to control target microorganisms in a food. In conducting such studies, one should also recognize that the factors could have additive, synergistic, or even adverse (neutralizing) effects when used in combination.

## Current Status

These aspects have been studied in a rather limited way. Results of some of these studies are briefly described here.[1–5]

### *Low-Heat Processing*

Low heat (≤100°C) does not kill many pathogenic and spoilage bacterial spores. They can be heat activated, which leads to germination and outgrowth. However, if the pH of the food is reduced to 4.5 or $NO_2$ and NaCl are added, the heat-shocked spores will not germinate.

### *Low Storage Temperature*

*Clostridium botulinum* grows at 35°C (95°F) at an $A_w$ of 0.95. However, if the storage temperature is reduced to 20°C (68°F), it does not grow unless the $A_w$ is increased to 0.97. Similarly, *Lis. monocytogenes* grows at 25°C (77°F) in a broth containing 6.5% NaCl in three days but fails to grow under similar conditions at 14°C (57.2°F).

### *Low pH*

*Clo. botulinum* grows at pH 7.0, 37°C (98.6°F), and an $A_w$ of 0.94; however, when the pH is dropped to 5.3, no growth is observed, even at an $A_w$ of 0.99. A *Salmonella* strain grows at pH 5.8 at an $A_w$ of 0.97, but when the pH is reduced to 5.0, an $A_w$ of 0.99 is required for growth. *Clo. botulinum* produces a toxin during incubation at 16°C (60.8°F) in 28 days at pH 5.5, but at pH 5.2 under the same conditions, no toxin is produced.

In using acid to reduce pH, it is important to recognize that organic acids, such as acetic, propionic, and lactic acids, are more effective than HCl and phosphoric acid. Also, acetic or propionic acids are more effective than lactic acids. Some acids, such as citric and phosphoric acids, act by both lowering pH and chelating divalent cations that are important for microbial functions.

### *Low $A_w$*

Some of the examples used show how a reduced $A_w$ interacts with other parameters in adversely affecting microbial growth. *Sta. aureus* can grow at an $A_w$ of 0.86 and high pH. However, it fails to grow even at an $A_w$ of 0.93 (in the presence of NaCl) at pH 4.6, a pH that favors growth of this bacterium at higher $A_w$. Similarly, *Sta. aureus* grows at 12°C, pH 7.0, and an $A_w$ of 0.93. However, if the $A_w$ is reduced to 0.90, it will not grow under the same conditions.

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It is also important to recognize that at a low $A_w$, some microorganisms may develop resistance to the killing effect of heat.

### Modified Atmosphere

In vacuum-packaged ($N_2$ or $CO_2$) foods, growth of aerobes is prevented and that of facultative anaerobes can be reduced. But there is a concern that vacuum packaging can selectively facilitate growth of anaerobic spoilage and pathogenic bacteria. Thus, suitable combinations of other factors, such as low pH, $A_w$, or preservatives, must be used with modified atmosphere to control their growth.

### Preservatives

Some preservatives, such as NaCl and BHA, act synergistically to increase the antimicrobial action of sorbates. Organic acids are also effective at low pH because of higher concentrations of undissociated molecules. Some preservatives may not be effective at higher pH, and some may lose potency during storage. Bacteriocins may be destroyed by proteolytic enzymes present in the food (raw food). The bactericidal effect of bacteriocins can be enhanced when used in combination with acids, SDS, and EDTA.

### Hydrostatic Pressure Processing (HPP)

The antibacterial effect of ultrahigh hydrostatic pressure can be increased by incorporating mild heat, slightly low pH, bacteriocins, lysozyme, and chitosan.[4] Spores are difficult to destroy unless very high pressure is used (~700 MPa). However, after low-pressure treatment (≥100 MPa), the spores may be induced to germinate, which can then be killed by higher pressure, mild heat, bacteriocins, or other antibacterial agents (see Chapter 40).

## Conclusion

Many of the control methods listed, such as low heat, low pH, low $A_w$, low temperature, and hydrostatic pressure, induce sublethal stress among the surviving cells and spores. Cells and spores injured by one parameter become sensitive to other parameters and are killed in their presence. Sublethal injury to microbial cells and spores and increased susceptibility of injured cells and spores to one or more preservation methods can play important roles in controlling microorganisms by a combination of factors.[6]

It will be important to study the beneficial effect of combining different parameters at different levels to determine their antimicrobial efficiency against target microorganisms in food systems. The hurdle concept is based on the scientific concept that when two or more methods are used together the combinations are more effective even at a much lower treatment level. However, because of the large number of possibilities, actual product challenge studies may not be possible for all. Instead, at the initial stage, computer simulation by using multifactorial parameters can be designed that take into consideration the characteristics of a food. Then a limited number of selected parameters can be studied by product inoculation. These methods are now being developed (see Appendix A).

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**QUESTIONS**

1. Describe the principle of using a combination of factors, or the hurdle concept, in the preservation of "fresh-like" foods.
2. In combining two or more factors to preserve foods, what criteria need to be considered?
3. Give three examples to show how the combination of factors has been found to control the growth of foodborne pathogens.
4. Spores of *Clostridium laramie*, associated with the spoilage of roast beef at refrigerated temperatures, were not destroyed by a UHP treatment of 80,000 psi for 20 minutes. In contrast, when the spores were subjected to a UHP treatment at 20,000 psi for 10 minutes and then treated with a bacteriocin containing biopreservatives and incubated at optimum temperature, no growth was observed. However, the pressurized samples without bacteriocin had growth. Explain the possible causes for the difference.
5. How can microbial sublethal injury be advantageously used to control microbial growth in food preserved by the hurdle concept?
6. Take a trip to your nearest supermarket and select six refrigerated foods that have been preserved (to control microbes) by using the hurdle concept.

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# MICROBIAL DETECTION AND FOOD SAFETY VII

This section addresses topics related to methods and practices to improve food safety and to reduce foodborne illnesses. These topics are considered either relatively new in food microbiology or taught in special and advanced food microbiology courses or in laboratory courses. Thus, these topics can be used as additional reading materials:

Chapter 42: Conventional, Immunological, Molecular, and Biosensor-Based Detection Methods
Appendix A: Predictive Modeling of Microbial Growth in Food
Appendix B: Regulatory Agencies Monitoring Microbiological Safety of Foods in the United States
Appendix C: Hazard Analysis Critical Control Points

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# Chapter 42

# Conventional, Immunological, Molecular, and Biosensor-Based Detection Methods

## Introduction

Food safety and emerging food biosecurity issues are important to consumers, food manufacturers, and food producers alike. Though foodborne pathogen statistics show a slight decline in the number of cases, outbreaks and product recalls continue to exert a huge economic burden on the producers. Microbial populations in foods, food ingredients, and the food contact environment normally constitute many different species coming from different sources. The total microbial population in a food varies greatly depending upon the level of sanitation used at all phases of production, the degree of abuse that leads to microbial growth, and the processing and preservation methods used to kill and prevent growth of microorganisms. Similarly, contamination of a food by specific types or species of microorganisms is dependent on the presence or source of these microorganisms and their entrance into the food, mostly as a result of poor sanitation during handling and processing. Microbiological examination of foods and food ingredients helps one assess their safety to consumers, their stability or shelf life under normal storage conditions, and the level of sanitation used during handling. In addition, the microbiological load and type can be important in determining whether a food and food ingredient meet acceptable standards, specifications, and guidelines. Microbiological evaluation of raw materials also provides important information about the heat-processing parameters that would be necessary to meet the microbiological standard, guideline, or specification of a product. Finally, microbiological evaluation of a food, food ingredient, and environment helps determine possible sources of a specific microbial type in a food and, in the case of a thermally processed food, the source and nature of post-heat treatment contamination. Despite all precautions, pathogens are found in ready-to-eat products, which is a serious concern because these products do not receive further treatment before consumption. In fact, several foodborne outbreaks occurred because of consumption of undercooked or minimally processed ready-to-eat meats (hotdogs, sliced luncheon meats, and salami), dairy products (soft cheeses made



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with unpasteurized milk, ice cream, butter, etc.), or minimally processed fruits (apple cider, strawberries, raspberries, cantaloupe, etc.) and vegetables (sprouts, lettuce, spinach, etc.). Food animals and poultry are the most important reservoirs for many of the foodborne pathogens, including *Salmonella*, *Campylobacter*, *Listeria*, or *Escherichia coli* O157:H7. Animal byproducts, such as feed supplements, may transmit pathogens to other animals (e.g., *Salmonella*, Prion associated with bovine spongiform encephalopathy [BSE] or mad cow disease). Application of untreated manure onto farmland can also contaminate soil or water with the eventual contamination of fruits and vegetables. Seafoods are another potential source of foodborne pathogens, including *Vibrio* species, *Listeria monocytogenes*, and Hepatitis A. Infectious doses of many of these pathogens are very low (~10–1000 cells). Detection technologies, both traditional and rapid, are able to detect such low numbers, but they are lengthy and laborious. Furthermore, ongoing concern with intentional administration of harmful microorganisms or toxins in food or water supplies demands significant improvement in detection technologies for a fast time to result with a high degree of accuracy. Modern biosensor technologies are showing some promising results in pathogen detection and principles, and application of some selected technologies are presented later in this chapter.

## Methods Used

The methods used for the microbiological evaluation or detection of foods, food ingredients, and environments are broadly grouped as quantitative and qualitative methods.[1–4] The quantitative methods are designed to enumerate or estimate directly or indirectly the microbial load in a test material. It should be recognized that none of the quantitative methods that are used now enumerate or estimate "total microbes," "total bacteria," or "total viable population" (terms used by many) present in a food although they can be obtained for a pure culture of a microbial strain growing in a sterile liquid or a solid medium. Rather, each method enumerates or estimates a specific group among the total microbial population that is normally present in a food, which grows or multiplies preferentially under the conditions or methods of testing. These include composition of an enumeration medium, temperature and time of incubation and oxygen availability during incubation, pH, and treatments given to a sample prior to enumeration and estimation. Examples of some of the quantitative methods used are total aerobic plate counts (TAPC) (or standard plate counts, SPC, for dairy products), anaerobic counts, psychrotrophic counts, thermoduric counts, coliform counts, *Staphylococcus aureus* counts, and yeast and mold counts.

In contrast, qualitative methods are designed to determine whether a representative amount (a sample) of a food or a certain number of samples in a batch of a food contain a specific microbial species among the total microbial population or not. These methods are used to detect the possible presence of certain foodborne pathogens, especially those capable of inflicting high fatality rates among the consumers. *Salmonella*, *Clostridium botulinum*, *Escherichia coli* O157:H7 and other Shiga-toxin producing *Esc. coli* (STEC), and *Listeria monocytogenes* in ready-to-eat foods are some that fall into this group.

## Standard and Recommended Methods

Many methods and variations of different methods that can be used for quantitative and qualitative detection of microorganisms in foods are reported in the literature. However, it is desirable to use methods that have been approved by the regulatory agencies. They can be either standard or recommended methods. In the United States, for the microbiological examinations of milk and

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milk products, one needs to use the methods that have been designated as standard by the regulatory agencies. For other foods, however, specific microbiological methods have been recommended or approved by the regulatory agencies. Although it is advisable to use such a method, there is no mandatory requirement. Some of the publications in the United States that are approved by the regulatory agencies are *Standard Methods for the Examination of Dairy Products*, *Standard Methods for the Examination of Water and Waste Water*, *Standard Methods for the Examination of Seawater and Shellfish*, *Compendium of Methods for the Microbiological Examination of Food* (all four are published by the American Public Health Association, Washington, D.C.), and *Bacteriological Analytical Manual of Food and Drug Administration* (prepared by the FDA and published by the Association of Official Analytical Chemists, Arlington, VA). The last two publications include recommended methods. All these books are revised, when necessary to incorporate the new methods and to update old methods, by different groups of experts. Many other countries have similar publications for standard and recommended methods. Similar books are published by international organizations, such as specific branches of the World Health Organization and the Food and Agricultural Organization (FAO) of the United Nations, and the International Standards Organization (ISO) publishes standard methods for foodborne pathogens in food and foodstuffs.

A food microbiology course invariably contains a laboratory component. The methods described in one or more of these books for the microbiological examination of food, food ingredients, and environments can be used in the laboratory. This will help the students or interested individuals become familiar with the standard and recommended methods approved by the regulatory agencies in the United States. Some of the methods are briefly discussed here. Details of these methods are available in the books listed above and references listed at the end of this chapter.

## Sampling for Microbial Analysis

### *Sample and Sampling Plan*[4–6]

The microbiological quality of a batch or a lot of a food, which could be liquid, solid, or semisolid, is determined by testing a small portion of the total amount. This tested portion is called a sample, and it must be the true representative of the total mass. It is very important to develop and implement an effective sampling plan; otherwise, even the most sensitive testing method will not provide information about the microbiological quality of a food. This is particularly important for the highly virulent pathogens, such as *Salmonella* or *Esc. coli* O157:H7 or a potent toxin, such as botulinum. In addition, different laboratories testing the same product but with samples that are collected differently could give different results, thus making it difficult for regulatory agencies to decide whether to accept or reject a lot to ensure safety to the consumers.

Several statistical sampling plans have been described in standard or recommended methodology books. Four of these are briefly discussed here.[3,4]

1. *Single attribute plan*: In this plan, a lot is sampled once and tested for the bacterium or bacteria of interest. The results are used to determine if a lot should be rejected or accepted (on the basis of the presence or absence of *Esc. coli* O157:H7). The sample size or number of samples ($n$) to be tested is dependent on the batch or lot size ($N$). Defective unit(s) (C) of $n$ is dependent on the microbial type.
2. *Multiple attributes plan*: Initially based on the lot size ($N$) the number of samples ($n_1$) are tested. If the defective unit(s) ($C_1$) is above the acceptance level, a second sampling is taken ($n_2$), tested, and analyzed for defective units ($C_2$) to decide to accept or reject the lot.

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3. *Three-class attribute plan*: Microbial levels are divided into three groups: ≤ *m* is acceptable; > *m* but ≤ *M* is marginal; and > *M* is unacceptable. Number of units ($C_1$) allowed between > *m* and ≤ *M* among a number of samples tested (*n*) is defined for a microbial type.
4. *Plan for low level of contamination*: This is important for some highly potent pathogens. Units, depending upon the lot size, are tested to determine the presence or absence of a pathogen in the unit to make a decision for acceptance or rejection of a lot.

### *Sampling Procedure*

The individual collecting the samples should know why the samples are being collected, that is, what type(s) of testing will be conducted, such as for APC, coliforms, or a specific pathogen. The person should also have information about the product if it has been implicated in a foodborne outbreak and the nature of the product (liquid, solid, or semisolid; frozen; bulk or single units). The sample should be collected aseptically using proper sanitary measures to prevent any contamination and in large enough volumes to allow repeat analysis, if needed. Following collection and until tested, the samples should not be handled to avoid growth or death of microorganisms. If the product is frozen, samples should be kept frozen until analyzed. Otherwise they can be stored at 0°C–4°C. Each sample should be labeled to identify date, time, nature of sample, types of analysis to be conducted, and the person(s) who collected the sample. The samples should be transported to the laboratory under proper conditions to avoid microbial contamination, growth, or death.

The laboratory that will test the samples should examine the conditions, such as temperature, appearance, and the sampling information, and note the time of receiving the samples. Once received in good condition, a sample should be tested as soon as possible. Standard or recommended methods should be used for the preparation of the sample and the procedures of testing for a specific microorganism or microbial group(s). The unused portion of the sample should be stored under proper conditions until the results are available. In some specific situations, a sample might need to be preserved for a considerable period of time (e.g., in an outbreak involving a lawsuit). The results should be recorded immediately and properly in permanent form.

## Quantitative Methods for Microbial Enumeration in Foods

### *Direct Enumeration*

#### *Microscopic Counts*

Either stained cells under a bright field or live cells under a phase contrast microscope can be counted and, using an appropriate microscopic factor, these counts can be expressed as microscopic counts per milliliter or gram of the food sample. However, viable and dead cells cannot be differentiated by this method. In addition, a sample must have large numbers (≈$10^6$/mL, g) of microorganisms for effective use of this method.[4] Foods that have particles cannot be effectively tested for enumeration microscopically. A commercially available LIVE/DEAD BacLight stain kit allows one to count fluorescent-labeled live gram-positive or gram-negative bacteria under a fluorescence microscope.

#### *Colony-Forming Units (CFU) in Nonselective Agar Media*

Aliquots from selected dilutions of a serially diluted sample (generally based on the number expected in a food; see Chapter 4) are either pour-plated or surface-plated using nonselective

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media, such as plate count agar (PCA), tryptic soy agar, nutrient agar, brain-heart infusion agar, and others. Different media can give different results. However, PCA is recommended for colony-forming unit (CFU) determination of several groups. The temperature and time of plate incubation required for the colonies to develop differs for the microbial groups being enumerated. For SPC, it is 32°C for 48 hours; for APC, it is 35°C for 48 hours; and for psychrotrophic counts, it is 7°C for 10 days or 10°C for seven days. The same procedure with specific modifications can be used to determine thermophilic, thermoduric, and anaerobic groups present in a food sample. The specific group(s) to be tested depends on their relative importance in a food. For a vacuum-packaged refrigerated food, the most important groups will be psychrotrophic, anaerobic, and facultative anaerobic groups (see Chapter 21).

### *CFU in Nonselective Differential Chromogenic Media*

A nonselective medium is supplemented with an agent capable of differentiating the colonies produced by specific group(s) of microorganisms that differ in metabolic or physiological characteristics from one another in the population by producing chromogenic pigments. pH indicators or oxidation-reduction indicators are often used in the medium. Thus, the colonies of cells capable of metabolizing lactose to lactic acid are differentiated from those that do not ferment lactose by growing them in an agar medium supplemented with lactose as a carbon source and a pH indicator, such as bromocresol purple. The lactose-fermenting colonies will be yellow, and the others that cannot ferment lactose, but grow, will be white against a purple background. Differential methods are also used in specific media for the enumeration of proteolytic, lipolytic, and pectinolytic microbial groups in a food.

### *CFU in Selective Agar Media*

A medium can be supplemented with one or more selective or inhibitory agents, such as antibiotics, salts, and so forth, and used by pour or surface plating of serially diluted samples. In the presence of such agents, only the microorganisms resistant to them can grow. Incubation conditions to stimulate colony formation differ with the organisms being studied. Enumeration of aciduric bacteria in a medium at pH 5.0, yeasts and molds in a medium at pH 3.5, and *Clostridium perfringens* in the presence of cycloserine are examples of selective enumeration of specific groups present in a food. Halophilic and osmophilic microorganisms can also be enumerated similarly by specific selective procedures.

### *CFU in Selective-Differential Chromogenic Agar Media*

In this method, a medium is supplemented with one or more selective agents to allow selective growth of specific resistant microbial group(s) while inhibiting growth of other sensitive associative microorganisms. In addition to selective agent(s), a medium is also supplemented with agent(s) that enable each type among the selective microbial groups to produce colonies with pigments (chromogenic) that differ in characteristics from one another. Violet red bile agar for coliforms, KF-azide agar for *Enterococcus* spp., V-J agar or Baird-Parker agar for *Sta. aureus*, and media recommended for the enumeration of some pathogens (e.g., *Yersinia enterocolitica*, *Campylobacter jejuni*, *Salmonella enterica*, *Lis. monocytogenes*, *Clo. perfringens*, *Aeromonas hydrophila*) are selective as well as differential agar media. Because of the presence of one or more selective compounds, they allow selective growth and colony formation of several closely related species. The differential

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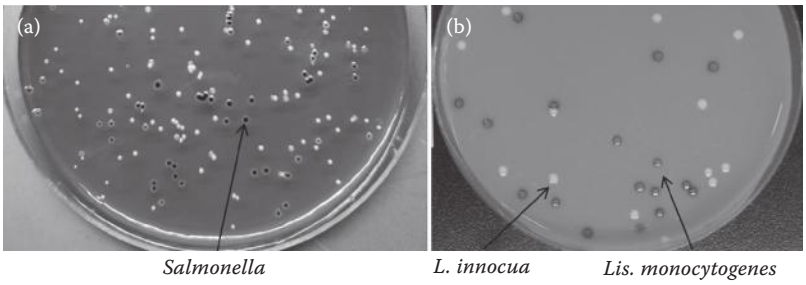



**Figure 42.1 (See color insert.) (a) Isolation of *Salmonella enterica* from raw chicken rinse using XLD (Xylose lysine deoxycholate) agar. Typical salmonella colonies appear as pink with a dark center. (b) Isolation of *Listeria monocytogenes* (pathogen) and *Lis. innocua* (nonpathogen) on RAPID'L.mono medium.**

agents then help to differentiate these species or groups from one another by their specific colony characteristics. Figure 42.1 shows the growth of a *Salmonella* colony on a XLD (xylose lysine deoxycholate) agar plate producing a pink colony with a dark center while non-salmonella produced white colonies. Examples of some selective differential chromogenic media for three major foodborne pathogens are presented in Table 42.1.

**Table 42.1 Examples of Selective Differential Agar Media with Antimicrobial Components for Three Major Foodborne Pathogens**

| *Pathogen* | *Selective Differential Media* | *Selective Agents* | *Inhibitory Action* |
|---|---|---|---|
| *Salmonella enterica* | XLD (Xylose lysine deoxycholate) agar | Sodium desoxycholate<br>Sodium thiosul/ph/fate<br>Ferric ammonium citrate | Coliforms, *Enterococcus*, *Shigella* |
| *Esc. coli* O157:H7 | CT-SMAC (Cefixime and tellurite–sorbitol McConkey agar) | Bile salt<br>Crystal violet<br>Cefixime and tellurite | Gram positives and other gram negatives, CT inhibits nonsorbitol fermenting *Esc. coli* |
| *Listeria monocytogenes* | MOX (modified Oxford medium) | Cefotetan<br>Acriflavin<br>Colistin sulphate<br>Cycloheximide<br>Fosfomycin | Gram-negatives, *Enterococcus*, yeast and molds |
| | PALCAM (Polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol agar) | Lithium chloride<br>Acriflavin<br>Ceftazidime<br>Polymyxin B | Gram-negatives, *Enterococcus*, yeast and molds |

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## Indirect Estimation

### Dilution to Extinction in Nonselective Broths

This method consists of serial dilution of a sample and transfer of an aliquot (usually 1.0 mL or 0.1 mL) to a final 5 mL or 10 mL of a nonselective broth, such as tryptic soy broth. This is followed by incubation of the tubes at a specific temperature for a specific period of time, which depends on the specific microbial group being investigated. The tubes are then examined for the presence or absence of growth (from the turbidity of the broth). Using the highest sample dilution that gave growth and assuming that this tube had one to nine viable cells of the group of interest, microbial numbers per milliliter or gram sample are estimated. The estimated numbers, however, can vary widely from the actual numbers. This method is not used much for the microbiological estimation in food.[4]

### Most Probable Number (MPN) in Selective Broth

In this method, aliquots from a serially diluted sample are inoculated in a broth (in tubes) having one or more selective agents that facilitate growth of selected microbial groups present in a food. Generally, three or five broth tubes in each dilution and a minimum of three consecutive dilutions are used. After incubation at a recommended temperature and time, the broth tubes in each dilution are scored for the presence and absence of growth. From the number of tubes showing growth in each of the three successive dilutions, the number of viable cells of the specific microbial group can be estimated from the available statistically calculated tables. This method also gives wide variation. MPN methods are used quite often for the estimation of coliforms and fecal coliforms in foods and water using brilliant green lactose bile broth and E-C broth, respectively.[3,4] A commercial automated MPN analysis system (TEMPO®) is now available for food quality assurance.

### Dye Reduction Test

This method is based on the principle that some dyes, such as methylene blue and resazurin, are colored in oxidized states but colorless under reduced conditions. This change can occur as a result of microbial metabolism and growth. It is assumed that the rate of reduction during incubation of a specific concentration of methylene blue added to a food is directly proportional to the initial microbial load in the food. However, as microbial groups in a mixed culture in a food differ in the rate of metabolism and growth and ability to reduce the environment, this method is not considered very accurate and effective with different foods. This method is generally used to determine the microbiological quality of raw milk.

## Enumeration of Injured Microbial Groups by Selective Media[7]

Sublethally injured coliforms and pathogenic bacterial cells, when enumerated by selective agar media, may die as a result of their developed sensitivity to selective agents in the media and thus cannot be detected (see Chapter 10). They are first allowed to repair the injury in nonselective media (broth or agar) for a short period, which enables the cells to regain resistance to the selective agents. Following repair, they can be exposed to selective media. For the enumeration of coliforms in food that may contain injured coliforms, the diluted sample can first be pour-plated in nonselective PCA and incubated for one to two hours at 25°C–35°C, thus enabling the repair of the cells. Double-strength violet red bile agar can then be overlaid on the plates and the plates incubated at 35°C for 24 hours for selective growth of coliforms.

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### Dilution Scheme, Plating, Incubation, Selection of Plates for Counting CFU, and Reporting Results

The standard and recommended method books listed above have detailed descriptions on these steps. They should be followed as accurately as possible. The average of CFU from duplicate or triplicate plates from a selected dilution should be used to report the counts of the specific microbial group per mL or g or $cm^2$ of the food sample. This data should be used in the proper interpretation of microbiological quality of the food.

## Qualitative Methods to Isolate Microorganisms in Foods

### Isolation of Pathogens[3,8,9]

The main objective of this method is to determine if a sample does or does not contain viable cells or spores of a specific pathogen. Foods are tested for several pathogens, such as *Salmonella*, *Esc. coli* O157:H7, Shiga-toxin producing *Esc. coli* (STEC), *Lis. monocytogenes*, *Vibrio cholerae*, and *Shigella* spp., by the specific isolation procedure when necessary. For other pathogens, such as enteropathogenic *Esc. coli*, *Yer. enterocolitica*, and *Cam. jejuni*, isolation procedures are not generally used; instead enumeration procedures are used.

An isolation procedure generally contains several steps, such as nonselective preenrichment followed by selective enrichment and then testing on an agar medium containing selective and differential agents. It is assumed that a food normally contains a low population of a pathogen as compared to the associative microorganisms, and the pathogens could be in injured state. The food sample (e.g., 25 g) is first preenriched in a nonselective broth (225 mL) and incubated for the injured cells to repair and then multiply in order to reach moderately high numbers (along with many other associated microorganisms). An aliquot is then transferred from the preenrichment broth to a selective enrichment broth and incubated. It is expected that, during incubation, the specific pathogen and closely related microorganisms will selectively grow to a high number while many of the associated microorganisms will not grow. A small amount (≈0.01 mL) of the enrichment broth is then streaked on the surface of a prepoured selective-differential agar medium plate, which is then incubated for a specified time for the colonies to develop. From the differential colony characteristics, the presence of a specific pathogen can be tentatively established.

This is generally considered a presumptive test. For confirmation, the cells from the characteristic colonies are purified and examined by the recommended methods for biochemical reaction profiles, serological reaction against a specific antibody, an immunochromatographic lateral flow assay, or by the polymerase chain reaction (PCR) discussed below. Isolation of a pathogen using the conventional methods can take 10–12 days, depending upon a particular species.

## Test for Bacterial Toxins in Foods

*Sta. aureus*, *Clo. botulinum*, *Bacillus cereus*, *Vibrio parahaemolyticus*, and molds, while growing in foods, are capable of producing toxins and causing intoxication or food poisoning among the consumers. Specific methods have been developed to test the presence of toxins in the suspected foods.[3,10]

To detect *Sta. aureus* enterotoxin in foods, recommended methods are to initially extract the toxin from 100 g of the food and then to concentrate the toxin in 0.2 mL sterile water or 0.2 M saline. The presence of toxin in the concentrated extract is then assayed against a specific antibody

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by the microslide precipitation method. This method is sensitive at the level of 0.05 μg enterotoxin per milliliter of the extract (food poisoning probably can occur from the consumption of less than 1.0 μg of *Sta. aureus* enterotoxin A). Methods, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and reverse passive latex agglutination assay (RPLA), can also be used in place of the microslide precipitation method. Biosensor-based surface plasmon resonance (SPR) sensors and fiber optic array sensors can detect this toxin in 1–20 ng quantities.

To detect botulinum neurotoxin produced by *Clo. botulinum* strains, the recommended procedure is to first extract toxin from a food. To activate toxin of nonproteolytic types (B and E), a portion of the extract is then treated with trypsin. Both trypsinized and nontrypsinized (for proteolytic types A and B) extracts in 0.5-mL portions are then injected into mice intraperitoneally (two mice for each sample). Heated (100°C for 10 minutes) extracts are injected similarly (control). The mice are then observed up to 48 hours for botulism symptoms and death. Typical botulism symptoms in mice, in sequence, are ruffling of fur, rapid and gasping breathing, weakness of limbs, and death from respiratory failure.

Specific methods for testing toxins of some other pathogenic bacteria and molds have also been developed and listed in the recommended books as well as available from commercial vendors.

## Rapid Methods and Automation

Conventional culture-based methods are considered a gold standard for pathogen detection. These methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in foods as discussed above. It consists of five steps involving preenrichment, selective enrichment, selective plating for colony identification, a biochemical presumptive test, and a serological confirmation test. The preenrichment step is beneficial because this step not only increases the populations of the target organism but also allows recovery of sublethally injured or stressed bacteria resulting from exposure to processing and storage conditions, namely heating, drying, freezing, cooling, preservatives, and so forth. Injured microbes, under favorable environments, are capable of recovering in food and causing disease in humans.

The culture method is highly sensitive, relatively inexpensive, and can give both qualitative and quantitative information on the number and the nature of the microorganisms.[8–13] However, it is labor intensive and lengthy, requiring 7–10 days. To overcome these difficulties, different rapid methods, many of which are automated, have been developed to detect microbial loads, foodborne pathogens, and their toxins.[3,8,11] Some of these methods have been approved by the regulatory agencies. Furthermore, rapid methods are highly desirable for use in the food processing plant for monitoring pathogens in raw materials, ready-to-eat food products, and to verify manufacturing process control. Bacteria-specific generic rapid methods are also needed for monitoring cleaning and hygiene practices employed in a processing plant. In addition to being rapid, they are quite specific, sensitive, relatively accurate, and less labor intensive. However, their effectiveness in food systems with a low level of contamination needs further evaluation. Many new methods are constantly being developed, which differ mainly in technique. The principles, procedures, and applications in food of some of these methods are briefly presented. Exact methods and steps are provided by the commercial farms.

### *Metabolic Fingerprinting*[8,10]

Each species of bacterium, mold, or yeast has unique carbon or nitrogen requirements for growth and survival. When a panel of appropriate sugars (carbon source) or amino acids (nitrogen source)

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is provided, microbes will metabolize and produce byproducts, such as acid or ionic species, that will reduce a colorless indicator like tetrazolium salt to a purple product, generating unique metabolic fingerprint patterns. This method requires pure cultures and certain physiological properties, such as gram-staining, oxygen requirements, and growth temperature optima, before applying this technique for detection. Miniature biochemical test kits (API strips) are available commercially for different microorganisms. In a more advanced metabolic fingerprinting system, a 96-well plate containing multiple substrates, sugars, or amino acids are used, and the substrate utilization patterns are compared with the database for specific identification of an organism in the MicroLog system (Biolog, Inc.).

## *Immunoassays for Rapid Detection of Pathogens*[12,13]

Several rapid and automated methods have been developed that rely on the specific antigen-antibody reaction and production of agglutination, color formation from a chromogenic substrate, formation of an immunoband, or fluorescence. Pathogen diagnosis relies heavily on antibodies. Antibody-based assay methods are simple and less cumbersome and have and easy-to-interpret results. These methods allow detection of intact cells, their secreted toxins, or metabolic byproducts. However, this method may not be able to differentiate between live and dead cells although live cells are of concern in food safety. Active culturing coupled with immunoassay can overcome such a problem. In addition, microbes are routinely exposed to sublethal injury or stress conditions, such as acidic pH, osmotic stress (salt), antimicrobial preservatives, storage temperatures, and heat-shock conditions in food, which may alter morphology and affect physiology resulting in aberrant antigen expression and a weakened signal during antibody-based detection.

The success of the immunoassay depends on the availability of an antigen-specific antibody. More importantly, the knowledge of antibodies' binding affinity and avidity should be known before employing them for a specific application. Two types of antibodies are used commonly: polyclonal and monoclonal. Polyclonal antibodies (PAb) contain an assortment of antibody molecules having different cellular origins and made against different epitopes of the same antigen, and therefore they may show some cross-reactions with antigens from different microbes. To overcome this problem, PAb can be made epitope-specific by carefully designing an epitope-specific synthetic peptide. The peptide is then conjugated to a large carrier protein for immunization of animals, such as rabbits or goats, for antibody generation. The quality of such antibodies may vary from batch to batch, which may affect the end result. In contrast, monoclonal antibodies (MAb) are homogeneous and are highly specific because they are produced by a single, preselected B-lymphocyte clone that is fused with a cancerous immortal myeloma cell to form a hybridoma line. Immunoassay experiments designed carefully to employ both polyclonal and monoclonal antibodies in a sandwich format can provide the best signal and the highest specificity with reproducible data.

Antibodies are widely used in various assay formats, such as immunomagnetic separation methods for capture and concentration of microbes, enzyme or fluorescence immunoassays, and immunosensor applications, which are discussed below.

### *Immunomagnetic Separation (IMS)*[5,11,14]

Small magnetic particles coated with antibody molecules are used in the capture and concentration of target pathogens from a mixture by a process known as immunomagnetic separation (IMS) (Figure 42.2). The magnetic particles used for these applications are superparamagnetic; that is,

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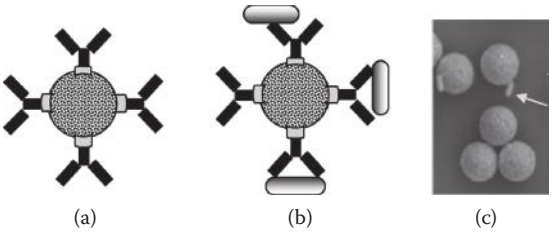



**Figure 42.2 Paramagnetic particles showing bacterial capture with antibody. (a) Paramagnetic particle coated with antibodies, (b) captured bacterial cells, (c) scanning electron microscopic picture showing captured *Listeria* cells (arrow).**

they only exhibit magnetic properties in the presence of an external magnetic field. The most common magnetic carriers are the Dynabeads® with diameter ranging from 1 to 2.8 μm. These are polystyrene-coated iron oxide. The streptavidin-coated beads are available for immobilization of a biotinylated antibody. They can be easily removed from a suspension by using a magnetic separator. Because there is no magnetic remnant, the particles are not attracted to each other and therefore can be easily suspended in a homogeneous mixture in the absence of any external magnetic field. IMS has two steps: First, the suspension containing the microbes of interest is mixed with magnetic particles for about 30–60 minutes, and then the tube containing the magnetic complex is placed in a magnetic separator, and the liquid is poured off. Second, the magnetic complex is washed several times with a buffer to remove unwanted contaminants, and then the cells are collected for further testing using nucleic acid–based PCR, immunoassay, or biosensor-based assays.

Immunomagnetic beads have been used for concentration and separation of various microorganisms from environmental samples and foods using the direct- or indirect-IMS methods. In the direct approach, the target organism is mixed with magnetic particles that are coated with antibodies specific to the organism. When the particles come in contact with the bacterial cells, they attach via the primary antibody. In the indirect approach, the primary antibody is added to the suspension and allowed to attach to the target organism. Then the magnetic particles, coated with a secondary antibody specific for the primary antibody, are added and allowed to attach to the primary antibody. The magnetic particle complexes are then separated using the magnetic concentrator. A flow-through Pathatrix® system is now available commercially in which beads are held in a compartment to capture target pathogens from recirculating fluid to increase capture efficiency from a large volume of samples. IMS has been used in conjunction with selective agar media plating, immunofluorescence assay, PCR, flow cytometry, and pathogenicity-based assays. The IMS method has been proven to be effective in capture of *Esc. coli* O157:H7, *Campylobacter*, *Salmonella*, and *Listeria* from various food matrices. Drawbacks of this method are nonspecific capture of nontarget bacteria and an inconsistent capture rate that may vary from 10% to 70% depending on the antibody or the intended target pathogen. Nevertheless, it is a powerful accessory tool in pathogen detection.

### *Reverse Passive Latex Agglutination (RPLA) Method*

This method has been developed to detect toxins of several foodborne pathogens, namely, *Sta. aureus*, *Clo. perfringens*, *Bac. cereus*, *Vib. cholerae*, and enterotoxigenic *Esc. coli*. The antibody of a specific toxin is immobilized on latex particles that are then mixed with a sample preparation

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suspected to contain a toxin (antigen) in wells of microtitration plates. If the specific toxin is present in the sample, a diffuse pattern will appear in the bottom; in its absence, a ring or button will appear.

### Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is one of the most common assays used for detection of pathogens such as *Salmonella*, *Lis. monocytogenes*, *Esc. coli* O157:H7, and *Cam. Jejuni*, or their toxins. Binding of antigens (pathogens or toxins) to primary antibody or other ligand molecules, such as receptor proteins, aptamers, etc., is quantitatively measured in a 96-well polystyrene plate (microtiter plate) by using a secondary antibody conjugated to an enzyme. Commonly used enzymes are horseradish peroxidase and alkaline phosphatase (ALP). Upon addition of substrates, such as *o*-phenyldiamine and tetramethyl benzidine (both are substrates for peroxidase and *p*-nitrophenyl-phosphate, a substrate for ALP), respectively, a positive color reaction is quantified by measuring the color intensity with a spectrophotometer. The amount of the enzyme present is estimated by the colorimetric determination of catalysis of the substrate and is proportional to the amount of antigen present. Three forms of ELISA assays are generally used: (a) indirect, (b) sandwich, and (c) competitive inhibition assay (Figure 42.3). In an indirect assay, the antigen is first immobilized on the polystyrene plate and reacted with a primary antibody. After washing, an enzyme-labeled secondary antibody is added and then allowed to bind to the primary antibody. (The secondary antibody is anti-animal host species–specific; that is, if the primary antibody is a mouse antibody, the secondary antibody is developed by immunizing rabbit or goat or sheep or any other animal with mouse antibodies.) Then a substrate solution is added for development of color. In "sandwich" ELISA, an antibody is first immobilized on the plate and is then allowed to capture the antigen. Then, a second pathogen-specific antibody labeled with an enzyme is added, forming a typical sandwich configuration. Antigen is required to have at least two binding sites for both antibodies. In competitive inhibition ELISA, bacterial cells or antigens are first immobilized in the wells. In separate tubes, the primary antibody is mixed with various dilutions of bacteria (or antigens) and added to the wells

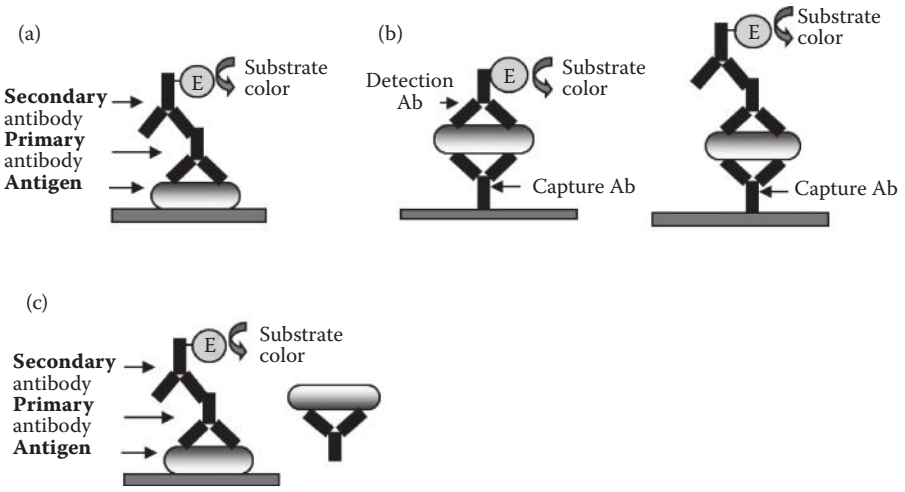



**Figure 42.3 Schematic diagrams for different formats of enzyme-linked immunosorbent assay (ELISA). (a) Indirect ELISA, (b) sandwich ELISA, (c) competitive ELISA.**

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containing the immobilized antigen. In this method, only the free-unbound antibody will bind the immobilized antigen. After washing, a secondary antibody-enzyme conjugate and substrate system are added for color development. The highest dilution of cells showing the minimum reaction, or equivalent to a background control, is considered positive. Generally, the detection limit for all ELISA assays ranges from $10^6$ to $10^7$ bacterial cells/mL. The presence of that high level of contaminants in a ready-to-eat product is not expected; therefore, an intermediate enrichment step is necessary to increase cell population. Several commercial ELISA assay kits are currently available for detection of foodborne pathogens from enriched food samples.[3,12]

### *Immunofluorescence Assay*

In principle, an immunofluorescence (IF) assay is similar to an ELISA assay. In IF, a fluorescently labeled detection antibody (against somatic or flagellar antigens of a pathogen) that emits fluorescence after forming a complex with an antigen on a glass slide or in a 96-well microtiter plate is used, which is detected by a fluorescence microscope, a digital camera, or a spectrofluorometer. The fluorescent markers used are rhodamine B, fluorescein isocyanate, and fluorescein isothiocyanate (FITC). The fluorescent antibody technique can be carried out by using two basic methods: The direct method employs binding of antigens with a fluorescent-labeled specific antibody. In the indirect method, the primary antibody is not coupled with the fluorescence, but instead, a species-specific secondary antibody is labeled with fluorescence. In the indirect method, the labeled antibody detects the presence of the primary antibody and antigen complex. The use of the indirect method eliminates the need to prepare a fluorescent-labeled antibody for each organism of interest.

### *Immunochromatographic Lateral Flow Assay*

The immunochromatographic lateral flow assay (also known as the "dipstick" assay) is a "sandwich" assay that is performed on a membrane rather than in microtiter wells as are used in ELISA (Figure 42.4). First, the captured antibody is immobilized on the membrane in a predefined area. A detection antibody, previously coupled with a color reactant (colloidal gold or latex particles), is incorporated into the nitrocellulose strip. A sample containing a specific antigen (microbe)

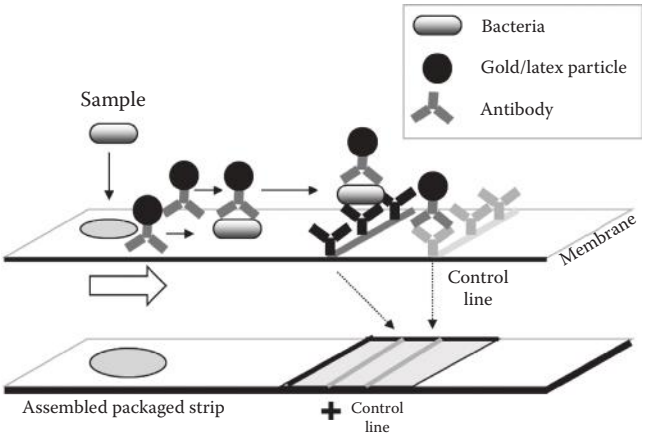



**Figure 42.4 Immunochromatographic lateral flow assay.**

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is added to the sample and allowed to bind to the colloidal gold or latex particle–conjugated antibody. This complex flows to the other end of the strip through a porous membrane by capillary action that contains two captured zones, one specific for the bacterial pathogen and another specific for unbound antibodies coupled to the color reagent (control line). The presence of only one (control) line on the membrane indicates a negative sample, and the presence of two lines indicates a positive result. The lateral flow assay gives results in 5–10 minutes, is easy to perform, is economical, and is suitable as an initial screening test.[12]

### *Flow Cytometry*

Flow cytometry permits rapid characterization of a cell population based on a number of parameters such as morphology, nucleic acid content, and surface antigenicity. Cells are labeled with appropriate fluorophores and passed rapidly in suspension on a cell-by-cell basis through a tunnel and detected by a laser beam. By analysis of interactions of each cell (light scatter or fluorescence) with the beam, a representation of the distribution of desired parameter within the population is acquired. A fluorescent-labeled antibody can be used to detect bacterial cells directly by flow cytometry. One of its disadvantages for use in food microbiology is the lack of discrimination between live and dead cells and interference with the food matrix. Application of reporter bacteriophage can overcome this problem by detecting only live pathogens (see below).

## *Bacteriophage for Detection of Pathogens[15–17]*

As discussed in Chapter 38, bacteriophages are viruses that infect and kill target bacteria. Thus it was used as a biocontrol to kill pathogens and spoilage bacteria. In recent years, however, there is an increasing emphasis on the use of bacteriophages as detection tools for foodborne bacterial pathogens because of their natural affinity toward its host. Moreover, bacteriophages are becoming attractive tools because of their ability to detect only the live pathogens, and only viable pathogens are of major concern in processed foods. Another advantage is that assays consisting of bacteriophages have a built-in amplification step because bacteriophages, after infection, can produce a large number of progeny phages in one to two hours (Figure 14.2). Bacteriophages have been applied for pathogen detection in various forms: Binding of phages with the target pathogen can be detected visually, spectrophotometrically, or by employing biosensor tools, some of which are described here:

## *Culture or Staining Methods*

Bacteriophages are currently being used for phage typing of bacteria. This is determined by measuring the turbidity of liquid culture spectrophotometrically or by monitoring the lysis of bacterial cells on lawn cultures on agar plates visually. Lysis of bacterial cells will result in less turbid bacterial suspension or a clearing zone (plaque formation) on a bacterial lawn (Figure 38.2).

Bacteriophages prelabeled with fluorescence dye or quantum dots are added to test samples, and binding of labeled phages to bacteria is monitored by flow cytometry or epifluorescence microscopy.

## *Reporter Phages*

In this approach, phages are genetically engineered to carry a "reporter gene," such as genes for bioluminescence (*lux*), green fluorescence protein (*gfp*), or β-galactosidase (*lacZ*). Once a bacteriophage infects a target pathogen host, the phage gene is integrated into the host genome and

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the gene products in the form of light, fluorescence, or β-galactosidase; enzymes are made, which are detected by a luminometer, fluorimeter, or spectrophotometer. Bacteriophages that are unable to infect a host bacterium will not produce any signal because they do not have the required machinery for gene expression. This approach is very efficient and was used for detection of low concentrations of *Esc. coli* O157:H7, *Salmonella*, *Lis. monocytogenes*, *Staphylococcus aureus*, *Bacillus anthracis*, *Yersinia pestis*, and *Mycobacterium tuberculosis*. The detection sensitivity was reported to be $10^2$–$10^6$ cells/mL, and the assay time is only one to three hours for most pathogens; however, it may take longer for slow-growing bacteria like *Mycobacterium*.

## Phages for Capture and Detection Using Biosensors

Intact bacteriophages or bacteriophage tail fiber proteins (endolysin) that bind to the host bacterial surface during infection are used to capture bacterial pathogens on paramagnetic beads or on the biosensor surface for detection. The tail fiber proteins, also called receptor binding proteins (RBPs), immobilized on paramagnetic beads are used to capture pathogens from food suspensions and are detected by plating on selective agar media plate or by using PCR (see below).

RBPs are also used as a substitute for antibodies to capture the target pathogen on biosensor platforms, such as surface plasmon resonance sensor (SPR), quartz crystal microbalance (QCM), etc. (see biosensor methods below).

## Bioluminescence Methods

A bioluminescence method measures the ATP content in a sample as an indirect measurement of microbial load. As only the viable cells retain ATP, the amount of ATP is regarded as directly related to the microbial load in the sample. Using the luciferin-luciferase (from firefly) system in the presence of $Mg^{2+}$, the ATP concentration in the lysed cells in a sample is measured. This method can detect as low as $10^{2-3}$ viable bacterial cells and about 10 yeast cells per gram or milliliter of a food. It can also be used to determine the microbial population on equipment surfaces. The method is very rapid, and several automated systems with methods to use them are now commercially available.

In another method, the genes encoding bacterial bioluminescence (*lux* gene) from luminous bacteria (e.g., *Vibrio fischeri*) have been cloned in some pathogenic, indicator, and spoilage bacteria important in food. The bacterial luciferase catalyzes oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde by molecular $O_2$ and emits light. As only live cells can produce light, the bacterial strains containing the cloned *lux* gene can be used to determine the effectiveness of methods employed to kill cells or to remove them from food and food environments very rapidly.[18] It is also being used to detect injured bacteria incapable of producing light but that are able to do so following repair of injury. Automated systems to measure light from the reaction are now commercially available.

Many other rapid methods, automated systems, and miniatures of traditional methods are now commercially available. Some have been discussed in more detail in the references included and can be consulted.

## Nucleic Acid-Based Methods

### Hybridization Method[19,20]

In this method, a DNA probe consisting of a 20- to 4000-nucleotide base sequence unique to a group of similar pathogens, such as *Salmonella* spp., is prepared. The unique sequence can

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be identified in the DNA or rRNA in the cells or from the amino acid sequence of a toxin produced by the bacterial group. The DNA probe can then be obtained from the cell DNA or synthesized from the nucleotides. The probes are radiolabeled with $^{32}P$, after hybridization, if autoradiography is used for their detection. In the nonisotopic colorimetric method, an enzyme (e.g., peroxidase) is bound to a specific protein (e.g., streptavidin), which, in turn, binds to specific ligands (e.g., biotin) on the DNA probe and can be used to produce a color reaction to aid in measurement.

Both isotopic and nonisotopic DNA probes specific for *Salmonella* and *Lis. monocytogenes* are commercially available and approved for use by the regulatory agencies. DNA probes for toxigenic *Esc. coli* and *Yer. enterocolitica* are also available. The commercial companies provide step-by-step procedures for each technique. In general, it involves an initial preenrichment step followed by an enrichment step (which can be just six hours or more) to facilitate growth of target bacteria; then lysis of cells; hybridization of the DNA probe with cell DNA or rRNA; capture of the hybridized DNA containing the probe; and measurement of either radioactivity or color production, depending upon the probe (e.g., if it is isotopic or nonisotopic). The methods are specific and relatively more rapid than the traditional methods.

### *Polymerase Chain Reaction (PCR)[21–23]*

The most widely used DNA-based method is the PCR. A gene segment specific for a pathogen is amplified several-fold by using a pair of specific primers (a small piece of DNA of about 20 base pair long). PCR steps include denaturation of double-stranded DNA, primer annealing, elongation of strands with a heat-stable *Taq* polymerase, and amplification (Figure 42.5). After amplification for 30–35 cycles, a single gene can be amplified to millions of copies, which are then separated in an agarose gel for visual identification. Multiplex PCR has been developed to increase the specificity of an assay by targeting multiple genes in a single reaction tube. The method has been developed for the detection of pathogens, such as *Lis. monocytogenes*, *Sal. enterica*, pathogenic *Esc. coli*, *Campylobacter*, *Yer. enterocolitica*, *Sta. aureus*, *Clo. botulinum*, *Bac. cereus*, and *Vib. vulnificus*.

A real-time quantitative PCR (Q-PCR) is becoming increasingly popular because it has some degree of automation. In this system, a fluorescent dye-labeled probe is allowed to hybridize with the target DNA; as the primer extension takes place, the endonuclease activity of the *Taq* DNA polymerase removes the probe, and the reporter dye is released, and the amplified PCR products are quantified by a built-in laser detector (Figure 42.6). The probe (~100 bp) is labeled with both a fluorescent reporter (R) and a quencher (Q) dye, and because of their proximate association, fluorescent activity is quenched under normal conditions. When the reporter molecule is physically removed from the quencher molecule, such as during primer extension (resulting from endonuclease activity), there is a fluorescent signal in the well. One of the major drawbacks of these PCR-based methods is that they cannot differentiate live cells from dead cells. To overcome this problem, a reverse transcriptase (RT)-PCR method has been developed in which messenger RNA (mRNA) that is transcribed only in the viable cells is used for amplification. Complementary DNA (cDNA) is synthesized from mRNA using a reverse transcriptase enzyme and then amplified using a standard PCR method. RT-PCR is also used to detect RNA viruses, such as Norovirus, rotavirus, and some other enteric viruses, responsible for food poisoning.

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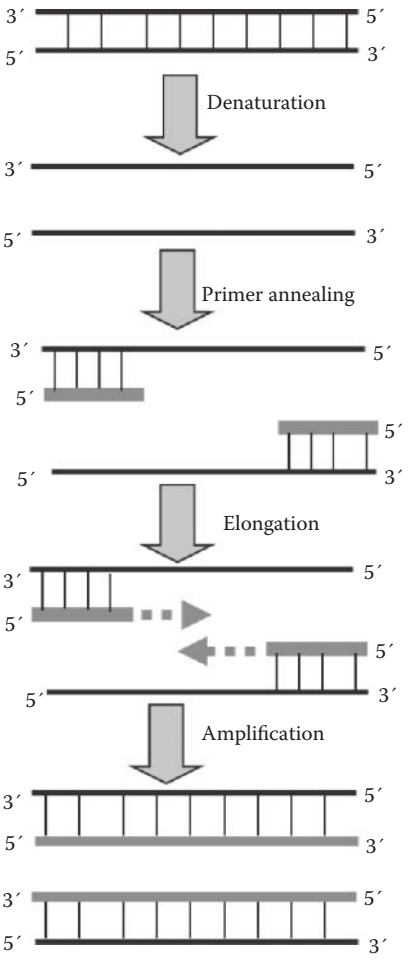



**Figure 42.5 Polymerase chain reaction assay.**

## *DNA Fingerprinting*[6]

DNA fingerprinting techniques have also been used for detection and identification of pathogens. Generally, the genomic fingerprinting methods require isolation of DNA from cells of pure isolated colonies, restriction enzyme digestion, and amplification or hybridization of fragments with appropriate primers or probes, which may take at least five to seven days to complete. An automated RiboPrinter® system, a compact robotic instrument, can perform all these tasks within eight hours. The DNA fingerprint pattern is then compared with the built-in database, and the precise identification is made for each bacterium. Other commonly used fingerprinting techniques include pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, amplified fragment-length polymorphism (AFLP), restriction fragment-length polymorphism (RFLP), rep-PCR, and multilocus sequence typing (MLST).

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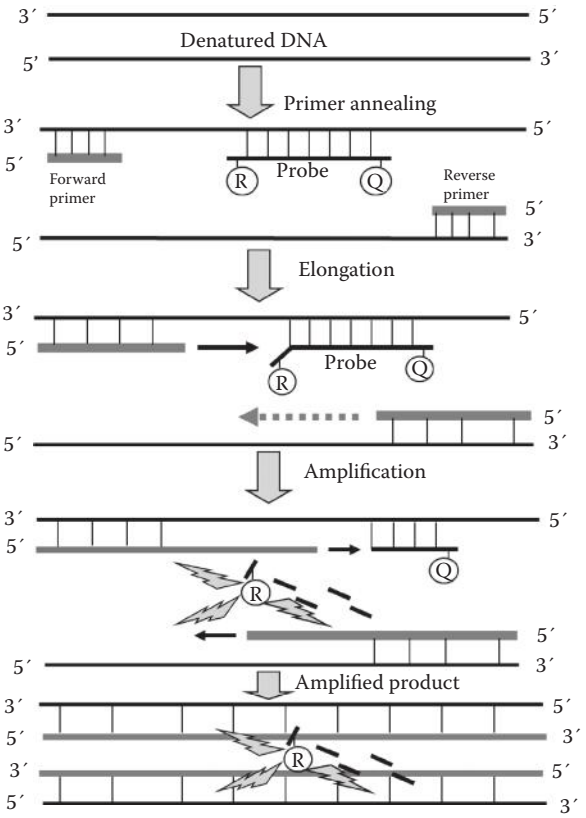



**Figure 42.6 Quantitative PCR assay for real-time detection of PCR amplified products. Probe is labeled with fluorescent reporter (R) dye and quencher (Q) molecule for optical detection of amplified product by a fluorescent detector in Q-PCR machine.**

## *Whole Genome Sequencing*[24]

It is now possible to sequence the whole genome of a pathogen very rapidly with relatively low cost because of the revolutionary development in DNA sequencing technology in recent years. A whole genome sequence allows rapid identification of a pathogen and also provides information related to its origin, evolution and genetic diversity and the presence of virulence genes. However, for genome sequencing of microbes, it is necessary to obtain pure isolated cultures from food matrices before sequencing.

## *Microarrays and Mass-Spectrometry*[14,25]

Detection of multiple pathogens in a single assay platform is highly desirable because of its potential for reducing cost for sample analysis and providing total microbiological safety/quality of a product. In DNA microarray, DNA probes targeting specific genes are first printed on the microarray chip and then exposed to test samples containing DNA. Complementary DNA present in the sample will hybridize with the probe in a spot, and the unbound or weakly bound DNA is removed by washing. The hybridized products are then detected and quantified by fluorescence.

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The DNA microarray has been used to detect *Lis. monocytogenes*, *Sal. enterica*, *Esc. coli* O157:H7, and *Cam. jejuni* individually or in combinations from several naturally contaminated fresh meat samples. Mass-spectrometry, such as matrix-assisted laser desorption/ionization (MALDI) time of flight mass spectrometry (TOF MS), generates a spectral signature for individual pathogens based on protein profiles and can be applied to detect multiple pathogens. This system also requires isolated pure cultures from food samples for analysis.

### *Pathogenicity Assay*[26–28]

Laboratory rodents, such as mice, guinea pigs, rats, and rabbits, are widely used models to study the virulent property of foodborne pathogens. To determine whether a food isolate is pathogenic, an infectious dose or a lethal dose is assessed in animal models. Animal models, though ideal for determination of a virulence property, is very expensive and may not be suitable for routine use. Moreover, for ethical reasons, their use sometimes is discouraged. Alternatively, mammalian cells have been routinely used for analysis of the pathogenic potential of foodborne pathogens. Various cancerous immortal cell lines that are used for pathogen testing are currently available. Not all cell lines are sensitive to a pathogen; thus one has to select a cell line that has been shown to be sensitive. For example, the pathogenic potential of *Lis. monocytogenes* has been determined using enterocyte-like Caco-2, macrophage J774, fibroblasts, and hybrid lymphocyte Ped-2E9 cell lines. Vero cells (monkey kidney cell line) have been used for Shiga toxin–producing *Esc. coli* cultures. A Chinese hamster ovary (CHO) cell line and lymphocyte Ped-2E9 cells have been used for detection of *Bac. cereus* toxins.

Cytopathogenic effects are measured by various methods. Light and electron microscopy have been used for qualitative analysis of cytopathic effects for characteristic membrane damage, cell lysis, clumping, elongation, vacuole formation, flocculation, and loss of surface molecules. Quantitative cytotoxicity assays, such as the Trypan blue exclusion test, allow staining of dead cells (blue appearance) while viable cells with intact membranes exclude stain. Dead or viable cells are then counted under a microscope for determination of the cytotoxicity effect. The ALP release assay, lactate dehydrogenase release assay, and MTT (3-[4,5-dimethyl thiazolyl-2]-2, 5-diphenyl-tetrazolium bromide) assay (metabolic staining) are also used for determination of cytotoxicity. Furthermore, adhesion, invasion, plaque formation, and apoptosis analysis are also used to determine the cellular mechanism of pathogenesis for foodborne pathogenic microorganisms.

## Biosensors for Pathogen Detection[11,13,14,28–31]

Biosensor-based tools continue to capture the imaginations of researchers for their potential in sensitive detection of pathogens in automated or semiautomated instruments in near real time. Biosensor-based technologies are increasingly being used in methods to detect foodborne pathogens as an alternative to conventional methods. Biosensors are devices that detect biological or chemical complexes in the form of antigen—antibody, enzyme—substrate, or receptor—ligand, which are placed in close proximity to a transducer that generates an electrical, optical, or mass-change signal.

Pathogen detection by biosensor tools is centered on four basic physiological or genetic properties of microorganisms: substrate utilization, phenotypic expression analysis of virulence or physiological or structural markers by antibodies, genetic analysis, and interaction of pathogens with eukaryotic cells (cytopathogenic effects). Many current popular commercially available rapid methods use conventional culture-based methods coupled with automated or semiautomated nucleic acid–based, antibody-, or substrate utilization–based methods to obtain results in 24–72

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hours. Interestingly, many of the modern-day biosensor-based methods are developed utilizing one of the above four principles or their combinations. However, antibody-based immunosensor methods are very common and offer the most versatility and convenience.

Pathogen detection using biosensors from food samples are challenging because of the complex nature of food, which consists of fats, proteins, carbohydrates, minerals, and preservatives with different acidities, salts, and colorings. Application of nanobiotechnology to detect pathogens from such a complex system presents numerous challenges. This is further complicated by the fact that in a food the populations of target microorganisms are normally very low compared to the indigenous flora. Therefore, exceptionally rational strategies need to be employed to detect such low numbers of pathogens directly from food. Varieties of biosensor-based methods have been and continue to be developed: electrochemical (impedance and amperometric), optical (light scattering, fiber optic, and SPR), thermometric (thermister and pyroelectric), and mass-based (piezoelectric and surface acoustic) methods. Among these, optical sensors appear to be very popular because of their sensitivity, available instrumentation, and relative ease of data interpretation.

## *Fiber-Optic Biosensors*[32–35]

A fiber-optic biosensor is a sensitive optical method that has been used for detection of foodborne as well as microbial and chemical biowarfare agents. A polystyrene or glass waveguide in the form of a fiber or glass slide is used for such application. This method utilizes the total internal reflection (TIR) property of light when it travels through the waveguide and generates a boundary of evanescent waves on the surface of the waveguide. Fluorescent molecules present in the evanescent field are excited and emit light that returns through the fiber in high-order modes and is detected by a fluorimeter. A fiber-optic sensor utilizes a sandwich immunoassay configuration, in which, first, a pathogen-specific capture antibody, receptor protein, or DNA or RNA aptamer is immobilized on the surface of the waveguide. Then the sample is injected into the manifold containing the fiber and allowed to bind to the target. After a washing step, a fluorescence-labeled detection antibody is added to form a sandwich with the antigen. The fiber is then connected to a laser source/detector (Figure 42.7). The laser light (635 nm) travels through the waveguide, generating an evanescent

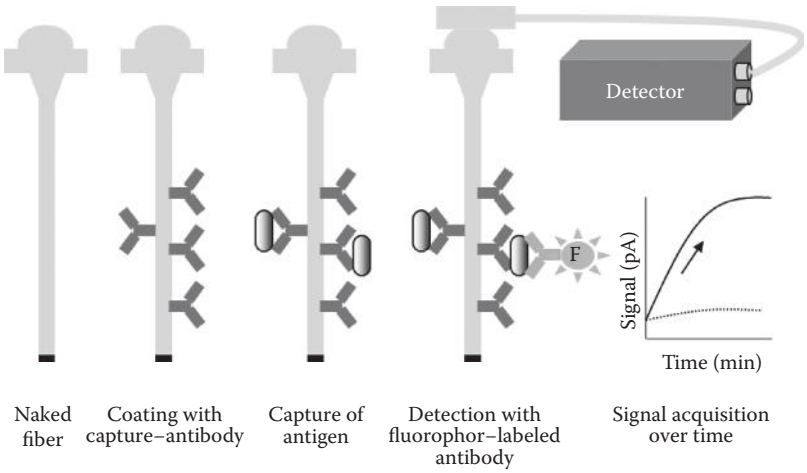



**Figure 42.7 Fiber-optic sensor for detection of foodborne pathogens.**

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wave that excites fluorophor molecules located in the antigen-antibody complex. Once excited, the fluorophors emit light, and the light intensity is proportional to the amount of antigen present, which is then detected by the detector. The common fluorophor molecules that are used in fiber-optic applications are Cyanine 5 and Alexa-fluor 647, and their respective excitation and emission wavelengths do not overlap so that they could be easily detected by a fluorimeter. A semiautomated fiber-optic device manufactured by Research International, Inc., called RAPTOR, is available, which is built with a sample injection port, microfluidic channels, laser diodes, and a computer interface for data analysis. The device accepts a coupon containing four fibers; thus a maximum of four pathogens can be detected at one time. Moreover, the fibers can be reused until a positive response is recorded.

A fiber-optic sensor has been used successfully for detection of *Sta. aureus* enterotoxins, *Bacillus anthracis* spores, *Franciscella tularensis*, *Bacillus globigii* spores, *Clo. botulinum* toxin, *Yersinia pestis*, *Brucella melitensis*, and vaccinia virus. Foodborne pathogens, such as *Lis. monocytogenes*, *Cam. jejuni*, *Salmonella*, and *Esc. coli* O157:H7, are detected from different food samples singly or in a multiplex format. Mycotoxins, such as ochratoxin A, fumonisin B, aflatoxin $B_1$, and deoxynivalenol, are also detected in an array biosensor in multiplex format. Generally, the detection limit for bacterial cells is $10^3$–$10^4$ cfu/mL, and toxins are detected in the nanogram quantities (0.5–20 ng/mL).

### *Surface Plasmon Resonance Sensor*[29,36]

A surface plasmon resonance (SPR) sensor is a powerful tool that can quantify the binding kinetics of two molecules without the need for any fluorescent tag. SPR is a phenomenon that occurs during optical illumination of a metal surface and is used for biomolecular interaction analysis. In this method, antibodies or receptors (receptor binding proteins, RBP) are immobilized on the surface of a thin film of a precious metal, such as gold (resonant layer), deposited on a reflecting surface of an optically transparent waveguide. The sensing surface is located on top of or below a high index-resonant layer and a low index-coupling layer. When light passes through the waveguide, it causes an internal total reflection at the surface of the waveguide. At a certain wavelength in the red or near-infrared region, the light interacts with a plasma or cloud of electrons on the high index-metal surface, and the resulting resonance effect causes a strong absorbance. The exact wavelength of this absorption depends on the angle of incidence, the metal, the amount of capture molecules immobilized on the surface, and the surrounding material. The presence of antigens interacting with the antibody causes a shift in the resonance to longer wavelengths, and the amount of shift is proportional to the concentration of bound molecules (Figure 42.7).

Advantages of SPR-based sensors are that they allow real-time or near real-time (below 30 minutes) detection of binding events between two molecules. The detection system is label-free, and thus it eliminates additional reagents, assay steps, and time. The sensor can be reused for the same analyte. In such a case, the bound analyte is desorbed by changing the pH of the solution. It is highly sensitive and it can detect molecules in the nanogram range. Several commercial SPR systems are available: BIACORE, Spreeta, SPR spectroscope, Optrel GbR, Reichert SR7000, and IAsys.

SPR-based sensors have been used for detection of foodborne pathogens and toxins. In most applications, this system shows promising results with toxins while signals with whole cells are inconsistent, and depending on the instrumentations, some are unable to detect whole cells. Staphylococcal enterotoxin and cells of *Esc. coli* O157:H7, *Salmonella enterica* ser. Typhimurium, *Yer. enterocolitica*, and *Lis. monocytogenes* are detected by SPR (Figure 42.8).

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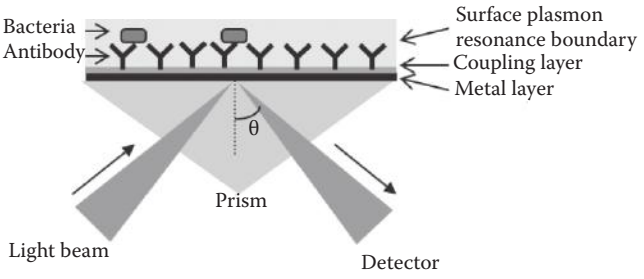



**Figure 42.8 Surface plasmon resonance (SPR)–based detection of pathogens and toxins.**

## *Electrochemical Immunosensor*[37]

Electrochemical immunosensors are extensions of the conventional antibody-based enzyme immunoassays (ELISA) in which catalysis of substrates by an enzyme conjugated to an antibody produces products in the form of pH change, ions, or oxygen consumption, which generate electrical signals on a transducer. Potentiometric, capacitive, and amperometric transducers have been used for such applications. In amperometric detection, for example, ALP conjugated to an antibody hydrolyzes *p*-nitrophenyl phosphate to produce phenol, which is then detected by voltammetry. In light-addressable potentiometric sensors (LAPS), the capture antibody is first immobilized on a membrane or magnetic beads for capture of target cells. In a sandwich format, a fluorescent-labeled detection antibody is allowed to react with the target cells. A urease-labeled antifluorescent antibody is then added. In the presence of urea, ammonia ($NH_3$) is produced, which changes the pH of the solution on the n-type silicon chip coated with a pH-sensitive insulator that records the voltage change. These sensors are very sensitive and have been used for detection of *Salmonella* and *Esc. coli* O157:H7 in 30–90 minutes.

## *Piezoelectric (PZ) Biosensor*[38]

Piezoelectric (PZ) biosensors detect changes in the mass on the surface of a quartz crystal in a quartz crystal microbalance (QCM) sensor. Antibodies or receptors are used for specific capture of pathogens, which increases the mass of the crystal, thereby changing the resonance frequency when an oscillating electric field is applied across the device. The frequency variation is measured by a quartz crystal analyzer. PZ has been used for detection of Staphylococcal enterotoxins and cells of *Salmonella* Typhimurium, *Salmonella* Paratyphi, *Lis. monocytogenes*, *Bac. cereus*, and *Esc. coli* O157:H7, *Vibrio* spp, and *Campylobacter jejuni*.

## *Impedance-Based Biochip Sensor*[39–41]

The impedance microbiology concept has been in existence for more than a century; however, its popularity grew only in the mid-'70s. Impedance is based on changes in conductance in a medium resulting from the microbial metabolism of inert substrates into electrically charged ionic compounds and acidic byproducts (e.g., amino acids, lactic acids, and acetic acids). This causes a change in the electrical impedance (resistance to flow of an alternating current) and conductance of the medium. By measuring these two parameters, bacterial growth in a medium as a

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function of time at a given temperature can be monitored. Impedance detection time (IDT) is the time required by microorganisms initially present in a food to reach $\geq 10^6$/mL. It is inversely proportional to the initial microbial load; a higher IDT means a lower initial load. From the IDT and the slope of the curve, the initial load as well as generation time of a microbial population can be calculated. The major advantage of this system is that it allows detection of only the viable cells, which is a major concern in food safety. At present, impedance instruments are able to detect $10^5$–$10^7$ bacteria per milliliter. Several commercially available systems, such as the Bactometer® (bioMérieux), Malthus AT analyzer (Malthus Instruments), BacTrac, and μ-Trac microorganism growth analyzer (SyLab), are used for pathogen monitoring and quality assurance purposes. Bacterial contamination in milk has been analyzed using the Bactometer for quality assessment purposes. The impedance-based assay has been used for detection and enumeration of *Listeria*, *Campylobacter*, *Esc. coli*, *Staphylococcus*, and *Salmonella* from food samples. The impedance method has been accepted by the Association of Official Analytical Chemists, International (AOAC) as a first-action method. This macroscale method, however, lacks sensitivity and time-to-result is long, requiring 24–48 hours.

To improve sensitivity, miniaturized microfabricated electronic devices, such as semiconductor silicone chips referred to as "biochips," are developed. In this biology-based, microelectrical-mechanical systems (Bio-MEMS) device, the cells are electrophoretically confined into a nanoliter volume in a chamber, thus allowing rapid turnover of substrate by bacteria into electrically charged byproducts aiding rapid and sensitive detection of bacterial growth by impedance. *Lis. monocytogenes* cells were successfully detected at $2 \times 10^7$ cells/mL chamber with only a two hour incubation step. Pathogen-specific antibodies, receptor molecules, such as virulence proteins or bacteriophage receptor proteins, immobilized in the detection chamber can provide specificity for target pathogen. Pathogens are also detected on a microfluidic chip by PCR.

## *Fourier Transform Infrared Spectroscopy (FTIR)[29,42] and Raman[43] Spectroscopy*

Both FTIR and Raman spectroscopy generate bacterial spectral scans based on the molecular composition of the cell wall and the cytoplasm. Basically, infrared spectroscopy consists of the infrared source, sample, and the detector. When infrared radiation is absorbed or transmitted through the sample to the detector, it generates a "scan" or "fingerprint" profile. A library of spectral scans can be generated for different bacterial species and strains, which can be used for future comparison. This method requires a transfer of cells (biomass) from the growth media to the IR-reflecting surface for spectral collection. It is a nondestructive label-free rapid method, and future identification depends on the available spectral library. FTIR has been used for classification or identification of several foodborne pathogens: *Yersinia*, *Staphylococcus*, *Salmonella*, *Listeria*, *Klebsiella*, *Escherichia*, *Enterobacter*, *Citrobacter*, and so forth.

Raman spectroscopy is also a label-free detection system and uses a diode laser (785 nm), which, upon interaction with microbial cells, generates Raman scatter, known as inelastic scattering. Typically, the Raman signal is very weak, and to amplify the signal, surface-enhanced Raman spectroscopy (SERS) has been configured using biorecognition molecules conjugated to gold or silver nanoparticles to specifically bind to target microorganisms or toxin molecules. Raman is used for detection of *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, *Francisella tularensis*, *Brucella abortus*, *Lis. monocytogenes*, *Esc. coli* O157:H7, and *Salmonella*.

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## Light Scattering[28,44,45]

Light-scattering technology differentiates samples based on refractive index, size, shape, and composition. Light scattering dates back many decades and has been used for many years in the semiconductor industry to detect defects on the semiconductor chips (wafers). When an illuminated light from a polarized monochromatic light source shines on a sample (bacteria, for example), scattered light forms distinct signature patterns that could be used for identification and detection of bacteria (Figure 42.9). The ability of a light-scattering system to detect a single cell in suspension is reported but with limited success.

Recently, a novel elastic light-scattering system, BARDOT (BActerial Rapid Detection using Optical scattering Technology), using a diode laser (635 nm) has been built to detect and identify bacterial colonies growing on solid agar plates. BARDOT generates unique scatter images for different bacterial genus, species, and even serovars. It is a noninvasive, label-free, and rapid method, requiring less than a minute for bacterial colony identification. In this system, when a laser shines on the center of a bacterial colony, it generates a distinctive and reproducible scatter pattern that is captured by a digital camera (Figure 42.9). The scatter images are stored in the image library and image analysis software allows identification of the test organism. Furthermore, the versatile discriminatory power of BARDOT is possible because of the availability of various selective and differential solid agar media for different microorganisms. BARDOT was used for detection and identification of foodborne pathogens *Lis. monocytogenes*, *Salmonella enterica*, *Vibrio* spp., *Bacillus* spp, and *Esc. coli* from food. BARDOT is also a useful tool for bacterial community analysis in produce or food products and to assess effectiveness of sanitation or hygienic practices during food processing.

## Cell-Based Sensors[11,27,28,46,47]

Cell-based sensors are defined as when a cell—prokaryotic (bacteria) or eukaryotic (mammalian cell)—serves as a transducer by emitting a specific signal during interaction with the analyte of interest (Figure 42.10). This system generates signals in seconds to minutes in the presence of an analyte. The cell-based assay reports the perturbations in the "normal" physiological activities of

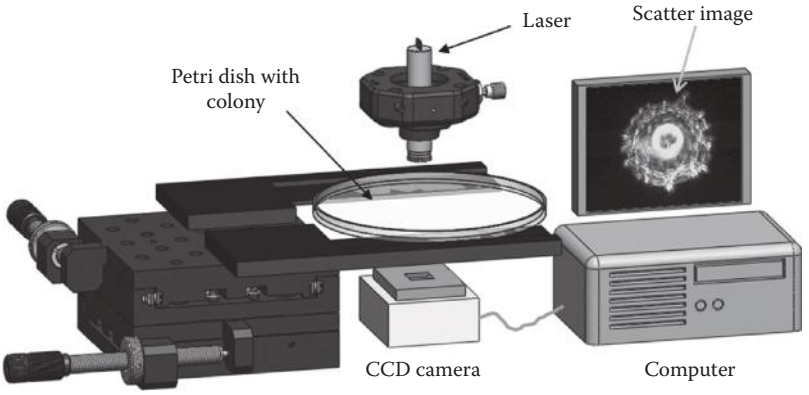



**Figure 42.9 Light scatterometer for bacterial colony identification. (Sketch was created by Huisung Kim.)**

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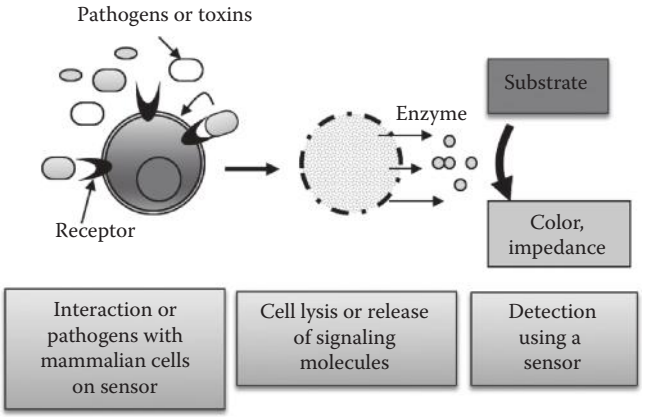



**Figure 42.10 Schematics of mammalian cell-based sensor. (Adapted from Bhunia, A.K., *The Science of Homeland Security*, Amass, S.F., Bhunia, A.K., Chaturvedi, A.R., Dolk, D.R., Peeta, S., and Atallah, M.J., Eds., Purdue University Press, West Lafayette, IN, 2006; pp. 109–149.)**

mammalian cells as a result of exposure to an "external" or environmental challenge, such as the presence of pathogens or toxins in clinical, environmental, or food samples, including *Lis. monocytogenes* and *Esc. coli* and toxins. Some of the cell-based assays also utilize the metabolic responses of cells (e.g., cyanobacteria) to detect biological products, like oxygen and herbicides in water.

A novel B cell-based sensor was genetically engineered to express cytosolic aequorin, a calcium-sensitive bioluminescent protein, and pathogen-specific surface antibodies. When a pathogen binds to the surface antibodies, it generates a specific bioluminescent signal, and it has been shown to be able to detect very low numbers of pathogenic bacteria, like *Esc. coli* O157:H7, *Bacillus anthracis* spores, and *Yersinia pestis* in minutes. Artificial cells, also known as "liposomes," made up of a lipid bilayer with specific receptors carrying a fluorescent-tagged reporter molecule inside, can serve as a sensitive sensor for detection of spore-forming toxins. Toxins first bind to a receptor and then disrupt the membrane, allowing the release of the fluorescent reporter, which could be detected by a spectrofluorometer. The liposome can be stored in ambient conditions for up to five months without the requirement for a cell culture facility, and it is able to detect nanogram quantities of toxin in 30 minutes, and moreover this system can eliminate the requirement for a cell culture facility. Mammalian cell-based biochips have also been used for detection of allergens and viruses. For example, the cultured basophilic mast cell line (RBL-2H3), on interaction with an allergen, releases a fluorescent-bound histamine, which is measured by a photomultiplier tube attached to a microscope.

## Conclusion

Microbial culturing methods are irreplaceable for microbiological analysis of food to monitor product quality and safety. Advances in molecular methods have benefited pathogen detection and identification in foods; however, these methods still lag behind for analysis of spoilage-causing microorganisms to assess shelf life or fermentative microorganisms in finished products. Biosensor-based methods are in the early stage of their development; some of them are proven to be fast and sensitive and have the potential to be built as an automated hand-held device for onsite

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pathogen testing. Eventually, this technology will be able to make the lab-on-a-chip concept a reality. In most applications, a biosensor device may find its application as a first-action screening tool for quick results in few hours, but for confirmation, we may still have to rely on conventional culture methods. Many of the biosensor platforms discussed above have demonstrated the proof of concept by testing with pure bacterial cultures or toxins; however, only some select biosensors are tested thoroughly with real-world food samples for their robustness, sensitivity, and cross-reactivity. In the future, efforts should be directed toward improvement in the applicability of most promising sensors for their effectiveness to detect foodborne pathogens from real-world food and environmental samples. We may be a few years away from its routine usage in food safety, food biosecurity, or food quality assessment applications. Biosensor technologies are also becoming powerful tools in biomedical applications in probing the human body for early diagnosis of malignant cancer, cardiovascular disease, diabetes, and so forth. As we continue to embrace this exciting field of science and technology, we will find that one day this will be an integral part of human life, and it will impact our lifestyle.

### QUESTIONS

1. Provide a general outline for isolation of foodborne bacterial cells from meat and poultry.
2. What selective enrichment broth and selective and differential agar media are used for isolation of *Salmonella* and *Esc. coli* O157:H7?
3. Discuss various methods used for separation of bacteria from food matrices.
4. Under what circumstances is the MPN method used for enumeration.
5. What is the basic principle of a biosensor, and discuss some of the advantages and disadvantages of this technology.
6. Discuss the basic operating principles of a fiber-optic biosensor and surface plasmon resonance biosensor.
7. How does a cell-based sensor work, and what physiological property of a pathogen is detected in this sensor?

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# Appendix A: Predictive Modeling of Microbial Growth in Food

## Importance

Every year, more than 12,000 new food products are introduced to U.S. consumers in addition to more than 100,000 existing food items. Some of these products obviously have limited shelf life because of the possibility of microbial growth under the conditions in which they are stored and handled following production. The initial microbial population in many of these products may include both spoilage and pathogenic types. To ensure stability and safety, it is important that proper control measures are used to prevent growth of the microorganisms from the time of production to the time of consumption of these products.[1,2]

Among the thousands of new products marketed every year, some are definitely produced to satisfy consumers looking for foods that are healthy, natural, low-fat, low-salt, tasty, convenient, do not contain harsh preservatives, are not given harsh treatment, have long storage life, and are safe. To produce these new types of foods (such as the new-generation refrigerated foods) with desirable safety, stability, and acceptance quality, the influence of many parameters, such as different ingredients at different concentrations under different processing and storage conditions and at different $A_W$, pH, and other intrinsic and extrinsic factors, on microbial growth and destruction in foods has to be determined.

It is almost impossible to conduct such microbiological studies for each product by the methods traditionally used to ensure safety and stability of food products. However, with the aid of computers, mathematical models can be developed to determine the influence of combinations of several parameters on microbial growth. Although they may not be accurate, they can be effective to obtain first-hand information very rapidly, helpful to eliminate many of the less important possibilities, and select a few that appear more promising. This information can then be used to conduct a modest traditional study that is feasible both experimentally and economically.



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## Traditional Methods

Several methods are used to determine the influence of control parameters on growth or toxin production, or both, by targeting microorganisms in foods. Three of these methods are briefly described here.[3,4]

### *Challenge Studies*

In this method, a food is first inoculated with the microorganisms that are expected to be the major causes of loss of stability or safety, or both, under retailing conditions. A realistic number of microbial cells or spores are used as inoculum. The product is then stored under conditions in which it is expected to be stored normally and then examined for the duration of storage for microbial numbers (growth) or toxin production.

To determine effective control measures against the target microorganisms, a large number of variables need to be studied. These include ingredients used, intrinsic and extrinsic factors, possible abuse, and others. As a result, the study can be quite complicated, time-consuming, and costly.

### *Storage Studies*

A normal product is stored under its proper storage conditions and, at selected intervals, samples are examined for the level of spoilage and pathogenic microorganisms that are expected to be normally present in the product and grow under the storage conditions. The results can be used to predict the expected shelf life and safety under the suggested storage conditions. However, this study will not provide information if the product is temperature abused even for a short time. In addition, if some of the samples being tested do not, by chance, contain the spoilage and pathogenic microorganisms normally expected to be present, then the results are of no practical value.

### *Accelerated Shelf Life Studies*

For products expected to have a relatively long shelf life, accelerated tests provide rapid information about their storage stability. The products are generally held at a higher temperature in order to increase the rate of microbial growth and accelerate the spoilage. However, foods normally contain mixed microflora that differ in optimum growth rate at different temperatures, and spoilage of a food is generally caused by a few species, among those present, that grow optimally at a given condition. To make the results valid, in designing this study, the temperature should be increased only to a level that does not affect the growth of the microorganisms of interest.

## Predictive Microbiology

Several mathematical models have been developed to predict growth of pathogenic and spoilage microorganisms in foods from the data generated by studying microbial growth rate at different pH, $A_W$, temperature, and preservative concentrations in laboratory media. The availability of suitable computers has helped in the rapid analysis of multifactorial data. Two kinetic-based models are discussed here. They indicate the effect of culture parameters on the growth rate of a microorganism, especially at the lag and exponential growth phases.[1–4]

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### Square Root Model

This is based on the linear relationship between the square root of the growth rate and temperature, and is expressed as: $\sqrt{r} = b(T - T_{min})$, where $r$ is rate of growth, $b$ the slope of the regression line, $T$ the temperature of food, and $T_{min}$ the theoretical minimum temperature of growth. The model has been further expanded to include the effects of temperature, pH, and $A_W$ on growth rate:

$$\sqrt{r} = b\sqrt{[A_W - A_{W\ min}](pH - pH_{min})(T - T_{min})}$$

where $A_W$ and pH are water activity and pH of a food, respectively, and $A_{Wmin}$, $pH_{min}$, and $T_{min}$ are minimum or lower limits of $A_W$, pH, and temperature, respectively, for growth.

In general, this model is quite effective when one or two parameters are used. However, its effectiveness is reduced when a variety of parameters, such as pH, organic acid, modified atmosphere, $A_W$, preservatives, salts, and others are used in combination to control microbial growth.

### Sigmoidal Model (Gompertz: USDA Model)

This model has been developed by the U.S. Department of Agriculture (USDA) to predict microbial growth in a food environment containing many controlling parameters. It has been tested in laboratory media to determine the growth rate of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., *Shigella flexneri*, *Escherichia coli* O157:H7, and *Aeromonas hydrophila* under variable temperatures, pH, $A_W$, NaCl and $NaNO_2$ concentrations, and aerobic and anaerobic atmospheres. The growth curves were statistically fitted using nonlinear regression analysis in conjunction with Gompertz functions. The results were then analyzed to develop this model. It gives the lag time, the maximum growth rate constant, and the maximum microbial load directly from nonlinear regression of the numbers versus time data. For these four parameters, the asymmetric sigmoidal equation is

$$N = A + C\exp(-\exp[-B(T - M)])$$

where $N$ is $\log_{10}$ colony-forming units (CFUs) per milliliter at time $t$, $A$ the initial $\log_{10}$ CFUs/ml, $C$ the $\log_{10}$ CFU difference between time $t$ and initially, exp is exponential, $B$ the relative growth rate at $M$, and $M$ the time at which growth rate is maximum. This model has been extensively used because of its simplicity and overall effectiveness.

## Conclusion

Predictive microbiology has generated tremendous interest because of its many advantages, most important of which is the quick availability of computer-generated information for both simple and complex studies. Interactions between different intrinsic and extrinsic factors and the importance of changing a parameter on growth and survival of microorganisms can be determined very quickly. However, current models have been designed by using laboratory media. Thus, their effectiveness in different food systems is not exactly known. Limited studies have shown that in a food system (e.g., ham salad), the growth responses of *Listeria* spp. and *Salmonella* spp. obtained by

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predictive modeling and by actual experiment were favorable; whereas for *Sta. aureus*, the growth rate predicted by the model was lower than that obtained in actual experiments. Thus, at present, the predictive data needs to be adopted with caution. Model data can be used in initial studies by using large numbers of variables to eliminate a great majority of them. Experimental data can then be obtained from actual product studies by using limited numbers of variables. This will help reduce the time and cost and make the study more manageable and effective.

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# Appendix B: Regulatory Agencies Monitoring Microbiological Safety of Foods in the United States

## Food Safety Regulations

Annual sales of food in the United States are more than $400 billion. Before foods reach the consumers, they are processed and handled conservatively by more than 25,000 processors, 35,000 wholesalers, 250,000 retailers, 500,000 eating establishments, and in many millions of homes. If one adds to them the various types of pathogens that can cause foodborne diseases and their natural presence in food animals and birds, food-producing environments, and among food handlers it becomes quite apparent that the chances of a foodborne pathogen being present in a food are high. Yet the numbers of reported foodborne disease outbreaks and the cases per year are relatively low. A major reason for this could be the stringent food safety laws in the United States.[1,2]

Before 1906, there were no food laws in this country, and marketing of unsafe food was quite common. In 1906, the federal government passed the Pure Food and Drug Act and the Meat Inspection Act and gave the U.S. Department of Agriculture (USDA) the power of supervision over the laws. According to these laws, selling of unwholesome and unsafe foods and meat in interstate commerce was illegal. The Meat Inspection Act also required inspection of the slaughter and meat-processing facilities. However, as the burden of proof that a substance had been added to a food at an unsafe level was with the USDA, the laws were not very effective. In 1938, the federal government passed the Food, Drug, and Cosmetic (FD & C) Act and gave additional power to the Food and Drug Administration (FDA) to administer the law. This law prevents the manufacture and shipment of unsafe and spoiled food and food ingredients in interstate commerce as well as for export and import. It permits sale of foods that are pure, wholesome, safe, and produced in a sanitary environment. According to this law, contamination of certain foods with specific pathogens, such as *Clostridium botulinum* and *Salmonella* spp., is considered to be unwholesome, and selling such food is illegal. (At present, several other potential foodborne pathogens, such as *Escherichia coli* O157:H7 and *Listeria monocytogenes* in ready-to-eat foods, are given the same status.)



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In 1959, the Federal Poultry Product Inspection Act was passed. This act requires the inspection of poultry-processing facilities that ship products in interstate commerce.

The Wholesome Meat Act (1965) and the Wholesome Poultry Act (1968) were passed, requiring inspection of the processing facilities, even those engaged in intrastate commerce. These facilities were to be inspected by state inspectors in the same manner as the processors engaged in interstate commerce were inspected by federal inspectors. At the federal level, the inspection of meat, poultry and eggs, and milk remains with the Food Safety and Inspection Service (FSIS) branch in the USDA.[1,2]

In 2011, to improve food safety efforts even further, the Food Safety Modernization Act (FSMA) was enacted.[3,4] The major emphases in this Act include (i) preventive control plans, (ii) mandatory produce safety standards: science-based food safety approach, (iii) mandatory inspection for high-risk products, (iv) product tracing, (v) performance standards, (vi) third-party certification, (vii) certification for high-risk foods, (viii) increased inspection authority, (ix) mandatory recall authority when the samples show evidence for pathogen contamination and death associated with the food, and (x) suspension of registration of the offending food producer.

## The Agencies

### *Federal Agencies*

In the United States, several federal agencies are delegated the responsibilities to monitor and regulate the origin, composition, quality, quantity, safety, labeling, packaging, and marketing of foods. Two agencies, the FDA and USDA, are directly involved with the microbiological safety of foods, and their responsibilities in this regard are discussed here.[2] Other agencies are involved in areas not directly related to microbiological safety of foods. Some of these are the following:

1. *Bureau of Alcohol, Tobacco, and Firearms (ATF).* Responsible for enforcing the laws that cover production and labeling of all alcoholic beverages except wine.
2. *Department of Justice.* Conducts seizures of a product and criminal proceedings in case of violation of a food law.
3. *Department of Defense (DOD).* The DOD is involved when there is an attempt for intentional administration of pathogens or toxins in the food for bioterrorism purposes.[4]
4. *Environmental Protection Agency (EPA).* Determines safety and tolerance levels of pesticide residues in foods and establishes water quality standards for drinking water (not bottled water).
5. *Federal Trade Commission (FTC).* Regulates correct procedures of advertising of foods.
6. *National Marine Fisheries Service (NMFS).* Responsible for seafood quality, habitat conservation, and aquaculture production (not microbiological quality).
7. *Food and Drug Administration (FDA).* At present, the FDA is a part of the Federal Public Health Service in the Department of Health and Human Services. It is delegated with the power to administer the federal FD & C Act. In that capacity, the FDA ensures the safety and wholesomeness of all foods, except fresh meat, poultry, and eggs sold in interstate commerce. It also oversees the wholesomeness of all exported and imported foods (except for fresh meat, poultry, and eggs). The FDA also inspects the safety and wholesomeness of foods served on planes, trains, and buses. It also conducts research to improve detection

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and prevention of contamination of food; develops regulatory testing procedures, specifications, and standards; and enforces regulations on processing and sanitation of food and processing facilities. The FDA sets up specific microbiological criteria that contain specifications (toleration levels) for aerobic plate counts (APCs), coliforms, *Esc. coli*, coagulase-positive *Staphylococcus aureus*, pathogens, and mycotoxins in various foods. FDA inspectors inspect processing plants once each two to three years unless a processor is in violation and a food has hazard potential. The FDA also helps processors develop their effective sanitation programs and gives contracts to state regulatory agencies to inspect plants. For foreign countries interested in exporting food to the United States, the FDA sends inspectors to help improve their food quality according to U.S. regulations. In case of violations, the agency sends formal notice of violation to the offender and sets a date (usually within 10 days) by which the problem needs to be corrected. In case of failure, depending on the severity of the offence, the FDA is authorized to take legal actions, through the Department of Justice, which include seizure of products, destruction of products, and criminal prosecution of the offenders. This includes products produced in the United States as well as imported products. In addition to microbiological quality and safety of foods, the FDA is also responsible for testing foods for composition, nutritional quality, food additives, food labeling, and pesticide residues (some with other agencies). The FDA also conducts market basket surveys to test a certain number of foods (imported and produced in the United States) for safety and other qualities.

8. *United States Department of Agriculture (USDA).* Under the Wholesome Meat Act and the Wholesome Poultry Product Act, the Food Safety Inspection Service (FSIS) of the USDA is responsible for inspecting slaughtering facilities of food animals and birds and producing wholesome and safe meat, poultry, eggs, and egg products that are sold in interstate commerce or imported. The FSIS also sets up microbiological specifications, particularly pathogen levels, for some of these products and conducts research on food safety and quality. On the basis of eating quality, the USDA also grades vegetables, fruits, milk and dairy products, and meat and egg products either produced in the United States or imported.
9. *Centers for Disease Control and Prevention (CDC).* The CDC is a branch of the Department of Health and Human Services. In the event of a foodborne disease outbreak, this agency investigates the causative agent of the outbreaks as well as the sequence of events that resulted in the outbreak.
10. *U.S. Army.* The wholesomeness and safety of foods (rations) consumed by the U.S. military personnel are the responsibility of the Department of Defense. The microbiology branch at Natick Army Research facility has established necessary microbiological specifications for APCs, coliforms, *Esc. coli*, pathogens, and other microbial groups for different types of foods procured from outside suppliers.

## *State and Local Government Agencies*

Some of the branches in the State Department of Agriculture and Public Health are responsible for the safety of food sold in the state. They cooperate with the federal government agencies to ensure the wholesomeness and safety of foods produced and served in the state. State inspectors inspect restaurants, retail food stores, dairies, grain mills, and processing facilities on a regular basis. Some states have authority over inspection of the quality of fish and shellfish taken from state waters. These agencies, if necessary, can embargo illegal food products sold in the state. Federal agencies provide guidelines, when necessary, to state agencies for the regulations.

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### International Agencies

The Food Standard Commission of the Joint Food and Agricultural Organization and World Health Organization (FAO/WHO), in cooperation with different nations, helps develop international standards in the production, processing, and preservation of foods exported and imported. Codex Alimentarius (CA) is an international food regulatory agency formed by different nations. It helps develop uniform food standards for all countries to ease export and import of foods between countries.

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# Appendix C: Hazard Analysis Critical Control Points

## Introduction

The traditional method of examining microbiological safety, storage stability, and sanitary quality of a food is to test representative portions (or samples) of the final product for the presence of some pathogens (such as *Salmonella*) or the number or level of certain pathogens (e.g., *Staphylococcus aureus* and *Vibrio parahaemolyticus*), different microbial groups (e.g., aerobic plate counts, psychrotrophic counts, thermoduric counts, and yeasts and molds), and indicator bacteria (e.g., coliforms and enterococci) per gram or milliliter of product. The major disadvantage with these types of end-product testing is that they do not provide close to 100% assurance about the safety, stability, and sanitary quality of the products. This aspect is particularly important with respect to pathogenic bacteria, especially those that are highly potent. By analyzing samples from a batch of a product, according to the sampling plan and testing methods recommended by regulatory agencies (see Chapter 42), it is not possible to give a high degree of assurance (close to 100%) that the untested portion of the end product is free of pathogens. Under certain circumstances, such as foods consumed by the crew in a space mission or by military personnel engaged in an important mission, close to 100% assurance of safety of a food is required.

Hazard Analysis Critical Control Points (HACCP) was originally developed jointly by the Pillsbury Company, the National Aeronautics and Space Administration (NASA), and the U.S. Army Natick Laboratories to produce foods with a high assurance of safety for use in the space program. It has two components: (i) to identify or analyze the hazards (HA) associated with the production and processing of a specific type of food (i.e., which pathogens can be expected to be present in a food) and (ii) to identify critical control points (CCP) (i.e., the places during processing of a food where proper control measures need to be implemented in order to prevent any risk to consumers). It is regarded as a *systemic approach* to assure safety and as a better method than end-product testing.

Since the introduction of the concept in 1971, HACCP has undergone several changes according to specific needs. In 1980, the National Academy of Science suggested that food industries in the United States could use the HACCP principles to produce safer foods, and regulatory agencies could help develop programs suitable for a particular processing operation. Currently, the Food Safety and Inspection Service (FSIS) of the USDA advocates the use of HACCP in meat and poultry inspection programs during both the slaughtering and processing of carcasses for products.



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The Food and Drug Administration (FDA) has been developing HACCP programs that cover various products it regulates. Internationally, HACCP is used in Canada, Australia, New Zealand, and several European countries.

It seems that the HACCP concept can be used universally to develop food safety programs. This will help reduce problems associated with foodborne pathogens as well as global trade of foods.[1–4]

## HACCP Principle of the NACMCF

There have been some changes in the original HACCP system. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) has developed a document for the HACCP system.[1–3] It is expected that by implementing this system in the production and processing of food, the safety of the products can be assured. Though HACCP principles are universal, for each product, a separate HACCP plan needs to be developed. This system is briefly presented here.

### *Seven Principles of HACCP*

HACCP involves seven principles:

1. Conduct a hazard analysis to determine risks associated at all stages, from growing raw materials and ingredients to the final product ready for consumption.
2. Identify critical control points to control these hazards.
3. Implement conditions to control hazards at each critical control point.
4. Implement effective procedures to monitor control for each point.
5. Implement corrective measures to be taken if a deviation occurs at a point.
6. Implement effective record-keeping systems for HACCP plan activities.
7. Implement procedures to verify that the plan is working effectively.

### *Brief Description of the Principles*

#### *Principle 1*

The possible hazards that exist for a specific food or ingredient from pathogenic microorganisms and their toxins are determined. This is performed in two segments: (i) by ranking the specific food into six categories and (ii) by assigning each a risk category on the basis of ranking for hazard categories.

Ranking for six hazard categories is based on the following assessments for a food: (i) does it contain microbiologically sensitive ingredients; (ii) does the processing method contain a step that is effective in destroying the pathogen; (iii) does the processing method contain a step for post-processing contamination of the product with pathogens or their toxins; (iv) is there a possibility of abusing the product during subsequent handling (transportation, display at retail stores, and handling and preparation by the consumers) that can render the product harmful for consumption; and (v) is the product, after packaging, given an effective heat treatment before consumption?

The hazard characteristics of a food are based on the following:

*Hazard A*. A nonsterile food intended for consumption by high-risk consumers (infants, elderly, sick, and immunocompromised individuals).

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*Hazard B*. The product contains "sensitive ingredients" in terms of microbial hazard.
*Hazard C*. The process does not contain a step to destroy harmful microorganisms.
*Hazard D*. The product is subject to recontamination after processing and before packaging.
*Hazard E*. The possibility exists for abuse before consumption that can render the product harmful if consumed.
*Hazard F*. The product is not given a terminal heat treatment following packaging and before consumption.

A food is ranked according to Hazards A through F by using a plus (+) for each potential hazard. The risk category is determined from the number of pluses.

*Category VI*. A special category that applies to nonsterile food to be consumed by high-risk consumers.
*Category V*. A food that has five hazard characteristics: B, C, D, E, and F.
*Category IV*. A food that has a total of four hazard characteristics in B through F.
*Category III*. A food that has a total of three hazard characteristics in B through F.
*Category II*. A food that has a total of two hazard characteristics in B through F.
*Category I*. A food that has a total of one hazard characteristic in B through F.
*Category O*. No hazard.

### *Principle 2*

To control microbiological hazards in a food production system, it is necessary to determine a CCP. A CCP is any point or procedure in a specific food system where effective control must be implemented in order to prevent hazards. All hazards in a food production sequence, from growing and harvesting to final consumption, must be controlled at some point(s) to ensure safety. The CCP includes heat treatment, chilling, sanitation, formulation control (pH, $A_W$, and preservatives), prevention of recontamination and cross-contamination, employee hygiene, and environmental hygiene. Application of one or more CCPs will destroy hazardous microorganisms or prevent their growth and toxin formation in the product.

### *Principle 3*

To control microbiological hazards at each identified CCP, it is necessary to set up critical limits (such as temperature, time, $A_W$, pH, preservatives, and, in some cases, aroma, texture, and appearance). One or more critical limits may be necessary at each CCP, and all of them should be present to ensure that the hazards are under control.

### *Principle 4*

The CCP used for a specific food production must be monitored to determine whether the system is effective in controlling the hazards. The monitoring can be continuous, such as in the case of heat treatment (temperature and time) of a product. If continuous monitoring cannot be adopted for a processing condition, it can be done at reliable intervals. The interval for a food production has to be developed on a statistical basis to ensure that the potential hazards are under control. Most online monitoring is done by rapid chemical and physical methods. Generally, microbiological methods are not effective because of the long time required for testing. However, some current

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methods, such as the bioluminescence method, could be effective under some conditions. All results of monitoring must be documented and signed by persons doing the monitoring as well as by an official of the company.

### *Principle 5*

In case a deviation from the present HACCP plan is identified from the monitoring system in a food production operation, effective corrective actions must be taken to assure product safety. The corrective action must demonstrate that it is effective in controlling the potential hazards resulting from the deviation of CCP. The deviations as well as the specific corrective measures for each must be documented in the HACCP plan and agreed on by the regulatory agency before applying the plan. Also, the product produced by the new plan should be placed on hold until its safety has been ensured by proper testing.

### *Principle 6*

Records of the HACCP plan developed for a specific food production should be kept on file in the plant. In addition, documents of CCP monitoring and any deviation and corrective procedures taken should be kept at the establishment. If necessary, the records must be made available to the regulatory agencies.

### *Principle 7*

Verification systems should be established to ensure that the HACCP system developed for a specific food production system is working effectively to ensure safety to the consumers. Both the food producer and the regulatory agency have to be involved in the verification of the effectiveness of the HACCP in place. Verification methods include testing samples for physical, chemical, sensory, and microbiological criteria as established in the HACCP plan. The NACMCF adopted guidelines for HACCP and its application. In it, the wordings of the seven principles were condensed and the meanings kept unchanged. It also provided the specific definition of terms such as hazard, verification, and validation; included sections on prerequisites, training, implementation, and maintenance; and gave a detailed explanation of the application of the HACCP program.[5]

## Conclusion

In a biological hazard category, the main objective of the HACCP system is to provide a high degree (close to 100%) of assurance that a food ready to be consumed will be free of pathogenic microorganisms or their toxins. However, the same principle can be applied to design control of spoilage microorganisms in foods, especially for those expected to have extended shelf life. Currently, there is an increase in the production of vacuum- and modified-air packaged unprocessed and low-heat-processed food products, many with low-fat content and high pH, some with an expected shelf life of more than 50 days at refrigerated temperatures. There is evidence that many of these products show unusual types of microbial spoilage, and some are by species that were not known before (see Chapter 20). Although studies are limited, there is evidence that the incidence of such spoilage is on the rise. It would be commercially advantageous for the food industry to adopt HACCP not only for pathogens but to also control spoilage microorganisms. It is also logical to

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think about controlling microbiological problems as a whole, which would include both pathogens and spoilage microorganisms (i.e., total microbial quality, TQM). One cannot devise methods to control only pathogens or only spoilage microorganisms. The International Organization of Standardization (ISO) 9000 series standards can be used to supplement HACCP systems and develop effective process control and process assurance systems.[6] Finally, the HACCP system can be combined with the hurdle concept of food preservation for the effective control of pathogenic and spoilage microorganisms for foods that otherwise could not be produced commercially (see Chapter 41).[7] Although food safety is of primary importance in countries with abundant food supplies, food spoilage is equally important in many countries, especially developing countries, where large quantities of food are lost because of spoilage. In those countries, the HACCP concept to reduce food spoilage will be particularly important to increase food availability.

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