Methods for the Microbiological Examination of Foods

I. Culture, Microscopic, and Sampling Methods

- The examination of foods for the presence, types, and numbers of microorganisms and/or their products is basic to food microbiology.
- In spite of the importance of this, none of the methods in common use permits the determination of exact numbers of microorganisms in a food product.

The four basic methods employed for "total" numbers are as follows:

1. Standard plate counts (SPC) for viable cells
2. The most probable numbers (MPN)method as a statistical determination of viable cells
3. Dye reduction techniques to estimate numbers of viable cells that possess reducing capacities
4. Direct microscopic counts (DMC) for both viable and nonviable cells

Conventional standard plate count

- By the conventional SPC method, portions of food samples are blended or homogenized, serially diluted in an appropriate diluent, plated in or onto a suitable agar medium, and incubated at an appropriate temperature for a given time,
- After which all visible colonies are counted by use of a Quebec or electronic counter.
- The SPC is by far the most widely used method for determining the **numbers of viable cells or colony-forming units (cfu)** in a food product.

Conventional standard plate count….

When total viable counts are reported for a product, the counts should be viewed as a function of at least some of the following factors:

- Sampling methods employed
- Distribution of the organisms in the food sample
- Nature of the food biota
- Nature of the food material
- The preexamination history of the food product
- Nutritional adequacy of the plating medium employed
- Incubation temperature and time used
- pH, water activity (aw), and oxidationreduction
- potential (Eh) of the plating medium
- Type of diluent used
- Relative number of organisms in food sample
- Existence of other competing or antagonistic organisms

Homogenization of Food Samples

- The need to clean and store blender containers is obviated.
- Heat buildup does not occur during normal operational times (usually 2 minutes).
- The homogenates can be stored in the Stomacher bags in a freezer for further use.
- The noise level is not as unpleasant as that of mechanical blenders.

The Spiral Plater

- The spiral plater is a mechanical device that distributes the liquid inoculum on the surface of a rotating plate containing a suitable poured and hardened agar medium.
- The dispensing arm moves from the near center of the plate toward the outside, depositing the sample in an
- Archimedes spiral.
- The attached special syringe dispenses a continuously decreasing volume of sample so that a concentration range of up to 10,000:1 is effected on a single plate.

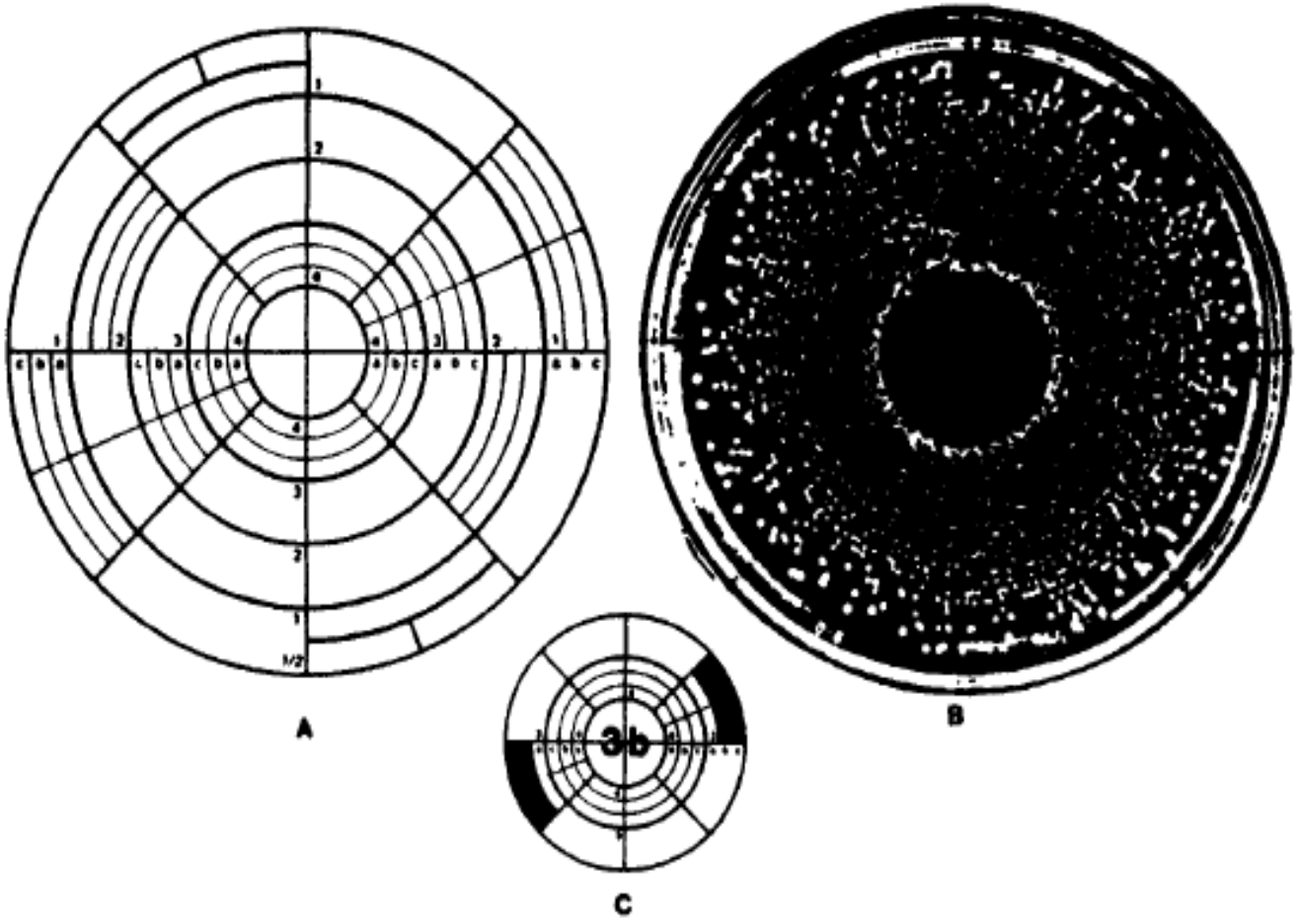


Figure 10–1 Special counting grid for spiral plater (*A*); growth of organisms on an inoculated spiral plate (*B*); and areas of plate enumerated (*C*). In this example, the inoculum volume was 0.0018 mL, counts for the two areas shown were 44 and 63, and the averaged count was 6.1×10^4 bacteria per milliliter. Courtesy of Spiral System Instruments, Bethesda, Maryland.

Direct Epifluorescent Filter Technique

- This membrane filter technique may be viewed as an improved modification of the basic method.
- The direct epifluorescent filter technique (DEFT) employs fluorescent dyes and fluorescent microscopy, and it has been evaluated by a number of investigators as a rapid method for microorganisms in foods. Typically, a diluted food homogenate is filtered through a 5-um nylon filter, and the filtrate is collected and treated with 2 mL of Triton X-IOO and 0.5 mL of trypsin. The latter reagents are used to lyse somatic cells and to prevent clogging of filters.
- After incubation, the treated fitrate is passed through a 0.6-um Nucleopore polycarbonate membrane, and the filter is stained with acridine orange.
- After drying, the stained cells are enumerated by epifluorescence microscopy, and the number of cells per gram is calculated by multiplying the average number per field by the microscope factor.
- Results can be obtained in 25-30 minutes, and numbers as low as around 6,000 cfu/g can be obtained from meats and milk products.

Microcolony-DEFT

- DEFT allows for the direct microscopic determination of cells; microcolony-DEFT is a variation that allows one to determine viable cells only.
- Typically, food homogenates are filtered through DEFT membranes, and the latter are then placed on the surface of appropriate culture media and incubated for microcolony development.
- A 3-hour incubation can be used for gram-negative bacteria and a 6-hour incubation for grampositives.
- The microcolonies that develop must be viewed with a microscope.
- For coliforms, pseudomonads, and staphylococci, as few as 10^3/g could be detected within 8 hours.

MICROSCOPE COLONY COUNTS

- Microscope colony count methods involve the counting of microcolonies that develop in agar layered over microscope slides.
- The first was that of Frost, which consisted of spreading 0.1 mL of milk-agar mixture over a 4-cm^2 area on a glass slide.
- Following incubation, drying, and staining, microcolonies are counted with the aid of a microscope.
- In another method, 2 mL of melted agar are mixed with 2 mL of warmed milk and, after mixing, 0.1 mL of the inoculated agar is spread over a 4-cm^2 area.
- Following staining with thionin blue, the slide is viewed with the 16-mm objective of a wide-field microscope.

AGAR DROPLETS

- The food homogenate is diluted in tubes of melted agar (at 45°C).
- For each food sample, three tubes of agar are used, the first tube being inoculated with 1 mL of food homogenate.
- After mixing, a sterile capillary pipette (ideally delivering 0.033 mL/drop) is used to transfer a line of 5 x 0.1-mL droplets to the bottom of an empty Petri dish. With the same capillary pipette, three drops (0.1 mL) from the first 9-mL tube are transferred to the second tube, and, after mixing, another line of 5 x 0.1-mL droplets is placed next to the first.
- This step is repeated for the third tube of agar. Petri plates containing the agar droplets are incubated for 24 hours, and colonies are enumerated with the aid of a 10x viewer.

DRY FILM AND RELATED METHODS

- A rehydratable dry film method consisting of two plastic films attached together on one side and coated with culture media ingredients and a cold-water-soluble jelling agent was developed by the 3M Company and designated Petrifilm.
- The method can be used with nonselective ingredients to make aerobic plate counts (APCs), and, with selective ingredients, certain specific groups can be detected.

MOST PROBABLE NUMBERS

- In this method, dilutions of food samples are prepared as for the SPC. Three serial aliquots or dilutions are then planted into 9 or 15 tubes of appropriate medium for the three- or five-tube method, respectively.
- Numbers of organisms in the original sample are determined by use of standard MPN tables.
- The method is statistical innature, and MPN results are generally higher than SPC results.

DYE REDUCTION

- Two dyes are commonly employed in this procedure to estimate the number of viable organisms in suitable products: methylene blue and resazurin.
- To conduct a dye-reduction test, properly prepared supernatants of foods are added to standard solutions of either dye for reduction from blue to white for methylene blue and from slate blue to pink or white for resazurin.
- The time for dye reduction to occur is inversely proportional to the number of organisms in the sample.
- Methylene blue and resazurin reduction by 100 cultures was studied in milk; with two exceptions, a good agreement was found between numbers of bacteria and time needed for reduction of the two dyes.

ROLL TUBES

- Screw-capped tubes or bottles of varying sizes are used in this method.
- Predetermined amounts of the melted and inoculated agar are added to the tube and the agar is made to solidify as a thin layer on the inside of the vessel.
- Following appropriate incubation, colonies are counted by rotating the vessel.
- It has been found to be an excellent method for enumerating fastidious anaerobes.

DIRECT MICROSCOPIC COUNT

- In its simplest form, the DMC consists of making smears of food specimens or cultures onto a microscope slide, staining with an appropriate dye, and viewing and counting cells with the aid of a microscope (oil immersion objective).
- DMCs are most widely used in the dairy industry for assessing the microbial quality of raw milk and other dairy products, and the specific method employed is that originally developedby R.S. Breed (Breed count).

Cultural Techniques

- Although there is clearly a place for the direct examination of a food for micro-organisms, a full microbiological examination usually requires that individual viable propagules are encouraged to multiply in liquid media or on the surface, or within the matrix, of a medium solidified with agar.
- A very wide range of media are available to the microbiologist and details of their formulation, and how they are used, may be found in a number of readily available books and manuals.

A selection of media commonly used in food microbiology

Medium	*Use*
Plate Count Agar	Aerobic mesophilic count
MacConkey Broth	MPN of coliforms in water
Brilliant Green/Lactose/Bile Broth	MPN of coliforms in food
Violet Red/Bile/Glucose Agar	Enumeration of Enterobacteriaceae
Crystal Violet/Azide/Blood Agar	Enumeration of faecal streptococci
Baird–Parker Agar	Enumeration of *Staphylococcus aureus*
Rappaport–Vassiliadis Broth	Selective enrichment of *Salmonella*
Thiosulfate/Bile/Citrate/Sucrose Agar	Isolation of vibrios
Dichloran/18% Glycerol Agar	Enumeration of moulds
Rose Bengal/Chloramphenicol Agar	Enumeration of moulds and yeasts
Cefixime/Tellurite/Sorbitol/MacConkey Agar	*E. coli* O157

II. **Physical, Chemical, Molecular, and Immunological Methods**

Table 11–1 Reported Minimum Detectable Levels of Toxins or Organisms by Physical, Chemical, and Immunological Methods of Analysis

Methods	*Toxin or Organism*	*Sensitivity*
Flow cytometry	*S. typhimurium* in milk	10^3/mL within 40 min, 10/mL after 6 h nonselective enrichment
Impedance	Coliforms in meats	10^3/g in 6.5 h
	Coliforms in culture media	10 in 3.8 h
Microcalorimetry	*S. aureus* cells	2 cells in 12–13 h
	S. aureus	Minimum HPR* ~10^4 cells/mL
ATP measurement	Beef carcass	10^2/cm^2 in ~5 min
Radiometry	Frozen orange juice flora	10^4 cells/g in 6–10 h
	Coliforms in water	1–10 cells in 6 h
Fluorescent antibody	Salmonellae	10^6 cells/mL
	Staph. enterotoxin B	~50 ng/mL
Thermostable nuclease	From *S. aureus*	10 ng/g
	From *S. aureus*	2.5–5 ng
Limulus lysate test	Gram-negative endotoxins	2–6 pg of *E. coli* LPS
Radioimmunoassay	Staph. enterotoxins A, B, C, D, and E in foods	0.5–1.0 ng/g
	Staph. enterotoxin B in nonfat dry milk	2.2 ng/mL
	Staph. enterotoxins A and B	0.1 ng/mL for A; 0.5 ng/mL for B
	Staph. enterotoxin C_2	100 pg
	E. coli ST_a enterotoxin	50–500 pg/tube
	Aflatoxin M_1 in milk	0.5 ng/mL
	Ochratoxin A	20 ppb
	Bacterial cells	500–1,000 cells in 8–10 min
	Aflatoxin B_1 in corn, wheat, peanut butter	6 ng/g
	Deoxynivalenol in corn, wheat	20 ng/g
Electroimmunodiffusion	*C. perfringens* enterotoxin	10 ng
	Botulinal toxins	3.7–5.6 mouse LD_{50}/0.1 mL
Micro-Ouchterlony	*S. aureus* enterotoxins A and B	10–100 ng/mL
	C. perfringens type A toxin	500 ng/mL
Lux luminescence	Various bacteria	~10 cfu/mL†
Passive immune hemolysis	*E. coli* LT enterotoxin	<100 ng
Aggregate-hemagglutination	*B. cereus* enterotoxin	4 ng/mL
Latex agglutination	*E. coli* LT enterotoxin	32 ng/mL
Single radial immunodiffusion	*S. aureus* enterotoxins	0.3 µg/mL
Hemagglutination–inhibition	Staph. enterotoxin B	1.3 ng/mL

continues

Table 11–1 Continued

Methods	*Toxin or Organism*	*Sensitivity*
Reverse passive hemagglutination	Staph. enterotoxin B	1.5 ng/mL
	C. perfringens type A toxin	1 ng/mL
ELISA	Staph. enterotoxin A in wieners	0.4 ng
	Staph. enterotoxins A, B, and C in foods	0.1 ng/mL
	Staph. enterotoxins A, B, C, D, and E in foods	≥1 ng/g
	Botulinal toxin type A	About 9 mouse LD_{50}/mL with monoclonal antibody
	Botulinal toxin type A	50–100 mouse i.p. LD_{50}
	Botulinal toxin type E	100 mouse LD_{50}
	Aflatoxin B_1	25 pg/assay
	Aflatoxin M_1 in milk	0.25 ng/mL
	Aflatoxin B_1	<1 pg/assay
	Salmonellae	10^4–10^5 cells/mL
	AFB_1	0.2 ng/mL (monoclonal)
	AFB_1	0.4 ng/mL (polyclonal)
	AFM_1	1.0 ng/mL (monoclonal)
	Fumonisins in feed	250 ng/g
	Zearalenone in corn	1 ng/g
	E. coli O157:H7 in ground beef	<1 cell/g
	C. perfringens enterotoxin	1 pg/mL
	AFB_1 in peanut butter	2.5 ng/g
	Ochratoxin in barley	1 ng/mL
Polymerase chain reaction (PCR)	*E. coli*	1–5 cells/100 mL H_2O
	E. coli	1 cell
	L. monocytogenes	1–10 cells
	V. vulnificus	10^2 cfu/g (oysters)
	C. perfringens	<1 cfu, 2–6 h
	Stx1 and Stx2 of *E. coli*	1 cell/g in 12 h
	C. *botulinum* toxins A to E	10 fg (~3 cells)
	Y. enterocolitica	10–30 cfu/g meat
Fluorogenic PCR-based assay	*E. coli*	0.5 cfu/g
Fluorescent PCR assay	*E. coli*	3 cfu/25 g
Immunomagnetic separation	*E. coli* O157:H7	<10^3 cfu/g
Ice nucleation	Salmonellae	ca. 25/g

*HPR, Exothermic heat production rate.
†cfu, Colony-forming units.

III. **METHODS FOR CHARACTERIZING AND FINGERPRINTING FOODBORNE ORGANISMS**

- A variety of methods are now in use for the purpose of characterizing or fingerprinting species and strains of organisms of interest in foods:
- serotyping
- phage typing
- nucleic acid probes
- polymerase chain reaction
- multilocus enzyme electrophoresis
- restriction enzyme analysis
- random amplication of polymorphic DNA
- pulsed field gel electrophoresis
- restriction fragment length polymorphism
- ribotyping