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The Effects of Microbial Inoculants on Bacterial Communities of the Rhizosphere Soil of Maize

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Abstract: The bacterial community of rhizosphere soil maintains soil properties, regulates the microbiome, improves productivity, and sustains agriculture. However, the structure and function of bacterial communities have been interrupted or destroyed by unreasonable agricultural practices, especially the excessive use of chemical fertilizers. Microbial inoculants, regarded as harmless, effective, and environmentally friendly amendments, are receiving more attention. Herein, the effects of three microbial inoculants, inoculant M and two commercial inoculants (A and S), on bacterial communities of maize rhizosphere soil under three nitrogen application rates were compared. Bacterial communities treated with the inoculants were different from those of the non-inoculant control. The OTU (operational taxonomic unit) numbers and alpha diversity indices were decreased by three inoculants, except for the application of inoculant M in CF group. Beta diversity showed the different structures of bacterial communities changed by three inoculants compared with control. Furthermore, key phylotypes analyses exhibited the differences of biomarkers between different treatments visually. Overall, inoculant M had shared and unique abilities of regulating bacterial communities compared with the other two inoculants by increasing potentially beneficial bacteria and decreasing the negative. This work provides a theoretical basis for the application of microbial inoculants in sustainable agriculture.

Keywords: microbial inoculant; diversity; key phylotype; rhizosphere soil of maize; sustainable agriculture; bacterial communities' structure; microbial functional diversity

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1. Introduction

Bacterial communities of rhizosphere soil are of vital importance to the growth of field crops and agricultural productivity [1]. Beneficial bacterial communities that are integrated into host plants contribute to the appreciating cycle of soil nutrients and high nutrient use efficiency [2,3]. The growth of beneficial bacteria and the reduction in pathogens result from the interaction between the rhizosphere and roots of their host plants [4], which can simultaneously promote the growth of crops and enhance induced systemic resistance in host plants against pathogens, soil-borne diseases, and other environmental stresses caused by abiotic factors [5]. Appropriate bacterial structure and functions, which are associated with microbial diversity, are the key drivers that can maintain the microbial ecosystem of agricultural soil and sustainable development of agriculture [6].

However, the bacterial structure and function have changed due to current unreasonable agricultural practices implemented by human beings, including intensive cultivation, years of continuous cropping, and overuse of chemical fertilizers [7,8]. Among them,

the overuse of chemical fertilizers has brought environmental problems to agricultural ecosystems by destroying the physiochemical processes of the soil [9,10], especially, the excessive use of nitrogen fertilizer resulted in soil acidification, environmental pollution, unbalance of nutrient [11]. Additionally, it could affect the absorption of phosphorus by plants [12]. Consequently, strategies that address these obstacles and amend the broken structure of the microbiome and maintain its beneficial functions are imperative.

Microbial inoculants, regarded as a new type of soil amendment, have been focused on, mediating the structure and function of microbial communities in the soil [13,14]. Previous studies have paid attention to the abilities of individual bacterial strain [15], such as growth promotion [16], disease resistance [17], and improvement of fertilizer use efficiency [18]. Different application forms of bacterial inoculants, including solid and liquid formations, were researched in order to apply to different conditions [19,20]. Furthermore, some studies explored the effects of inoculants on plant growth at different working concentrations of the bacterial inoculants [21]. In addition, some pioneers have explored the mixed applications of bacterial inoculants combined with organic fertilizers and micro- or medium-nutrient fertilizers [22]. Different application effects have resulted from the diverse bacterial types (different phylum, genus, and species) contained in the microbial inoculants applied to the agricultural soil [23,24]. Generally, one bacterial inoculant is considered viable if its positive effects are greater than negative. The development of microbial inoculants with more beneficial effects and as little negative effect as possible, or even with no manifest negative effects, has received close attention and has been advocated by many researchers.

In this study, the effects of different microbial inoculants (including one made of bacterial strains that were screened in our lab, and two commercial inoculants) on bacterial communities of rhizosphere soil were investigated. To carry out our research conveniently, maize (*Zea mays* L.) was selected as our experimental crop because it is one of the most important food crops, ranked third in the list of the top three cereal crops in the world, besides wheat and rice [25]. Maize is widely planted in Central America, Mexico, Africa, and northeastern China, accounting for 94% of all cereal crop consumption along with wheat and rice, which satisfies the vast need for nutrients and nearly half the caloric requirement of humankind [26]. Although many beneficial bacteria had been studied and their traits had been verified in laboratory and pot experiments, research about the application of them in field is still scanty, where the functional strains could not go well in practices, coming across some obstacles as the applied environment was too complicated [27,28]. Herein, the effects of three microbial inoculants on the diversity of bacterial communities and key phenotypes of microbiome in maize rhizosphere soil were investigated to explore the modulating effects caused by different inoculants.

2. Materials and Methods

2.1. Screening and Identification of Bacterial Strains

Two bacterial strains were isolated from samples of maize plant soil in Jilin Province, China, using LB (Luria-Bertani) medium (tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 20 g, H_2O 1000 mL, pH 7.0–7.2; sterilized at 121 °C for 20 min), and screened via solubilizing phosphate experiment using NBRIP medium (glucose 10 g, $Ca_3(PO_4)_2$ 5 g, $MgCl_2$ 5 g, $MgSO_4$ 0.25 g, KCl 0.2 g, $(NH_4)_2SO_4$ 0.1 g, H_2O 1000 mL, pH 7.0; sterilized at 115 °C for 30 min. The two strains were identified as *Citrobacter amalonaticus* (GenBank number: MW362493) and *Bacillus safensis* (GenBank number: MW362494), respectively.

2.2. Preparation of Three Inoculants for Application of Field Experiment

Inoculant M was prepared by mixing the two bacterial strains screened above. The two strains were cultured in LB medium at 28 °C for 18–24 h, and they were mixed together at a ratio of 1:1 for application. Inoculant A was offered by Genliyuan Microbial Fertilizer Co. LTD (Hebei, China) and Inoculant S was provided by Otaqi Biological Products Co. LTD (Beijing, China). Inoculants A and S were commercial and patented products. Inoculant A mainly contained species of Actinomycetes, Bacillus, and Saccharomyces, as well as

some undescribed nitrogen-fixing bacteria and photosynthetic bacteria, while inoculant S contained not only living organisms but also some micro-nutrient such as Cu, Fe, Zn, Mn, and so on. However, detailed information of their composition was unknown. Inoculant A and Inoculant S did not need to be cultured beforehand, and they could be used directly according to the usage described in Table 1.

Table 1. All treatments and instructions of the field experiment.

No.	Treatments	Instructions
1	CF	Urea, 600.00 kg ha^{-1}; Calcium Superphosphate, 1000.00 kg ha^{-1}; Potassium Sulfate, 240.00 kg ha^{-1}
2	D20N	Urea, 480.00 kg ha^{-1}; Calcium Superphosphate, 1000.00 kg ha^{-1}; Potassium Sulfate, 240.00 kg ha^{-1}
3	D40N	Urea, 360.00 kg ha^{-1}; Calcium Superphosphate, 1000.00 kg ha^{-1}; Potassium Sulfate, 240.00 kg ha^{-1}
4	CF + M	
5	D20N + M	Inoculant M, 75.00 dm^3 ha^{-1}
6	D40N + M	
7	CF + A	
8	D20N + A	Inoculant A, 75.00 dm^3 ha^{-1}
9	D40N + A	
10	CF + S	
11	D20N + S	Inoculant S, 75.00 dm^3 ha^{-1}
12	D40N + S	

CF: conventional amount of nitrogen fertilizer; D20N: decrease of 20% nitrogen against conventional amount; D40N: decrease of 40% nitrogen against conventional amount; M: Inoculant M; A: Inoculant A; S: Inoculant S.

2.3. Conditions and Treatment Design of Field Experiment

The field experiment was conducted at the Institute of Plant Protection, Jilin Academy of Agricultural Sciences (Gongzhuling, Jilin Province, China; 43°31′52″ N, 124°49′31″ E, Figure 1) in 2018. Soil conditions of the field experiment are listed in Table S1, and the local climate was monsoon climate of medium latitudes. Twelve treatments, including three levels of nitrogen fertilizer and the three inoculants mentioned above, were used in this study (Table 1). Each treatment had four replications, and the area of every replication was 29.44 m^2 (6.4 m × 4.6 m).

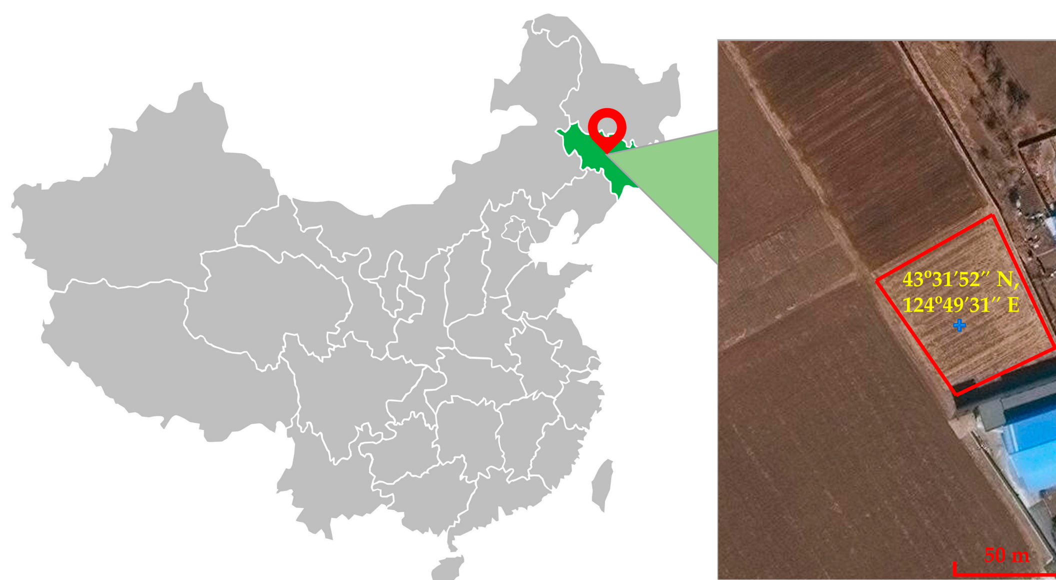

Figure 1. The location of field experiment. The area framed by the red line in the image was the experiment site. The numbers and letters in yellow color were the longitude and latitude of the experiment site.

The maize seeds ('Jidan 558') were provided by the Biological Pesticide Laboratory, Institute of Plant Protection, Jilin Academy of Agricultural Sciences. All the seeds were

sterilized in 10% H_2O_2 for 15 min, then washed in sterilized water three times [29], and then immersed in the different inoculants for 12 h. All seeds were sown on 30 April in a depth of 10 cm. Additionally, seeds were sown at a spacing between planting rows of 65 cm, and a spacing between plants of 23 cm. Thus, each replication had almost 150 maize plants.

2.4. Sample Collection

Plant samples were collected by the quadrat method, in which a 2 m^2 (2 m $\times$ 1 m) quadrat was utilized three times in each replication. Bulk soil used for physiochemical detection was collected when the plant samples were dug out. Soil laid in the hole of plant roots and soil dropping from roots were considered as bulk soil. Rhizosphere soil samples were collected after plants were carefully dug out with roots and gently shaken to discard excess soil. Only soil without any aggregates was regarded as rhizosphere soil, which was adhering to the roots very closely [30]. Soil sample was mixed by all collected quadrats in each replication, and quartering was used to acquire the appropriate amount of soil sample for further analyses, from which 0.5 g rhizosphere soil of each replication was used for DNA extraction.

2.5. DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

The MP DNA extraction kit (MP Biomedicals, LLC, Solon, OH, USA) was used for DNA extraction of rhizosphere soil samples according to the manufacturer's instructions. The V4-V5 region of the 16S rDNA gene was amplified from the bacterial DNA by PCR using barcode 515F (GTGCCAGCMGCCGCGG) and 907R (CCGTCAATTCMTTTRAGTTT) primers as described elsewhere [31]. The PCR amplification was tested by 1% agarose gel electrophoresis, colored by ethidium bromide for 40 min at 100 V.

2.6. Library Construction and Sequencing

The TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) was utilized in library construction, and the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and qPCR were utilized to quantify the libraries. Then, the libraries were sequenced at the Illumina MiSeq platform described as Zhang et al. [32]. All sequence data were submitted to the Sequence Read Archive (SRA: SRP297881) and are freely available at the NCBI (BioProject: PRJNA685114).

2.7. Data Processing and OTU Clustering and Annotation

All raw reads were treated by quality control and length trimming to achieve an accurate taxonomic assignment for each sequence. As a consequence, unqualified raw reads (including those containing ambiguous bases and those shorter than 200 bases) were removed along with primers and barcodes. Raw tags were generated from the qualified reads, which were assembled by FLASH (V1.2.7) (http://ccb.jhu.edu/software/FLASH/, accessed on 20 October 2020) [33]. Then, quality filtering of the tags was implemented by QIIME (V1.7.0) (http://qiime.org/scripts/split_libraries_fastq.html, accessed on 20 October 2020) [31]. The UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html, accessed on 21 October 2020) [34] was utilized to detect chimeras by checking the Gold database (http://drive5.com/uchime/uchime_download.html, accessed on 21 October 2020) [35], all chimeras were removed. Eventually, effective tags without chimeras were ready for further processing.

Subsequently, all the effective tags were clustered through Uparse (Version 7.0.1001) (http://drive5.com/uparse/, accessed on 24 October 2020) [36]. The effective tags were clustered into the same OTUs when their identity was no less than 97%. The OTUs with the highest frequency were chosen to be representatives of OTU sequences. The OTUs that only had one sequence were removed from the dataset because these special OTUs could be caused by sequencing errors. To further explore their functions, a representative sequence of each OTU was assigned to a taxonomic level using the RDP classifier [37].

MUSCLE (Version 3.8.31) (http://www.drive5.com/muscle/, accessed on 24 October 2020) was used to blasted all the OTUs, and MrBayes 3 was used to figure out the phylogenetic relationships. The comparison of the OTUs and bacterial communities under different treatments (different nitrogen application rates combined with different inoculants) were implemented by Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/, accessed on 25 October 2020) [38].

2.8. Bioinformatic Analyses

To explore the differences in richness and diversity of bacterial communities based on inner samples among different groups, after rarefaction, the OTU numbers and alpha diversity, which consisted of the observed species, Shannon, Simpson, Chao1, ACE, Good's-coverage, and PD_Whole Tree indices, were computed by QIIME. On the basis of phylogenetics, the PD_Whole Tree index was utilized to compute Faith's phylogenetic diversity metric. R software (Version 3.6.0, R Foundation for Statistical Computing, Vienna, Austria) was used to draw the rarefaction curves based on graphics, plot, and RColor-Brewer packages.

To further explore the differences in bacterial communities among all samples based on either inner or outer comparisons of different groups, beta diversity was implemented. Unifrac distance metrics were computed by software QIIME (Version 1.7.0) on the basis of an unweighted pair group method with arithmetic mean (UPGMA). The differences among all treatments were demonstrated through PCoA (principal coordinates analysis) and NMDS (nonmetric multidimensional scaling). Thereafter, PCoA, PCA (principal components analysis), and NMDS diagrams came from the vegan, ade4, and ggplot2 packages of R software (Version 3.6.0). To achieve a better perspective into the clustering of bacterial communities, the weighted (taking changes of relative taxonomic abundance into consideration), unweighted UniFrac metrics, and Bray–Curtis distance were utilized for the calculation of beta diversity [39]. Metastats analysis was performed at different taxonomic levels, using the permutation test between groups based on R software (Version 3.6.0) (The R Foundation for Statistical Computing, Vienna, Austria).

Since the alpha and beta diversities were explored, the key phylotypes of all treatments in the CF group were further researched via heatmaps, LefSe (LDA effect size) analysis, and histograms [40]. Heatmaps were operated and clustered by representative bacterial statistics of RDA (redundancy analysis)-identified OTUs. Thereafter, LefSe analysis was implemented by LefSe software on Novogene Platform (Beijing Novogene Technology Co., Ltd., Beijing, China). Histograms were drawn on the basis of the relative abundance of the top 40 species [41].

2.9. Statistical Analysis

Raw data were initially preprocessed by Microsoft Excel 2016, and the analyses of variance (ANOVA) were implemented using IBM SPSS statistics 25.0 software (SPSS, Inc., Chicago, IL, USA). Kruskal–Wallis test was used to calculate the p-value in usual analyses based on relative abundance of different taxa in all treatments. Tukey test was used to calculate the p-value in the analysis of Bray–Curtis distance. Permutational multivariate analysis of variance (PERMANOVA), based on vegan, was used for the comparison of bacterial communities of different treatments. Wilcoxon rank-sum test was used to measure the p-value in LefSe analyses. All diagrams and plots were drawn using Origin 2018 (OriginLab Corporation, Northampton, MA, USA) and R (Version 3.6.0), and all tables were drawn directly using Microsoft Word 2016. All data are presented as means $\pm$ standard deviation.

3. Results

3.1. Sequencing Results

Sequencing results of amplicon libraries contained samples from twelve treatments, provided 1,070,851 raw data, which was replaced by 1,067,020 after quality control with the

high-quality reads' average length of 374 bp. All high-quality reads were assembled, and OTUs were clustered from all qualified tags to study the species diversity of the treatments (Table S2).

3.2. Overview of Bacterial Taxonomic Composition

At the phylum level, the dominant phyla were Proteobacteria, Gemmatimonadetes, and Acidobacteria, accounting for 83.36%, 13.98%, and 2.66% of the total number of species, respectively. The top group at the class level was mainly composed of Gammaproteobacteria (with the number of 70.28%), unidentified Gemmatimonadetes (with the number of 13.98%), Alphaproteobacteria (with the number of 13.08%), and unidentified Acidobacteria (with the number of 2.66%). Xanthomonadales, unidentified Gammaproteobacteria, Gemmatimonadales, and Sphingomonadales were the dominant group at the order level, representing 32.82%, 27.02%, 13.98%, and 11.47%, respectively, followed by Aeromonadales (5.90%), Pseudomonadales (4.54%), unidentified Acidobacteria (2.66%), and Rhizobiales (1.61%). At the family level, Rhodanobacteraceae, unidentified Gammaproteobacteria, Gemmatimonadaceae, and Sphingomonadaceae formed the main group, with 32.82%, 23.75%, 13.98%, and 11.47%, while Aeromonadaceae (5.90%), Moraxellaceae (4.54%), Burkholderiaceae (3.28%), unidentified Acidobacteria (2.66%), and Beijerinckiaceae (1.61%) provided 17.99% of the total community in all treatments. When it occurred to genera, the abundances of *Chujaibacter*, unidentified *Gammaproteobacteria*, *Gemmatimonas*, *Sphingomonas*, and *Rhodanobacter* were higher than those of other genera (Figure 2).

3.3. Dissimilarity of Bacterial Communities in Different Treatments

The comparison of the OTUs of the different inoculants combined with different nitrogen application rates illustrated that the CF group (including treatment CF, CF.M, CF.A, CF.S) shared 1897 common OTUs, and that treatments CF, CF.M, CF.A, CF.S owned 339, 514, 284, and 234 unique OTUs, respectively. When it came to the D20N group (including D20N, D20N.M, D20N.A, D20N.S), 2121 common OTUs were shared. They had 365, 286, 323, and 227 unique OTUs in D20N, D20N.M, D20N.A, and D20N.S, respectively. In addition, 1717 common OTUs were shared in the D40N group including D40N, D40N.M, D40N.A, and D40N.S. The number of unique OTUs was 402, 238, 142, and 1584 in D40N, D40N.M, D40N.A, and D40N.S, respectively (Figure 3A).

The variation in the OTUs in the CF group increased from CF to CF.M, and then decreased from CF.M to CF.A and CF.S. The D20N group had a similar tendency as the CF group in terms of using or not using inoculants, but the difference between these two groups was that CF.M had more OTUs than CF.A, while it was opposite in the D20N group. However, the performance of OTUs in the D40N group decreased from D40N to D40N.A (via D40N.M) and increased at D40N.S (Figure 3B). Taking all of these results into consideration, although the bacterial communities showed different pattern in the D40N group compared with the CF and D20N groups, it was obvious that the diversity of bacterial communities tended to decrease with the utilization of inoculants except Inoculant M and Inoculant S, which were used in the CF and D40N groups, respectively. In the D20N group, the effect of inoculants on reducing the diversity of bacterial communities was weakened. Furthermore, the effects of Inoculant M on bacterial communities were the largest in the CF group based on the OTU richness.

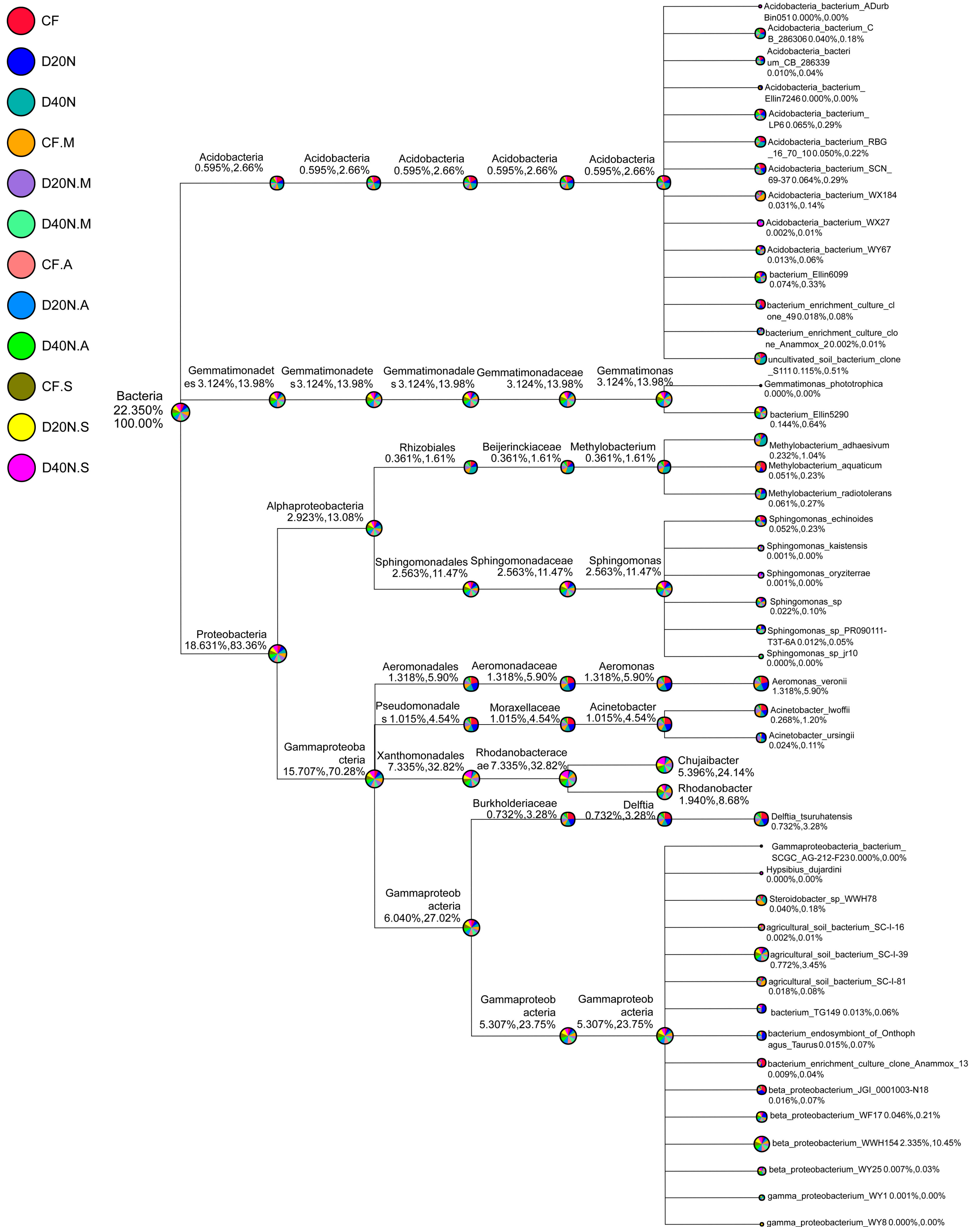

Figure 2. Taxonomic tree of bacterial communities of rhizosphere of maize from twelve treatments.

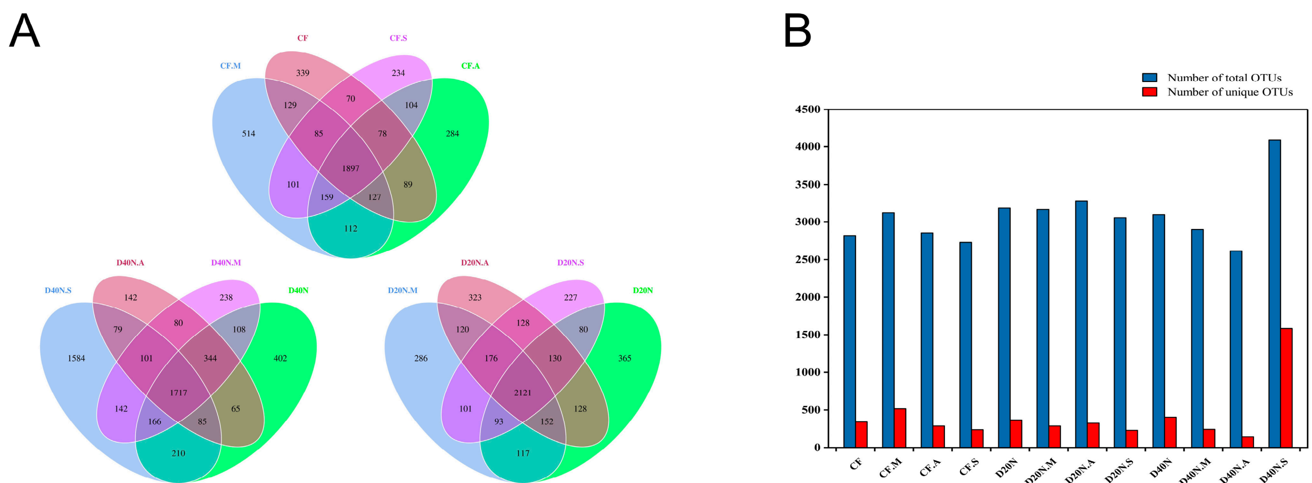

Figure 3. Comparison of bacterial communities in different treatments. (**A**) Venn diagram for treatments among different combinations. Every circle indicates a treatment, the numbers of OTUs shared between different treatments was interpreted with the number in the overlapping circles, while the number in the non-overlapping area represented the number of unique OTUs of the specific treatment. (**B**) Statistics of the OTU numbers in different treatments. Total OTUs referred to all the OTUs in a certain treatment. Unique OTUs were the ones (in a certain treatment) that were exclusive compared with other treatments.

3.4. Alpha Diversity

According to the results of the rarefaction curves, CF.M had the highest abundance of bacterial communities in the CF group (Figure 4A), whereas D20N had the highest abundance of bacterial communities in the D20N group (Figure 4B). When it came to the D40N group, the highest abundance occurred in D40N.S (Figure 4C), which was consistent with the results of OTU numbers (Figure 3B). The number of observed species was highest in D40N.S samples at 2449.25 ± 135.71, followed by D20N, D20N.M, and CF.M. The highest index value of Shannon appeared in D20N (8.98 ± 0.45), followed by D20N.M, and CF.M. On the contrary, the lowest Shannon index value was 8.43 ± 0.64, occurring in the D40N.S samples. D40N.S had the lowest Simpson value as well. In addition, D40N.S had the highest value for both the Chao 1 index and ACE index, followed by CF.M, D40N, and D20N.M, respectively, where the main difference was that CF.M was ranked second in the Chao 1 index, while D40N was ranked second in the ACE index. The PD_Whole Tree indices of all treatments ranged from 147.05 (D40N.A) to 202.65 (D40N.S). D20N, D20N.M, and CF.M were listed behind D40N.S, based on the PD_Whole Tree values. Compared with other indices of alpha diversity, little variation was found in Good's coverage of all treatments. The results indicated that different inoculants changed the alpha diversity of bacterial communities at different nitrogen application rates (p-value < 0.05, tested by Duncan multiple range test, DMRT).

In addition, a trend was found that the diversity of bacterial communities declined in most inoculant-applying treatments in the different nitrogen application rate groups, compared with their own control (CF, D20N, and D40N) in the corresponding groups. Two exceptions were discovered: one was CF.M in the CF group, and the other was D40N.S in the D40N group, whose diversity of bacterial communities was enhanced by Inoculant M and Inoculant S, respectively. The results of alpha diversity were consistent with the statistics of the OTU numbers and their Venn diagrams (Figure 3). The performance of Inoculant M in the CF group (CF.M) was different among all treatments (p-value < 0.05, tested by DMRT). As a consequence, in order to further explore the effects of different inoculants on bacterial communities, we focused on CF group (CF, CF.M, CF.A, CF.S) in the subsequent analyses.

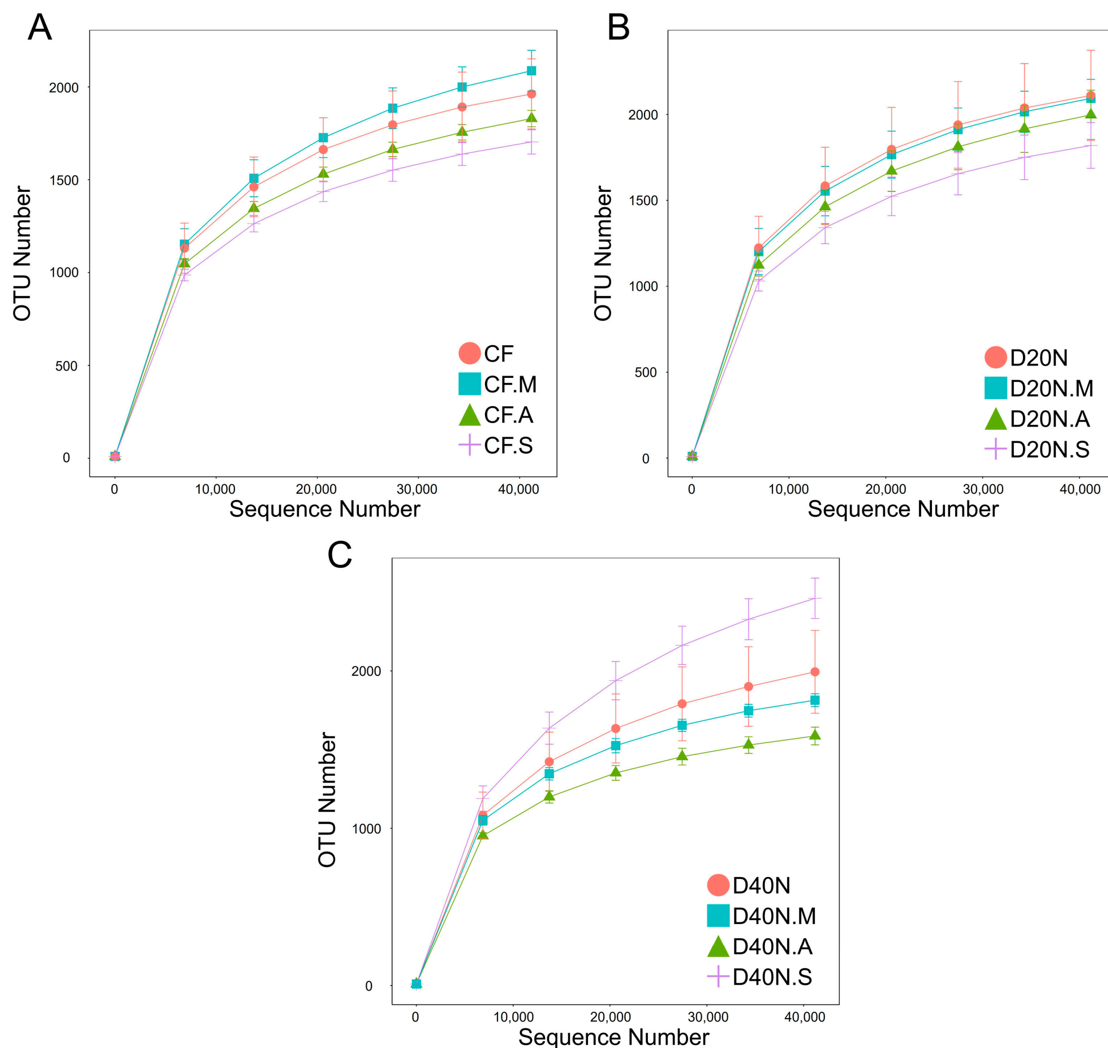

Figure 4. Rarefaction curves of bacterial communities in all treatments. (**A**) Rarefaction curve of treatments in CF group. (**B**) Rarefaction curve of treatments in D20N group. (**C**) Rarefaction curve of treatments in D40N group. CF: conventional fertilizer, D20N: decrease of 20% nitrogen, D40N: decrease of 40% nitrogen.

3.5. Beta Diversity

The results of the PCoA based on the unweighted Unifrac distances indicated that the bacterial communities of CF and CF.M were separate. Evident separations between the communities of CF.M and CF.A, CF.M and CF.S, CF and CF.A, and CF and CF.S exist (Figure 5A). The highest variations in the microbiome of different treatments represented a strong separation between different utilizations of inoculants and their control, except that the communities of CF.A and CF.S were clustered very well. The results of the PCA, which were plotted on the basis of OTU levels, showed a similar trend to that of PCoA (Figure 5B). NMDS analysis indicated that different microbial inoculants played an important role in shaping the bacterial communities in soil samples of the maize rhizosphere. The stress of NMDS analysis was 0.115, which is regarded as a good model in representing the differences among all treatments. There were high similarities in bacterial communities between the CF.A and CF.S samples, whereas they were both separated from CF.M and CF. The cluster of CF.M samples and CF samples were separated (Figure 5C). The Bray–Curtis distance demonstrated that the CF.M samples had the highest variation among all samples. A trend was detected where the diversity of the bacterial community in the CF.M samples was enhanced compared with CF, while CF.A and CF.S had little variation between each other (Figure 6). Interestingly, the bacterial communities, based on the Bray–Curtis

distances, were highly similar between CF.A and CF.S (p-value = 0.9031, through Tukey test), except for CF and CF.M (p-value = 0.0396, through Tukey test), CF and CF.A (p-value = 7.21 $\times 10^{-6}$, through Tukey test), CF and CF.S (p-value = 1.80 $\times 10^{-6}$, through Tukey test), CF and CF.A (p-value = 0.0046, through Tukey test), and CF.M and CF.S (p-value = 0.0010, through Tukey test), which illustrated that the bacterial communities of these treatments were different (Table S3). Additionally, based on the results of PERMANOVA, CF.M, CF.A, and CF.S samples had significantly different bacterial communities from the CF samples. Moreover, the bacterial communities of CF.M were significantly different from those of CF.A and CF.S, respectively, while the last two were similar (Table S4).

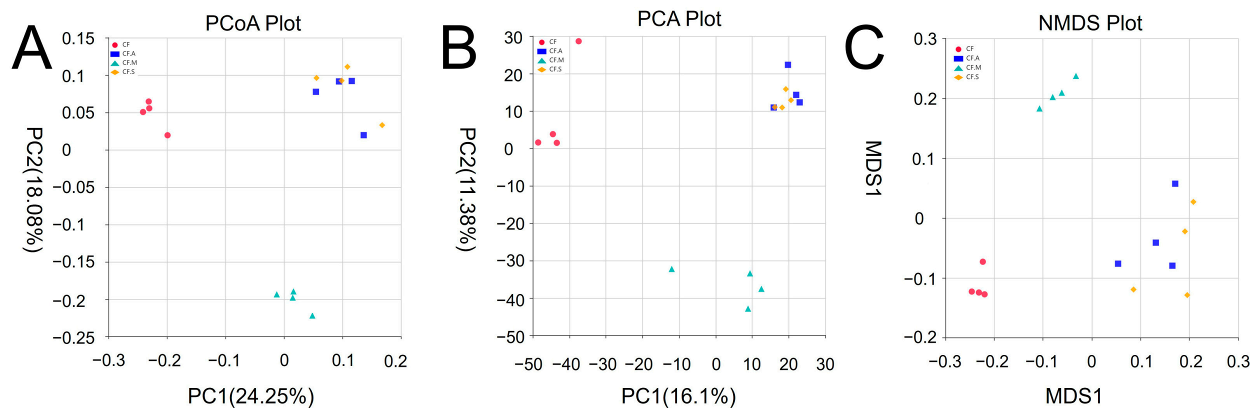

Figure 5. The beta diversity indices of the CF group (CF, CF.M, CF.A, CF.S) of the maize rhizosphere. (**A**) Principal coordinate analysis (PCoA) based on the unweighted Unifrac distances. (**B**) Principal components analysis (PCA) based on operational taxonomic unit (OTU) levels. (**C**) NMDS analysis results based on the unweighted Unifrac distances.

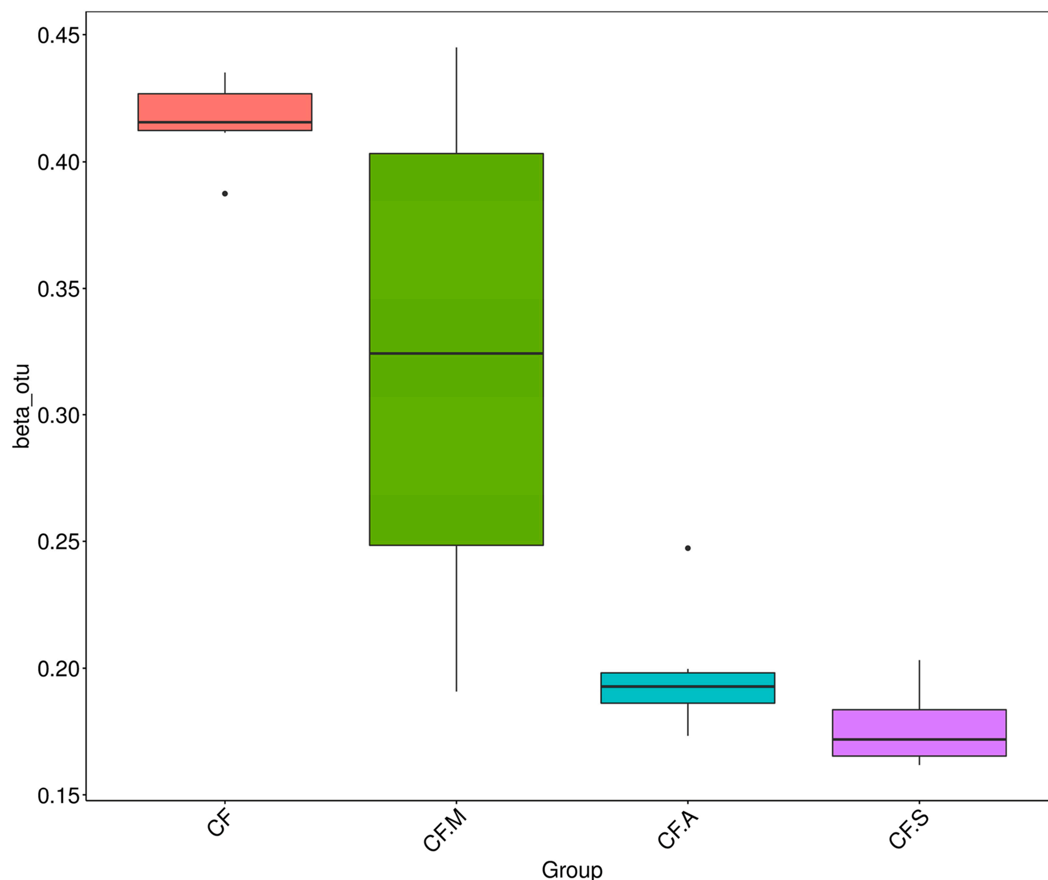

Figure 6. Boxplots of beta diversity based on the Bray–Curtis distances.

3.6. Inoculant M Mediated the Key Phylotypes of the Rhizosphere Microbiome of Maize

Since the effect of inoculant M on the bacterial community in maize rhizosphere soil was significantly different from that of inoculants A and S, and the control (CF), key phylotypes were explored to further understand the microbiome in maize rhizosphere soil and the specific changes of bacterial communities caused by different treatments. From the results of the heatmaps, it was obvious that the variations in species were similar between CF.A and CF.S, but was significantly different from CF at both the phylum and genus levels (Figure 7). Interestingly, the variation in the structure of the bacterial community in CF.M differed from that of CF.A, CF.S, and CF. The phenomenon was observed where the variation of key phylotypes in CF.M was between CF.A and CF.S, and CF at the phylum level (Figure 7A). At the genus level, key phylotypes in CF.M distinguished them further (Figure 7B).

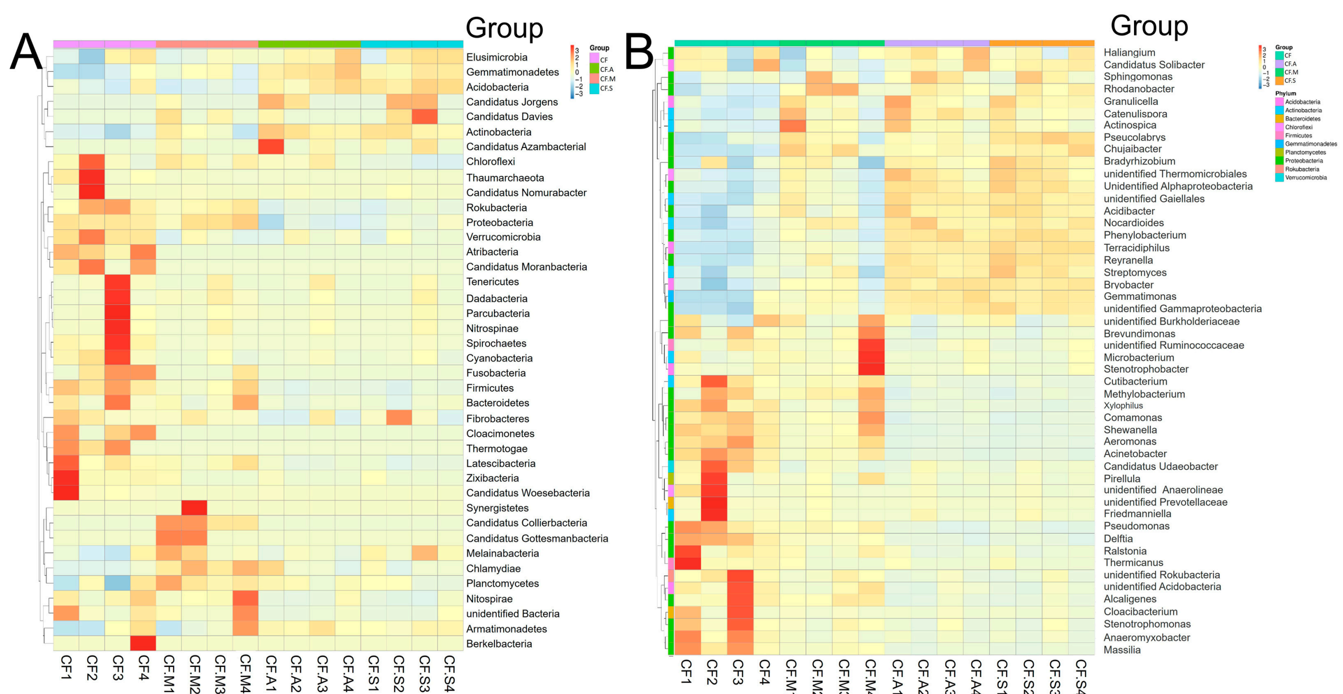

Figure 7. Heatmaps based on representative bacterial statistics of RDA-identified OTUs in the CF group (CF, CF.M, CF.A, CF.S). (**A**) Heatmaps based on representative bacterial statistics of RDA-identified OTUs in the CF group at the phylum level. (**B**) Heatmaps based on representative bacterial statistics of RDA-identified OTUs in the CF group at the genus level.

Different biomarkers were found in different treatments based on LefSe analysis (Figure S1 and Table S5). At the phylum level, the biomarker of CF was Firmicutes, while the biomarker of CF.M was Proteobacteria, the biomarkers of CF.A were Actinobacteria and Gemmatimonadetes, and the biomarker of CF.S was Acidobacteria. When it came to genus level, the biomarkers of CF were *Aeromonas* and *Acinetobacter*. In contrast, the biomarkers of CF.M were *Rhodanobacter* and *Chujaibacter*, and the biomarkers of CF.A and CF.S were *Gemmatimonas* and unidentified *Gammaproteobacteria*. In addition, we selected the top 40 species shared in all treatments of the CF group at the genus level to investigate the differences in relative abundance among these treatments (Figure 8). The abundance of *Pseudolabrys*, *Terracidiphilus*, *Granulicella*, *Phenylobacterium*, *Gemmatimonas*, and *Rhodanobacter* were increased in CF.M, CF.A, and CF.S, compared with CF. Nevertheless, the abundance of *Ralstonia*, *Xylophilus*, and *Comamonas* were decreased in CF.M, CF.A, and CF.S (Table S6).

Interestingly, we found that the relative abundance of the genus *Dietzia* was significantly (p-value < 0.05, through Kruskal–Wallis test) increased only in CF.M while the number of other three treatments was zero. Furthermore, the relative abundance of *Rhodovastum* was significantly (p-value < 0.05, through Kruskal–Wallis test) higher in Inoculant M than

CK whereas there was no significant difference between CF.A, CF.S, and CF (Table S7). When it came to *Granulicella*, the numbers had significant (p-value < 0.05, through Kruskal–Wallis test) differences between three inoculants treatments and CF, whereas there was no significant difference in relative abundance of *Granulicella* among these three inoculants. Additionally, both CF.A and CF.S had higher relative abundance of *Gemmatimonas* than CF.M (p-value < 0.05, through Kruskal–Wallis test), and that of CF.M was significantly higher (p-value < 0.05, through Kruskal–Wallis test) than CF (Figure 9).

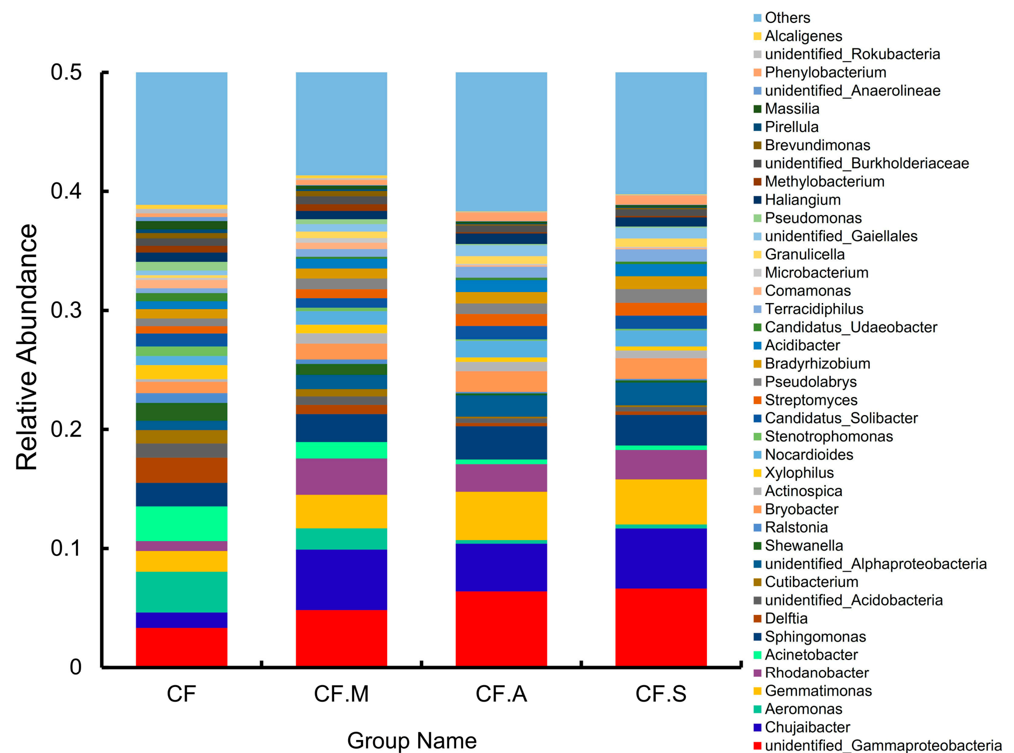

Figure 8. The relative abundance of top40 genera in the CF group.

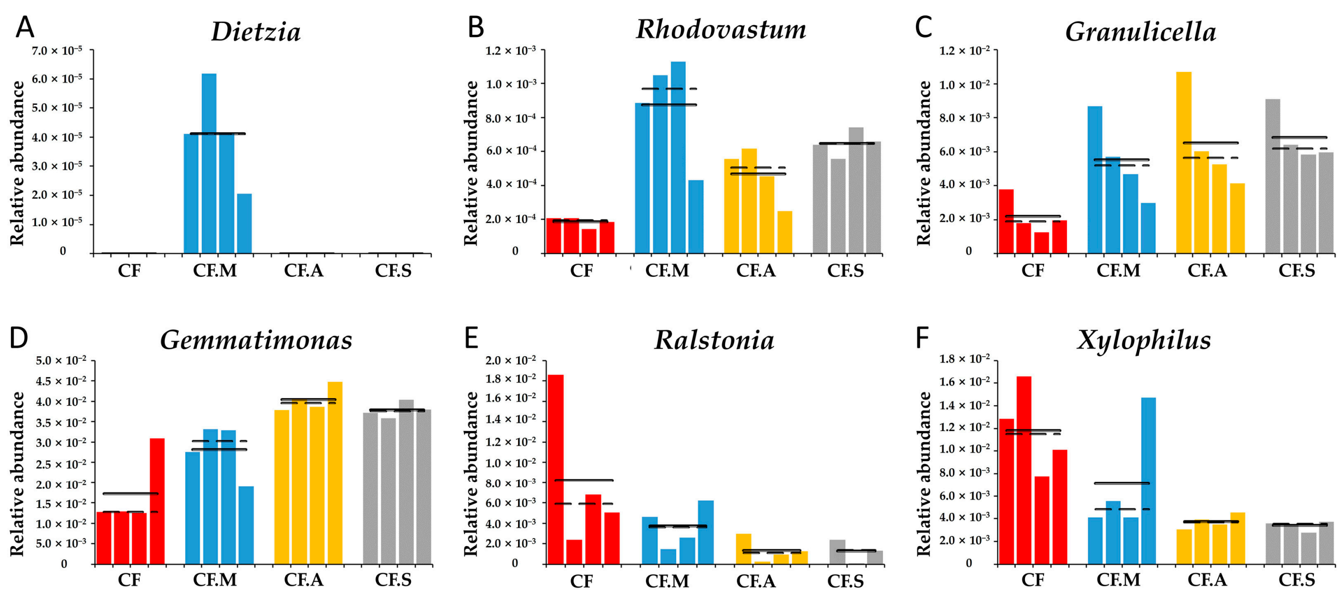

Figure 9. The relative abundance of *Dietzia* (**A**), *Rhodovastum* (**B**), *Granulicella* (**C**), *Gemmatimonas* (**D**), *Ralstonia* (**E**), and *Xylophilus* (**F**) in the rhizosphere microbiome based on results of LefSe analysis. Solid and dashed lines indicated the means and medians, respectively.

4. Discussion

The bacterial community of rhizosphere soil is known to be associated with the status of agricultural soil: whether it is nutrient efficient [42], whether the elements are conveniently available for plants [43], whether it is sufficient for fertility [44], and whether it is sensitive to pathogens [45]. Apart from reflecting the status of the soil, the bacterial community can influence and change the abiotic and biotic properties of soil [46,47] in return for the habitat (matter and energy) provided by their hosts [48]. With the negative variation in and destruction of bacterial communities resulting from unreasonable agricultural practices such as excessive use of nitrogen fertilizers [49], the physiochemical properties of soil have declined along with biotic factors [50]. To solve this issue, microbial inoculants have been focused on to mediate the microbiome adhering to the roots of host plants. Based on the urgent requirement of microbial regulators, inoculants with more specific and efficient abilities are being sought. It was found that these three inoculants modulated the bacterial communities to better structural and functional formations for maize production, compared with non-inoculant control. Moreover, among all results, the performance of Inoculant M in the CF group (CF, CF.M, CF.A, and CF.S) proved to be significantly different from that of Inoculants A and S based on OTU richness, species abundance, diversity analyses (alpha diversity and beta diversity), and key phylotypes analysis. This suggests that the regulatory effect of Inoculant M on the microbiome was unique to that of Inoculants A and S. Inoculant M is a promising modulator which can improve bacterial communities in maize rhizosphere soil for agricultural practice.

Combining the summaries of alpha (Table 2 and Figure 3) and beta diversity (Figure 5, Figure 6, Tables S3 and S4), Inoculant M could shape the bacterial community into a differential structure compared by other two inoculants [51]. Previous studies have shown different effects of single bacterial strains and inoculants consisting of several (mostly no more than five) bacterial strains on microbial communities [52–54]; however, few have paid attention to the comparison between simple inoculants (mainly consisting of single, two, or three strains) and commercial inoculants referring to complex compositions, and between commercial inoculants themselves. Zhong et al. found that different inoculants led to different assemblies of the microbiome [55]. However, in this study, Inoculant A and Inoculant S led to similar bacterial communities. One of the conjectures was whether the formulae of Inoculants A and S were homologous [56]. The responses of bacterial communities and plants to the application of microbial inoculants are dependent on plant and bacterial genotypes as well [57]. Another hypothesis regarding Inoculant A and Inoculant S was that the formulas could have been different, but were rich enough or sufficiently complex that they provided more than the fundamental requirement of the soil, which might eventually result in a similar microbiome. This hypothesis needs to be tested further by comparison of complex inoculants. Putting the similarity between CF.A and CF.S aside, Inoculant M had unique effects on shaping bacterial communities in the study.

Table 2. Statistic results of alpha diversity indices.

Sample Name	Observed Species	Shannon	Simpson	Chao1	ACE	Goods Coverage	PD Whole Tree
CF	$1953.75 \pm 213.81^{b,c,d}$	8.87 ± 0.31	0.99 ± 0.00^{b}	$2104.32 \pm 217.44^{b,c,d}$	$2235.96 \pm 223.64^{b,c,d}$	$0.99 \pm 0.00^{d,e}$	$182.44 \pm 15.05^{c,d,e}$
CF.M	$2076.50 \pm 125.05^{c,d}$	8.88 ± 0.11	0.99 ± 0.00^{b}	2293.59 ± 99.03^{d}	2474.94 ± 127.62^{d}	$0.99 \pm 0.00^{b,c}$	$186.97 \pm 9.66^{d,e}$
CF.A	$1820.25 \pm 57.16^{a,b,c}$	8.73 ± 0.09	0.99 ± 0.00^{b}	$1973.39 \pm 62.99^{a,b,c}$	2149.73 ± 73.83^{bc}	$0.99 \pm 0.00^{d,e}$	158.04 ± 4.42^{ab}
CF.S	$1705.75 \pm 82.42^{a,b}$	8.57 ± 0.06	0.99 ± 0.00^{b}	$1854.81 \pm 96.38^{a,b}$	$2009.90 \pm 117.01^{a,b}$	$0.99 \pm 0.00^{d,e}$	152.14 ± 4.74^{a}
D20N	2104.00 ± 299.20^{d}	8.98 ± 0.45	0.99 ± 0.00^{b}	$2237.29 \pm 310.51^{c,d}$	$2373.10 \pm 302.13^{c,d}$	$0.99 \pm 0.00^{d,e}$	196.57 ± 22.35^{e}
D20N.M	$2089.50 \pm 124.42^{c,d}$	8.90 ± 0.61	$0.99 \pm 0.01^{a,b}$	2252.00 ± 94.90^{d}	$2421.19 \pm 47.15^{c,d}$	$0.99 \pm 0.00^{c,d}$	192.34 ± 14.26^{de}
D20N.A	$2009.50 \pm 170.35^{c,d}$	8.84 ± 0.14	0.99 ± 0.00^{b}	$2186.80 \pm 173.65^{c,d}$	$2374.97 \pm 177.58^{c,d}$	$0.99 \pm 0.00^{c,d}$	$174.18 \pm 16.00^{b,c,d}$
D20N.S	$1821.00 \pm 154.70^{a,b,c}$	8.57 ± 0.12	$0.99 \pm 0.00^{a,b}$	$1964.32 \pm 162.53^{a,b,c}$	$2130.63 \pm 174.49^{b,c}$	$0.99 \pm 0.00^{d,e}$	$162.33 \pm 15.15^{a,b,c}$
D40N	$1988.75 \pm 308.07^{c,d}$	8.78 ± 0.34	0.99 ± 0.00^{b}	2274.14 ± 331.58^{d}	2494.88 ± 378.75^{d}	0.99 ± 0.00^{b}	$182.94 \pm 22.65^{c,d,e}$
D40N.M	$1814.75 \pm 52.69^{a,b,c}$	8.74 ± 0.14	0.99 ± 0.00^{b}	$1968.25 \pm 53.76^{a,b,c}$	$2130.81 \pm 75.93^{b,c}$	$0.99 \pm 0.00^{d,e}$	$159.83 \pm 5.75^{a,b}$
D40N.A	1587.75 ± 86.92^{a}	8.47 ± 0.12	$0.99 \pm 0.00^{a,b}$	1713.85 ± 102.57^{a}	1838.79 ± 113.34^{a}	0.99 ± 0.00^{e}	147.05 ± 6.84^{a}
D40N.S	2449.25 ± 135.71^{e}	8.43 ± 0.64	0.98 ± 0.02^{a}	2803.00 ± 38.66^{e}	3184.54 ± 67.32^{e}	0.98 ± 0.00^{a}	202.65 ± 7.91^{e}

All data in the text and tables are presented as means ± standard deviation (SD). Means followed by the same lower-case letter are not significantly different at the 5% level by DMRT (Duncan multiple range test).

To further understand the significantly different genera between different treatments, key phylotype analysis was implemented and discussed [58]. CF.M showed obvious different structure of bacterial community from CF and other two inoculants through heatmaps (Figure 7A,B). From the LefSe analysis (Figure S1 and Table S5), the genera whose LDA were larger than 4 were discussed as biomarkers of different treatments. It was reported that the biomarkers of CF, *Aeromonas* and *Acinetobacter* were severe pathogens [59,60]. When it came to the biomarkers of CF.M, CF.A, and CF.S, *Rhodanobacter* and *Gemmatimonas* were reported to have the ability to improve the circulation of nitrogen in soil. Little information about *Chujaibacter* could be found in the literature, but one investigation mentioned that it could survive in variable salinity conditions by degrading organic matter as a basis for utilizing *N*-acetylglucosamine [61]. Demonstrated by relative abundance statistics of the top 40 genera in all treatments (Figures 7B and 8), many beneficial genera were increased by Inoculant M, Inoculant A, and Inoculant S, such as *Pseudolabrys*, *Terracidiphilus*, *Granulicella*, *Phenylobacterium*, *Gemmatimonas*, and *Rhodanobacter*. Among them, *Pseudolabrys*, *Terracidiphilus*, *Granulicella*, and *Phenylobacterium* were found to have positive correlations with solubilizing phosphate in soil. *Pseudolabrys* had been reported to secrete naphthol-AS-BI-phosphohydrolase [62], *Terracidiphilus* and *Phenylobacterium* can both secrete alkaline phosphatase (ALP) [63,64], and *Granulicella* can produce ALP, acid phosphatase (ACP), and naphthol-AS-BI-phosphohydrolase simultaneously [65]. With all the enzymes mentioned above, the process of solubilizing phosphate can proceed smoothly. Additionally, Park et al. revealed that *Gemmatimonas* can denitrify and break down lignin and cellulose [66]. *Rhodanobacter* was found to participate in the process of denitrification by Van et al. [67] as well. The genera mentioned above were almost all beneficial bacteria associated with nutrient uptake, plant growth-promotion, and denitrification, which was partly consistent with the results of the LefSe analysis. Meanwhile, some negative bacteria (i.e., potential plant pathogens), including *Ralstonia* [68], *Xylophilus* [69], and *Comamonas* [70], were decreased by the three inoculants. Except for the common variations among three inoculants and CF, some special differences were explored in genera *Dietzia* and *Rhodovastum*, whose relative abundances were significantly (p-value < 0.05) increased only by Inoculant M (Figure 9). Bharti et al. found that Dietzia could promote the growth of wheat and protected wheat from salt stress by secreting various enzymes and other molecule organics [71]. Rhodovastum was reported to be a photo-organotrophic bacterium, which was regarded as a beneficial bacterium to plants [72]. Inoculant M modulated the key phylotypes of the microbiome not only by improving the beneficial bacteria as with Inoculant A and Inoculant S, but also enhanced some advantageous bacteria, whose variations were unique to the other two inoculants. This suggests that Inoculant M has unique functions in mediating bacterial communities of maize rhizosphere soil, which makes Inoculant M potentially applicable in maize production. It should be pointed out that the use of microbial inoculants will not cause an increase in production cost. As we all know, cost control is an important part of agricultural production, and the cost of implementing the technology is the basis for its application. The maize yields of CF.M, CF.A, and CF.S were all significantly higher (p-value < 0.05, tested by DMRT) than CF. Furthermore, the maize yield of CF.M was significantly higher (p-value < 0.05, tested by DMRT) than CF.A and CF.S, while there was no significant difference between CF.A and CF.S (Table S8). Nevertheless, the cost of treatment is hard to obtain, since the Inoculant A and Inoculant S were provided freely by corresponding companies for scientific research. We can only calculate the cost of Inoculant M, which is no more than 750 rmb ha^{-1}. Detailed cost accounting of using these microbial inoculants is currently needed, which will provide better application potential of this technology.

5. Conclusions

In this study, all three inoculants were able to shape the bacterial communities of maize rhizosphere soil into improving assemblies by increasing potentially beneficial bacteria and decreasing the harmful bacteria, as compared to the non-inoculant control. In particular, Inoculant M showed shared and unique abilities to modulate bacterial communities

compared with the other two inoculants, proving that Inoculant M is promising for application in agricultural practices in the future. This study provides data support for the mediation of the microbial community of maize rhizosphere soil by microbial inoculants and a theoretical basis for the application of microbial inoculants in the green, healthy, and sustainable development of agriculture. This article focused on the effects of different inoculants on bacterial communities of maize rhizosphere soil, moreover, the effects of these inoculants on fungal communities and nematode communities should be further researched.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11050389/s1, Figure S1: LDA (Linear Discriminant Analysis) plot of LefSe anlysis among different treatments. Table S1: Soil conditions of the field experiment, Table S2: Statistics of the sequencing results, Table S3: Bray-Curtis results, Table S4: PERMANOVA results of bacterial communities treated by different treatments, Table S5: Statistic results of LefSe analysis, Table S6: Relative abundance of top40 genera, Table S7: OUT table, Table S8: The yields of maize in different treatments.

Author Contributions: Y.D., J.L. and M.S. designed the experiment; M.S., Y.Z., Z.Z., K.D., J.P., H.L. and Q.L. performed the in vitro and field experiments; M.S. analyzed the data; M.S. and J.L. interpreted the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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