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Legume Species Alter the Effect of Biochar Application on Microbial Diversity and Functions in the Mixed Cropping System—Based on a Pot Experiment

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Abstract: Biochar application to legume-based mixed cropping systems may enhance soil microbial diversity and nitrogen (N)-cycling function. This study was conducted to elucidate the effect of biochar application on soil microbial diversity and N-cycling function with a particular focus on legume species. Therefore, we performed a pot experiment consisting of three legume species intercropped with maize: cowpea, velvet bean, and common bean. In addition, one of three fertilizers was applied to each crop: biochar made of chicken manure (CM), a chemical fertilizer, or no fertilizer. Amplicon sequencing for the prokaryotic community and functional prediction with Tax4Fun2 were conducted. Under the CM, Simpson's diversity index was higher in soils with common beans than those in other legume treatments. On the other hand, N-cycling genes for *ammonia oxidation* and *nitrite reductase (NO-forming)* were more abundant in velvet bean/maize treatment, and this is possibly due to the increased abundance of *Thaumarchaeota* (6.7%), *Chloroflexi* (12%), and *Planctomycetes* (11%). Cowpea/maize treatment had the lowest prokaryotes abundances among legume treatments. Our results suggest that the choice of legume species is important for soil microbial diversity and N-cycling functions in CM applied mixed cropping systems.

Keywords: mixed cropping; legume; microbial diversity; biochar; nitrogen cycling

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1. Introduction

Modern agricultural practices, including monoculture cropping systems and the intensive use of inorganic fertilizers, have led to soil degradation and a loss of genetic diversity in soil [1,2]. Among the different scales of biodiversity, the diversity of soil microorganisms is especially important for the stability of agricultural ecosystems because soil microbes can be considered the main drivers of the biogeochemical reactions that are critical for soil health and crop productivity [3]. For example, they are especially involved in biogeochemical processes essential for plant health and growth, including nutrient absorption, immune function, pathogen prevention, and stress tolerance [4,5]. Therefore, agricultural management systems that maintain or increase soil microbial diversity and functioning should be established.

Among the various agricultural practices that can potentially promote soil microbial diversity, the use of mixed cropping systems has received increasing attention. Legume-based intercropping systems have been reported to enhance soil microbial diversity and functions, including the mineralization of available phosphorus (P) and nitrogen (N) [6,7]. In particular, sweet maize (*Zea may* L.)/soybean (*Glycine max* L.) intercropping systems have been reported to promote the expression of key genes involved in N-cycling (e.g., ammonium oxidation, nitrite reductase, and nitrous-oxide reductase) [8]. Moreover, mixed cropping systems with field pea varieties have been demonstrated by Horner et al. [9] to

build stronger and larger co-occurrence networks in rhizosphere prokaryotic communities compared to monocropping systems. These studies have suggested that crop diversification, including mixed cropping systems, improves soil microbial diversity and nutrient availability in agricultural soil. However, outside of certain successful cases, not all intercropping systems improve soil microbial conditions and crop productivity. Previous research has indicated that microbial community composition and structure are often less affected by intercropping systems [10–12]. In addition, cereal/legume intercropping systems did not improve plant production compared with monocropping systems [13,14]. This inconsistency can be explained by interspecific interactions between legumes and cereals, as plant combinations in intercropping systems significantly alter soil microbial communities composition and relative abundance. In addition, the soil microbes in the rhizosphere of legume crops have a high diversity and are species-specific due to their variable N acquisition abilities through symbiosis with rhizobia [15]. However, few studies have yet examined how different legume combinations in mixed cropping systems may alter soil microbial community composition, structure, and functions (specifically, N-cycling).

In addition to the type of legume species, fertilizer is another common agricultural management technique that plays a major role in determining microbial community composition and functioning. The use of organic fertilizer often enhances the abundance and diversity of prokaryotes, when compared to inorganic fertilizer [16,17]. Among variable organic fertilizers, biochar has been reported by many previous studies to have positive effects on soil microbial diversity and functioning in mixed cropping systems [18,19]. Furthermore, the combined use of biochar and legume-based intercropping systems could alter the expression of microbial N-cycling genes, which is important for plant growth. For example, biochar application stimulates microbial *ammonium oxidation* and reduces gaseous N loss from soil by reducing the denitrification potential [20]. However, the effect of biochar on plant beneficial functions are reported as plant species-specific. For example, a number of studies reviewed by Kochaneck et al. [21] showed that the variability of plant species significantly impacted the soil microbial community in biochar-applied soil. Plant root exudates contain organic acids (citric acid, malic acid, and ethanoic acid) that promote biochar decomposition, with the amount and quality varying based on plant species [22]. Therefore, the difference in legume species used in mixed cropping might be responsible for the variable nutrient availability of biochar, which is important for the soil microbial community and N cycling function. Although most studies have examined the effects of biochar in comparison with monocropping systems, more studies are necessary to address the biochar effect in different intercropping systems.

This study elucidated legume species-specific effects of maize/legume mixed cropping on soil microbial community structure and their predicted functions, with a special focus on the interaction between legume species and fertilizer. The main hypotheses of this study were: (i) soil microbial community compositions are different among the legume species used in the mixed cropping system, and (ii) combined use of specific legumes and biochar promotes microbial diversity and enhances microbial N-cycling rates. To test these hypotheses, a greenhouse legume-maize mixed cropping experiment was set-up using three legume varieties commonly used in mixed cropping systems [23–25] and a chemical or biochar (carbonized chicken manure (CM)) as fertilizer. To test the impact of these factors on soil microbial communities structure, prokaryotic diversity and functional diversity were assessed using a 16S rRNA gene survey.

2. Materials and Methods

2.1. Soil Sampling

The soil used in this experiment was sampled from land that had been abandoned for 30 years to develop a low-nutrient-input system at a university farm located at the Field Science Centre for Northern Biosphere, Hokkaido University, Japan (43°04′ N, 141°20′ E). The properties of the soil are given in Table 1. The soil type was clay loam with 44.6% sand, 21.5% silt, and 33.9% clay.

Table 1. Chemical properties of soil and carbonized chicken manure (CM).

Chemical Properties	Soil	CM
Water content (%)	19.5 ± 0.1	19.1 ± 1.2
C/N	14.7 ± 0.2	10.0 ± 0.4
pH (1:10)	6.3 ± 0.0	10.4 ± 0.3
EC (μs/cm)	78.5 ± 3.5	12740 ± 793
Total nitrogen (%)	0.25 ± 0.01	4.06 ± 0.45
Total carbon (%)	3.9 ± 0.1	40.5 ±3.4
P g kg^{-1} soil	0.21 ± 0.01	36.8 ± 1.0
Ca g kg^{-1} soil	5.9 ± 0.3	134 ± 5
Mg g kg^{-1} soil	0.53 ± 0.01	15.1 ± 0.3
K g kg^{-1} soil	0.56 ± 0.01	45.3 ± 0.9

Errors represent standard deviation (n = 3).

2.2. Experimental Design and Soil Sampling

A pot experiment was performed in a greenhouse at the Graduate School of Hokkaido University. The sampled soil was air-dried and sieved with a 2 mm mesh and subsequently poured into Wagner pots (surface area = 1/5000 a, diameter = 16 cm, and height = 19 cm). Each pot contained 1.8 kg of air-dried soil. The experimental design, which was completely randomized and included three fertilizer treatments × four plant treatments (three mixed cropping treatments and a single maize treatment), was conducted in triplicate. Three common leguminous species used for legume/maize mixed cropping systems, namely cowpea (*Vigna unguiculata* (L.) Walp.), velvet bean (*Mucuna pruriens* (L.) DC.), and common bean (*Phaseolus vulgaris* L.), were selected for our study. One of the four types of plant treatment was then planted into each pot: (1) single maize (*Zea mays* L.; SM), (2) mixture of cowpea and maize (VM), (3) mixture of velvet bean and maize (MM), and (4) mixture of common bean and maize (PM). The pots received one of three fertilizer treatments, namely control ('Ctr') without fertilizer, chemical fertilizer containing P and K ('CF'), or biochar made from CM (50 g pot^{-1} of carbonized CM; 'CM'). The application rate for CF was 30 kg P ha^{-1} and 50 kg K ha^{-1}. Soil and CM chemical properties are described in Table 1. The application amount of CM was designed to optimize P uptakes and the growth rate of plants [26,27]. As well as CF, recommended amounts of P and K were applied according to previous studies [28,29]. Three replicates were performed for 12 treatments (3 fertilizer treatments × 4 crop types); therefore, 36 pots were prepared in total. During these treatments, maize, cowpea, velvet bean, and common bean were sprouted for 2 weeks in small pots filled with vermiculite before they were transplanted to Wagner pots. The temperature was maintained at 25 °C to 30 °C for the duration of the experiment. Plants were grown for 50 days after transplanting. This was in agreement with previous studies that showed that plant N demand was highest after 50 days [30,31].

The soil was sampled from each pot at 0 to 10 cm and passed through a 2 mm sieve to homogenize the sample and remove roots and stones. Then, it was stored at 4 °C or −20 °C for subsequent chemical analysis and DNA extraction, respectively. Legume roots were gently washed with water and then the nodule number per plant was visually counted. Harvested plants were dried in an oven at 65 °C for 3 days to determine their dry weight.

2.3. Chemical Property Analysis

Within one week after soil sampling, pH and extractable NH_4^+ and NO_3^- concentrations were measured with the following method: for soil pH, 6 g of soil was shaken for 30 min with 30 mL of Milli-Q water, and then the pH was measured using a pH sensor (AS800; ASONE Co., Osaka, Japan). To measure soil NH_4^+ and NO_3^-, samples were extracted with a KCl solution (2 mol L^{-1}) and then subjected to a colorimetric analysis using a flow injection analyzer system (ACLA-700; Aqualab Co., Ltd., Osaka, Japan).

2.4. DNA Extraction and 16S rRNA Sequencing

Using the same sampled soils, DNA was extracted with the NucleoSpin® Soil kit (Takara Bio, Inc., Shiga, Japan), following the manufacturer's instructions. The extracted DNA was subsequently amplified by polymerase chain reaction (PCR) targeting the V4 region of the 16S rRNA gene (amplicon size ~250 bp; forward primer 515F: 5′-GTGCCAG CMGCCGCGGTAA-3′ and reverse primer 806R: 5′-GGACTACHVGGGTWTCTAAT-3′). To perform PCR, 10 µL of AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 µL of the forward primer, 0.4 µL of the reverse primer, 8.2 µL of nuclease-free water, and 1 µL of DNA extract were mixed. The PCR cycles were as follows: first, 95 °C for 10 min, then 20 cycles at 95 °C for 30 s, then 57 °C for 30 s and 72 °C for 1 min, and finally 72 °C for 7 min. The PCR products were subsequently purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) according to the protocol provided. Purified PCR products were quantified with the QuantiFluor® ONE dsDNA system by a Quantus Fluorometer E6150 (Promega, Madison, WI, USA).

An additional PCR was performed on the original PCR products to add Ion-Torrent-specific barcodes. The 515F forward primer with the Ion Xpress Barcode Adapters Kit sequence and the 806R reverse primer attached to the Ion Xpress sequence of the Ion P1 adaptor were used (Thermo Fisher Scientific K.K., Tokyo, Japan). Amplicons from the first PCR were diluted to 2000 ng mL^{-1}, and 1 µL of each PCR product was subsequently mixed with 10 µL of AmpliTaq Gold® 360 Master Mix, 0.4 µL of forward primer, 0.4 µL of reverse primer, and 7.2 µL of nuclease-free water. The second PCR cycle was set to 95 °C for 10 min and then 5 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, followed by 72; °C, for 7 min. Products from the second PCR were purified following the same method outlined previously. The final length and concentration of the amplicons were confirmed using a Bioanalyzer DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA(Agilent Technologies, USA). The library was subsequently diluted to 50 pM and loaded onto the Ion 318 chip using Ion Chef Instruments with an Ion PGM Hi-Q Chef Solutions. The samples were sequenced on an Ion PGM Sequencer with Ion PGM Hi-Q View Sequence Solutions (Ion Torrent Life Technologies, Guilford, CT, USA). Sequence data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under accession number PRJNA743765.

2.5. Sequence Processing

The barcoded 16S rRNA gene sequences were denoised, quality-filtered, and assessed using the DADA2 algorithm implemented in Quantitative Insights Into Microbial Ecology (QIIME2; see Bolyen et al. [32]). Rarefaction was performed with minimal reads among all samples, and sequence data were subsampled to 41,095 sequences per sample. The R package Vegan was used to assess sequencing depth and to generate an alpha rarefaction curve (Figure S1). The rarefaction curve was then evaluated using the interval of a step sample size of 1000. Clustering of operational taxonomic units (OTUs) was performed at 99% identity and was conducted using the SILVA 123 database.

2.6. Measurement of Prokaryotes Abundance

To measure prokaryotes abundance, quantitative PCR (qPCR) was performed on extracted DNA and diluted 50 times with nuclease-free water. The 515F/806R primer pairs described above were used to amplify the V4 region of the 16S rRNA gene. For the standard curve, PCR products from the DNA extracted from the Ctr pots were used, purified with AMPure XP, and further diluted to five different concentrations. Samples were prepared with 10.4 µL of KAPA SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA, USA), 0.08 µL of the forward primer, 0.08 µL of the reverse primer, and 2 µL of diluted DNA extract. Nuclease-free water was added to achieve a final volume of 20 µL. CFX96 Touch Real (Bio-Rad Laboratories, Inc., Richmond, CA, USA) was used, and the cycling conditions were 95 °C for 30 s, 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, all followed by 95 °C for 1 min and then a subsequent ramp from 55 °C to 95 °C by 1 °C

increments for 10 s each. Ct values (threshold cycle) were calculated after quantifying the amplification results using qpcR R package.

2.7. Statistical Analysis

To quantify the diversity of soil microbial communities, the Shannon index and the Simpson index, which are used to estimate community α-diversity, were used. For each diversity index, a two-way ANOVA on prokaryotic community structure was performed using fertilizer treatments and plant species followed by a Tukey's Honest Significant Difference test (emmeans R package). In addition, a correlation test using the Pearson method was performed for each diversity index using the prokaryotic community structure as a correlate.

A nonmetric multidimensional scaling (NMDS) analysis of community structure dissimilarity based on the Bray–Curtis index was performed using the metaMDS function in the vegan package in R. The envfit function in the vegan package was used to illustrate significant correlations ($p < 0.05$) between soil chemistry and relative abundances of phylum (>0.1%), with NMDS values presented as vectors on an NMDS ordination. Differences in prokaryotic community structure between treatments were tested by permutational multivariate analysis of variance (PERMANOVA) with the factors 'plant' and 'fertilizer', using the adonis function of the vegan package in R.

Functional profiling of the prokaryotic community was conducted with the Tax4Fun2 R package [33]. The rarefied OTU table was used for Tax4Fun2 searches, and metagenome functional profiles were predicted against the Kyoto Encyclopedia of Gene and Genomes ortholog tables [34]. To evaluate biogeochemical reactions, 36 gene-coding enzymes related to the N metabolism pathway were selected [35,36] (Table S1) and visualized with 'ComplexHeatmap' R package.

For soil chemical property data, a two-way analysis of variance (ANOVA) was performed to investigate the effect of two main treatments (fertilizer and legume treatments) on soil pH, NH_4^+, and NO_3^-.

3. Results

3.1. Alpha Diversity

The impact of the interaction between legume species and fertilizer treatments was visualized using microbial diversity indices ($p < 0.05$). With the PM crop, a significantly higher microbial diversity (Simpson's index) was observed under CM treatment than the Ctr (Figure 1). In contrast, with the MM crop, there was a lower diversity with CM than in the Ctr treatment. There were no significant differences in the microbial diversity between the SM and VM crop.

Correlation tests between diversity indices (Shannon or Simpson) and prokaryote community structure (phylum or class level) showed the contribution of each microbial taxon on overall diversity (Table S2). At the class level, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* (each belonging to the phylum *Proteobacteria*), *Acidimicrobiia* (phylum *Actinobacteria*), and *Sphingobacteriia* (*Bacteroidetes*) positively correlated with diversity indices, and they were more abundant in PM and VM in CM-applied soil than MM. In contrast, *TK10* (*Chloroflexi*), the *Soil Crenarchaeotic Group* (*Thaumarchaeota*), and *Spartobacteria* (*Verrucomicrobia*) that were negatively correlated with diversity indices were more abundant in MM than other legume treatments.

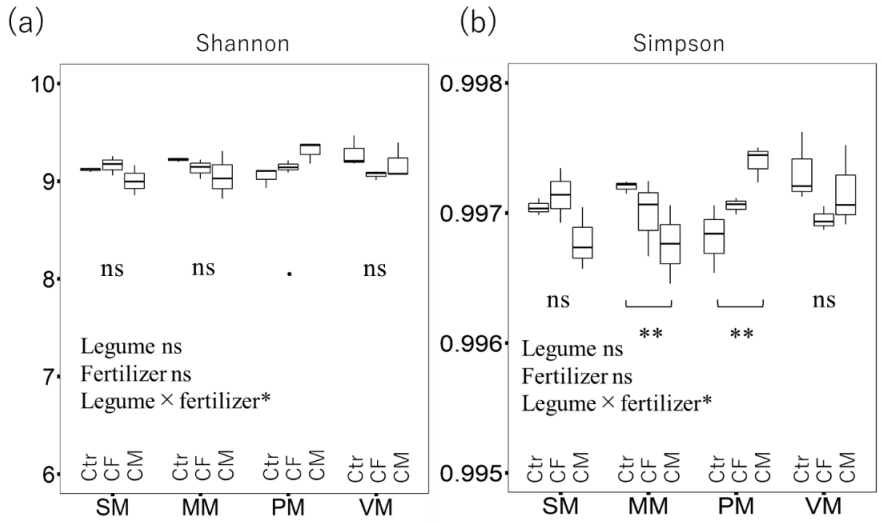

Figure 1. Box plot of bacterial OTUs alpha diversity indicating (**a**) the Shannon's Index and (**b**) the Simpson's index. Two-way ANOVA and Tukey–Kramer's pairwise comparison were performed on the calculated alpha-diversity indices: $p < 0.25$, * $p < 0.05$, and ** $p < 0.01$. The abbreviations of legume treatments were as follows: single maize, SM; maize cropped with velvet bean, MM; maize cropped with common bean, MP; maize cropped with cowpea, VM. Fertilizer treatments were abbreviated as no fertilizer, Ctr; chemical fertilizer, CF; carbonized chicken manure, CM.

3.2. Soil Microbial Community Abundance and Structure

Prokaryotic community structure was significantly affected by legume treatments (PERMANOVA, $p < 0.001$), the fertilizer treatments ($p < 0.001$), and their interaction ($p < 0.01$). NMDS plots based on a Bray–Curtis dissimilarity index showed that CM-treated plots clustered separately from CF and Ctr, which clustered together (Figure 2). For CM-treated soils, the variability among legume treatments was higher than in other fertilizer treatments, whereas for CF-treated pots, microbial beta diversity was highly similar. Moreover, microbial communities under MM treatments clustered separately compared to other legume treatments in the CM treatment ($p < 0.001$, Figure S3). However, the distinct clustering by legume species was not clearly observed within the Ctr and CF treatments. Among the soil chemical properties, pH and NO_3^--N concentration were significant factors in the NMDS ordination. Based on a two-way ANOVA with the relative abundances of prokaryotes phyla as a response variable, *Thaumarchaeota*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, *Proteobacteria*, and *Gemmatimonadetes* contributed the most to changes in community structures, and their relative abundance was influenced by the interactions between legume species and fertilizer treatments (Figure 3; Table 2). Under CM treatment, *Thaumarchaeota*, *Verrucomicrobia*, *Chloroflexi*, and *Planctomycetes* were significantly more abundant in MM than other legume treatments. Comparing fertilizer treatments, *Thaumarchaeota*, *Verrucomicrobia*, *Chloroflexi*, and *Planctomycetes* were more abundant in the CM treatment, whereas *Proteobacteria* was more abundant in the Ctr and CF treatments.

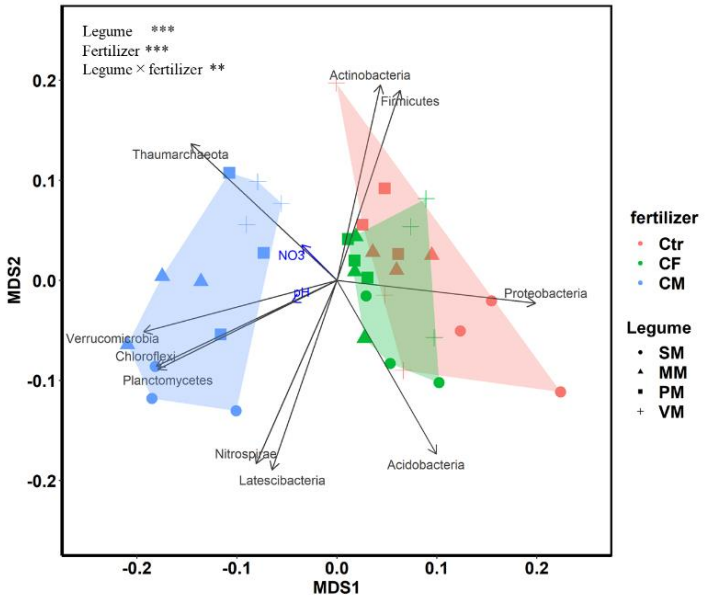

Figure 2. Nonmetric multidimensional scaling plot based on Bray–Curtis dissimilarity (stress = 0.158). The color represents the fertilizer. The shape indicates the legume type. The legend in the upper left shows the significant result of the the Permutational multivariate analyses of variance (PerMANOVA) test for legume and fertilizer treatments. Asterisks show *p*-values (** $p < 0.01$, and *** $p < 0.001$).

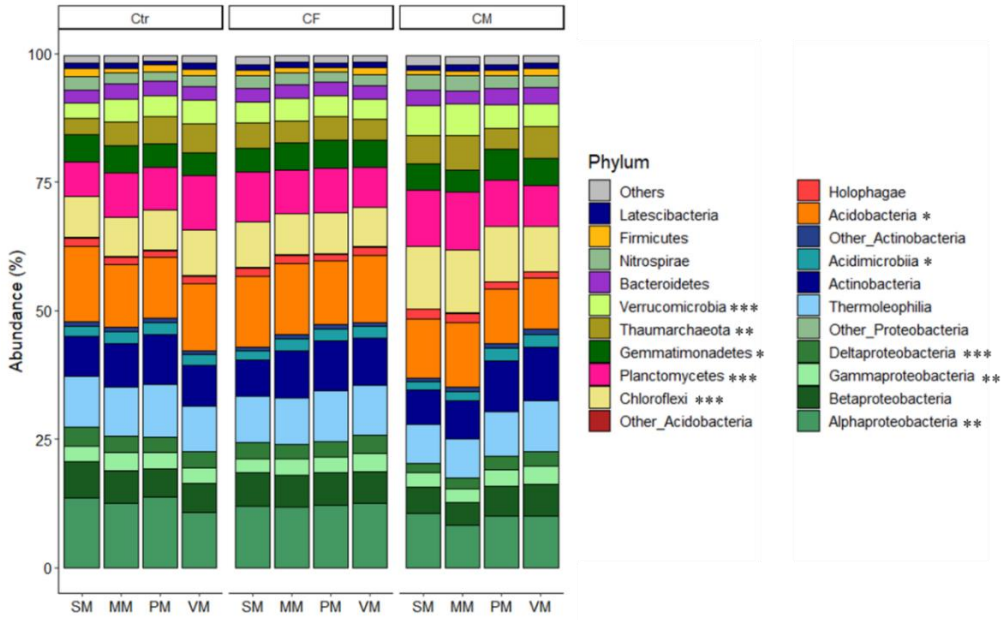

Figure 3. Relative abundance (%) of the main phyla and classes. Phyla with relative abundance > 10% (*Acidobacteria*, *Actinobacteria*, and *Proteobacteria*) are separated into classes. Phyla and classes with relative abundance < 0.1% are subsumed into 'Others'. Asterisk show *p*-value of two-way ANOVA for legume and fertilizer treatments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Legume treatments: SM, MM, PM, and VM; fertilizer treatments: Ctr, CF, and CM.

Table 2. The relative abundance of prokaryotes at the phylum level showed significant interactions between legume species and fertilizer treatments.

Treatment	Relative Abundance (%)					
	Thaumarchaeota	*Gemmatimonadetes*	*Chloroflexi*	*Planctomycetes*	*Verrucomicrobia*	*Proteobacteria*
Ctr						
SM	3.2 ^{a}	5.2 ^{a}	7.8 ^{ab}	6.8 ^{a}	2.9 ^{a}	27 ^{b}
MM	4.7 ^{ab}	5.3 ^{a}	7.6 ^{a}	8.5 ^{b}	4.4 ^{b}	26 ^{ab}
PM	5.2 ^{ab}	4.7 ^{a}	7.8 ^{ab}	8.2 ^{ab}	4.2 ^{b}	25 ^{b}
VM	5.7 ^{b}	4.4 ^{a}	8.7 ^{b}	11 ^{c}	4.6 ^{b}	27 ^{b}
CF						
SM	4.9 ^{a}	4.6 ^{a}	8.8 ^{b}	9.8 ^{b}	4.1 ^{a}	24 ^{a}
MM	4.2 ^{a}	5.2 ^{a}	8.0 ^{ab}	8.5 ^{ab}	4.4 ^{a}	24 ^{a}
PM	4.7 ^{a}	5.4 ^{a}	7.9 ^{ab}	8.7 ^{ab}	3.9 ^{a}	24 ^{a}
VM	4.2 ^{a}	5.3 ^{a}	7.7 ^{a}	7.6 ^{a}	3.8 ^{a}	26 ^{a}
CM						
SM	5.5 ^{ab}	5.1 ^{ab}	12 ^{c}	11 ^{b}	5.9 ^{b}	20 ^{ab}
MM	6.7 ^{b}	4.3 ^{a}	12 ^{c}	11 ^{b}	6.2 ^{b}	17 ^{a}
PM	4.0 ^{a}	6.0 ^{b}	11 ^{b}	9.1 ^{a}	4.6 ^{a}	22 ^{b}
VM	6.2 ^{b}	5.3 ^{ab}	8.7 ^{a}	7.9 ^{a}	4.5 ^{a}	23 ^{b}

The results from multiple pairwise comparisons are shown as different letters, indicating significant differences between treatments ($p < 0.05$).

3.3. 16S rRNA Gene Abundance

Crop type had a significant effect ($p < 0.01$) on the prokaryotes absolute abundance, based on qPCR and when averaged across fertilizer types, but fertilizer type had no significant effect (Table S4). The prokaryotes abundance in the SM crop was significantly higher than in the VM ($p < 0.05$). However, there was no correlation between prokaryotes abundance and diversity.

3.4. Gene Function Prediction by Tax4Fun2

Two-way ANOVA analysis indicated that the gene abundances coding for *ammonium oxidation*, *carbamate kinase*, *glutamate dehydrogenase*, *nitrate reductase*, *nitrite oxidoreductase*, and *nitrite reductase (NO-forming)* were significantly influenced by the interaction effects of legume and fertilizer treatments (Figure 4, Table S1). Among fertilizer treatments, CM altered the abundance of many of the genes of interest. In particular, the MM crop (with CM) showed the highest abundance of *glutamate dehydrogenase*, *carbamate kinase*, *ammonium oxidation*, and *nitrate reductase (NO-forming)* genes. However, other fertilizer treatments (Ctr and CF) did not show a significant difference among legume treatments, although the abundance of glutamate dehydrogenase and *nitrite reductase (NO-forming)* genes was higher in the mixed cropping systems than in SM. *Nitrogenase* gene abundance, which was expected to be affected by the absence of legumes, did not show any significant difference between legume and fertilizer treatments.

3.5. Soil Chemical Properties

A significant increase in soil pH was observed in the CM treatment (Table 3) compared to other treatments, regardless of legume treatments. The CM also had a high concentration of salt-based ions, such as potassium, sodium, and calcium (Table 1). Additionally, the application of CM significantly increased NO_3^--N concentration ($p < 0.001$), but there was no significant difference in NH_4^+-N concentration between treatments.

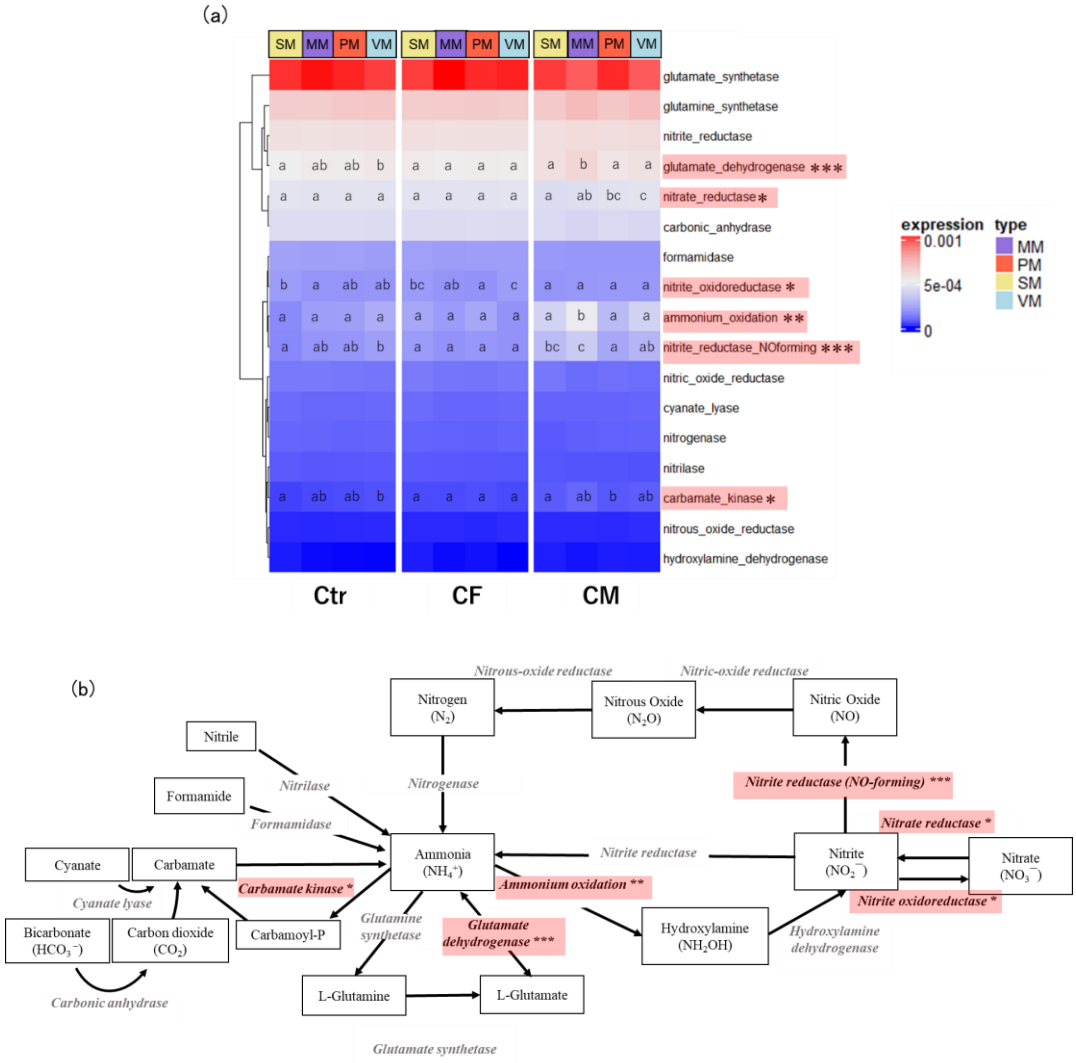

Figure 4. (**a**) Predicted prokaryotic genes coding N metabolism pathway from KEGG database. Asterisk show *P* value of two-way ANOVA interaction for legume and fertilizer treatments (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Different letters in the table indicate significant ($p < 0.05$) pairwise differences among legume treatments. Individual KEGG codes are described in Table S1. (**b**) N metabolism pathway (ko00910) and enzymes based on Kyoto Encyclopedia. The genes highlighted in red indicate significant pathways for the two-way ANOVA interaction.

3.6. Legume Nodulation and Plant Biomass

A higher number of nodules was observed in PM than in other legume treatments, regardless of fertilizer treatment (Table S3). With CF application in particular, more leguminous nodulation was observed in PM and MM. However, legume nodulation counts did not correlate with plant biomass. With CM application, MM had the highest biomass (13.9 g) among mixed cropping systems (10.1 and 9.5 g in VM and PM, respectively).

Table 3. Soil pH, nitrate, and ammonium content after plant cultivation.

Treatment		pH (H_2O)	NO_3^--N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})
SM				
	Ctr	6.7 ± 0.03	8.2 ± 1.6	4.7 ± 0.30
	CF	6.7 ± 0.04	7.1 ± 0.4	3.4 ± 0.75
	CM	7.6 ± 0.09	15.9 ± 2.4	5.4 ± 1.22
MM				
	Ctr	6.7 ± 0.02	10.9 ± 4.2	4.2 ± 2.64
	CF	6.7 ± 0.02	6.3 ± 3.3	4.7 ± 1.37
	CM	7.3 ± 0.10	19.7 ± 6.2	5.1 ± 2.48
PM				
	Ctr	6.7 ± 0.02	12.8 ± 2.2	6.0 ± 2.80
	CF	6.6 ± 0.02	7.9 ± 3.2	5.7 ± 0.98
	CM	7.2 ± 0.12	35.0 ± 19.3	2.6 ± 0.37
VM				
	Ctr	6.7 ± 0.02	10.6 ± 3.2	4.5 ± 1.35
	CF	6.7 ± 0.03	6.7 ± 3.1	4.9 ± 0.68
	CM	7.2 ± 0.08	30.8 ± 13.2	5.4 ± 2.22
Two-way ANOVA			*p*	
Legume		<0.001	0.45	0.92
Fertilizer		<0.001	<0.001	0.90
Legume × fertilizer		<0.01	0.91	0.39

The expressed plant species included single maize, SM; maize cropped with velvet bean (*Mucuna pruriens* (L.) DC.), MM; maize cropped with common bean (*Phaseolus vulgaris* L.), PM; maize cropped with cowpea (*Vigna unguiculate* (L.) Walp.), VM. Fertilizer applied with the control, Ctr; chemical fertilizer, CF; carbonized chicken manure, CM. Two-way ANOVA was performed to examine the effects of the interactions between plant species and fertilizer treatments. *p* values are shown at the bottom of the table.

4. Discussion

4.1. Changes in Microbial Diversity and Community after Biochar Application

Among the fertilizer treatments, CM clearly changed the structure of the prokaryotic community (Figures 1 and 2). The application of CM also altered soil pH (Table 3), which is well-known to strongly influence microbial community and abundance in soils [37,38]. Overall, *Thaumarchaeota*, *Verrucomicrobia*, *Chloroflexi*, and *Planctomycetes* were more abundant under CM application. In previous studies, the abundance of *Thaumarchaeota* and *Verrucomicrobia* was positively correlated with soil pH [39,40], and *Chloroflexi* and *Planctomycetes* became more abundant with the enrichment of labile carbon and aromatic compounds [41]. Thus, the chemical property and carbon content of CM might explain the larger impacts on soil prokaryotic communities when compared to chemical fertilizer.

The degree that differences in community composition increased with CM application varied among legume treatments. It is possible that the amount and quality of plant root exudates affect soil microbiomes. Some chemical compounds (e.g., aromatic organic acids) released from plant roots can enrich specific microbial species and activities that are beneficial for plant growth [42]. Furthermore, the physical properties of biochar itself may influence plant exudate availability on microbial community and diversity in the intercropping system because of absorption of the root exudates onto biochar's surface or its porosity. Liao et al. [43] indicated that the presence of biochar stimulated the assimilation of plant-delivered carbon by members of *Firmicutes* and *Bacteroidetes* in a legume-based intercropping system. In support of this previous study, *Bacteroidetes* increased in PM and VM under CM application in our study (Table S2). Thus, biochar might increase or decrease specific groups of prokaryotes when applied to particular legume species.

Under CM application, the prokaryotic community became more diverse in PM crops but less diverse in MM crops (Figure 1). This result contradicts a previous study that demonstrated that biochar application has a positive effect on soil microbial diversity in mixed crops [44]. With PM treatment, members of *Proteobacteria* and *Bacteroidetes* (i.e.,

Betaproteobacteria, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Sphingobacteriia*) that were significantly positively correlated with Shannon and Simpson diversity indices became more abundant. Both phyla are highly abundant in rhizosphere soil [45,46]. In contrast, CM applied to MM crops increased the relative abundances of *Thaumarchaeota*, *Verrucomicrobia*, *Chloroflexi*, and *Planctomycetes*, which were negatively correlated with diversity indices. Thus, our results indicate that specific combinations of legumes and maize that are used with biochar can be vital for determining the diversity of prokaryotes in soils.

4.2. Soil Microbial Functions Related to the N Cycle

Functional prediction analysis with Tax4Fun2 indicated that CM treatment enhanced N metabolism functioning (e.g., *ammonium oxidation* and *nitrite reductase (NO-forming)*), especially in MM treatment (Figure 4). Biochar application was similarly reported to enhance ammonia-oxidizing archaea and bacteria in rotated cropland [47], as well as increase *nitrite reductase* gene abundance [48]. Soil pH has been considered a critical environmental factor impacting *ammonium oxidation* and *nitrite reduction*, with an optimal range of pH 7 to 8 [49]. Therefore, in the present study, the increase in soil pH through biochar application (pH 6 to 7; Table 3) was a possible cause of enhanced N-cycling. In addition, we used biochar made of chicken manure that contains a higher N content (4.06%) compared to biochar made from other materials such as sugar cane bagasse (1.8%) and rice straw (1.3%) [50]. Thus, we note that the biochar used in the current study altered not only soil pH and physical conditions, but also N availability in soils (Table 3).

Within CM treatments, there was enhanced *ammonium oxidation*, *nitrite reductase (NO-forming)*, *carbamate kinase*, and *glutamate dehydrogenase*, particularly in MM compared to the other legume treatments. A higher abundance of those genes indicates a faster conversion of organic-N to ammonium or nitrate, which then becomes available for use by the plant. As N is a primary element for plant growth, we observed the highest plant biomass in MM treatment (Table S3). Our results are consistent with previous studies showing the improvement of crop productivity in the maize/velvet bean intercropping system compared to mono-cropping systems [23,24]. These studies indicated that weed reduction by cover crop can cause increased harvest; however, to our knowledge, little information is available regarding the interaction of velvet bean used in intercropping systems and prokaryotic N-cycling function. It should be mentioned that Tax4Fun2 is not an actual measurement but a functional prediction tool based on 16S rRNA gene sequences. Future work on shotgun metagenomics would be necessary to examine these predicted observations. However, the present study may provide basic knowledge for further metagenomic studies of soil prokaryotes and N-cycling gene expression in mixed cropping systems.

While comparing the community compositions at the phylum level, *Thaumarchaeota*, *Chloroflexi*, and *Planctomycetes* were more abundant in CM-applied soils than other fertilizer treatments (Table 2). Among the legume treatments, these taxa were most abundant in MM. *Thaumarchaeota*, archaea ubiquitously present in a wide variety of ecosystems, may contribute to the increase in ammonia-oxidizing functioning in a soil community [51]. In addition, members of *Planctomycetes* can perform anaerobic oxidation of ammonium to di-nitrogen via the anammox pathway, which might correspond to greater denitrification functioning [52]. Moreover, *Chloroflexi* is often associated with carbohydrate and amino acid degradation [53]. Thus, consistent with the hypotheses, the use of CM in a specific legume-combined mixed cropping system (i.e., MM) enhanced the N-cycling gene abundance with an increase in microbial taxa involved in N-cycling.

Similar to previous studies, CF treatment facilitated legume nodulation in MM and PM more than in other fertilizer treatments due to its phosphorus and potassium content [54] (Table S3). In leguminous nodules, N-fixation is performed by members of the *Alphaproteobacteria* and *Betaproteobacteria* classes (e.g., *Rhizobium spp*. and *Paraburkholderia spp*.) [55]. However, we found no significant differences in *nitrogenase* gene abundance or in the abundance of *Alphaproteobacteria* and *Betaproteobacteria* between fertilizer types.

4.3. Effects of the Legume Varieties and Biochar Application on Soil Microbes

In this study, the variability of legume species significantly changed the effect of biochar application on the diversity, community, and N cycling function of soil prokaryotes. A recent study indicated that after biochar application, the microbial community was strongly affected by plant species [21,56], in agreement with our observation. Research on eight different legume accessions and the rhizosphere microbiome showed only 0.7% OTUs that were shared across all treatments, indicating a strong legume species-specific effect on the rhizosphere microbial community [57]. In addition, different legume species are known to have different types of genes that host specific rhizobium because of their co-evolution with symbiotic microorganisms [15].

In mixed cropping systems, the competition and complementary relationship of nutrients between cereals and legumes are key to promote microbial activity, which is important for P and N acquisition. For example, nutrient deficiency caused by mixed cropping systems facilitated the nodulation of legume and mycorrhizal transfers of P and N [58,59]. Additionally, coexisting maize can facilitate the nodule formation of legumes with flavonoids (signaling compounds for rhizobia) contained in root residues [60]. Biochar has a positive influence on the maize root exudate production and promotes the N fixation and N transfer from legume to maize [61], suggesting the complex effect of biochar application in the association with legume species and maize on the rhizosphere microbial community. Thus, future investigations should consider the effect of biochar in the context of the combination of crop species.

5. Conclusions

Our results demonstrated that the choice of legume species in the intercropping system is an important factor controlling the effect of biochar application on soil microbial diversity, community, and functions. The combination of biochar and common bean enhanced microbial diversity with the increase in the abundance of *Proteobacteria* and *Bacteroidetes*. On the other hand, biochar combined with velvet bean altered the soil microbial genes for *ammonia oxidation* and *nitrite reductase (NO-forming)*, but not the prokaryotic diversities. The increase in the relative abundances of *Thaumarchaeota*, *Planctomycetes*, *Verrucomicrobia*, and *Chloroflexi* was identified as a potential cause for the alteration of the N-cycling functional genes in velvet bean treatments. These results suggest that consideration of the legume-species-specific effect is necessary to optimize the positive effect of biochar on microbial diversity and functions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12101548/s1, Table S1: Selected genes were tested by two-way ANOVA, Table S2: Relative abundances (%) of phylum and classes, Table S3: Dry matter of plant shoots and roots, Table S4: 16S rRNA gene abundance, Figure S1: Rarefaction curve of all samples, Figure S2: Nonmetric multidimensional scaling plot based on Bray–Curtis dissimilarity for each fertilizer treatments.

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