Thirty-one years of rice-rice-green manure rotations shape the rhizosphere microbial community and enrich beneficial bacteria

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A B S T R A C T

Green manure rotation is commonly used to increase soil fertility and improve crop yield. However, the effects of this management practice on the underground microbial ecosystem and the indirect impact on the aboveground crop growth have not been systematically analysed. In this study, we investigated the rice rhizosphere and bulk soil microbial community in a 31-year-old field experimental site treated with different green manures and rice rotations using both 16S rDNA high-throughput sequencing and quantitative PCR approaches. Four treatments have been setup in this experimental site since 1982, including a rice-rice-winter fallow treatment as a control and three green manure rotation treatments: rice-rice-Chinese milk vetch, rice-rice-rape and rice-rice-ryegrass. The qPCR results showed that the bacterial abundances in the rice rhizosphere of the green manure rotation treatments were all significantly higher than in the winter fallow (p < 0.05), but no significant differences were found among those three green manure rotation treatments. Moreover, a-diversity analysis revealed that green manure rotations decreased the microbial diversity (Shannon and Simpson indexes) and richness (Chao value) in the rice rhizosphere. Permutational Multivariate Analysis of Variance based on b-diversity revealed the microbial community was significantly switched in rice rhizosphere after long-term green manure rotation (p < 0.01). Additionally, the soil and plant characteristics contributed almost equally to the rhizosphere bacterial community based on a partial CCA-based variation partitioning analysis. At the genus level, the well-known plant-growth-promoting rhizobacteria Acinetobacter (31%–41%) and Pseudomonas (14%–28%) were the preponderant groups in green manure rotation treatments but accounted for only 4.4% and 2.5% in the winter fallow treatment. Overall, long-term rice-rice-green manure rotation shaped the microbial community in the rice rhizosphere; in particular, some beneficial bacteria, Acinetobacter and Pseudomonas, accumulated in the rhizosphere of green manure treatments.

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1. Introduction

The application of green manure to paddy soil is considered a good management practice in agricultural production systems because it can increase soil sustainability by reducing soil erosion, ameliorating soil physical properties (MacRae and Mehuys, 1985), and increasing the soil organic matter, fertility (Biederbeck et al., 1998) and nutrient retention (Dennis et al., 2010; Drinkwater et al., 1998). It can also reduce the occurrence of plant diseases and insect pests (Larkin and Griffin, 2007; Naz et al., 2015). In addition, a cover crop such as milk vetch with a low C/N ratio may be a more favourable green manure since it minimizes the impact of CH4 emissions and increases the rice productivity (Kim et al., 2012). For example, it was reported that a 28-year rice-green manure rotation increased rice yields by 18%–27% (Gao et al., 2013). Biological processes such as plant litter decomposition, gas
emissions, soil fertility transformation and crops nutrition absorption require soil microbial activity; however, the knowledge about the effect of green manure rotations on microbial communities is still limited.

It is well known that soil microbial diversity is critical for maintaining the sustainability of an agricultural production system (Bending et al., 2004), but only a few studies have examined the effect of green manure on soil microbes by classical soil biological methods. Tejada et al. (2008) found that the soil microbial biomass, soil dehydrogenase, urease, β-glucosidase, phosphatase and aryl-sulfatase activities were significantly increased by amendment with green manure. Piotrowska and Wilczewski (2012) came to the similar conclusion that green manure rotation was a useful management practice for enhancing soil biological activity as evaluated by enzymatic activity. Stark et al. (2007) found that the addition of green manure improved the microbial biomass and activity and changed the soil microbial community significantly during the early days of the experiment. The soil microbial population was improved by a legume green fallow and cereal cropping system after 6 years based on a cultivable colony-count method (Biederbeck et al., 2005). A 26-year long-term planting of winter green manure significantly improved the soil microbial biomass carbon, microbial biomass nitrogen and enzyme activities in red-dish paddy soil (Yang et al., 2011). Zhang et al. (2013) revealed significant effects of a long-term rotation of rice with milk vetch as a green manure on the endophytic microbial community using both culture-dependent and clone library methods. All of these studies suggested that soil amendment with green manure increases the soil microbial activity. However, these existing studies mainly used indirect methods such as biomass and enzyme activities or low throughput methods such as DGGE and clone library. All of these studies suggested that soil amendment with green manure increases the soil microbial activity. However, these existing studies mainly used indirect methods such as biomass and enzyme activities or low throughput methods such as DGGE and clone library. Overall, the impact of green manure treatment on bacterial population dynamics and crop yields remains unclear.

The rhizosphere is the critical zone of soil surrounding the plant roots (Dennis et al., 2010). Microbes are more active in this “hot spot” in terms of higher abundance, more interactions and greater genetic exchange (Bulgarelli et al., 2013). The rhizosphere also attracts a variety of plant-associated bacteria from the soil environment, such as plant growth-promoting Rhizobacteria (PGPR) (Compart et al., 2010). Rhizosphere microbial communities are more closely associated with plant growth than those in bulk soil. Members of the genera Bacillus, Pseudomonas, Enterobacter, Acinetobacter, Burkholderia, Arthrobacter and Paenibacillus are the common PGPRs in the rhizosphere (Jha et al., 2009; Karagoz and Ates, 2012; Santojo et al., 2016; Trotel-aziz et al., 2008). The application of green manure has been shown to improve soil quality and crop yields; however, the effects on the microbial communities in the rhizosphere are still unknown.

The application of green manures to rice fields is a traditional agricultural practice in China. Long-term green manure and rice rotation experiments were set in the Key Field Monitoring Experimental Station for Red Soil Eco-environment of the Ministry of Agriculture, Qyang County in Hunan Province (26°45′42″N, 111°52′32″ E). The soil is a typical red soil in the south of China, also known as a Ferralic Cambisol, that originally developed from Quaternary red clay, with the characteristics of low pH and poor fertility. Four treatments were established in 1982, specifically rice-rice-winter fallow (WF), rice-rice-Chinese milk vetch (MV), rice-rice-rape (RP) and rice-rice-ryegrass (RG), with these three green manure plants (Leguminosae, Cruciferae and Gramineae) commonly grown in the rice field. Each treatment was composed of three replicate plots of 37.5 m² (2.5 m × 15.0 m) and separated by a 60 cm cement bund. The initial soil pH was 6.5, the organic matter content was 20.1 g kg⁻¹, and the contents of total nitrogen, total phosphorus, total potassium, alkali-hydrolysable nitrogen, available phosphorus and available potassium were 0.94 g kg⁻¹, 0.66 g kg⁻¹, 11.5 g kg⁻¹, 156 mg kg⁻¹, 7.2 mg kg⁻¹ and 176 mg kg⁻¹, respectively. From 1982 to 2013, the total fertilizer (basal plus topdressing fertilizer) applied to the early and late rice of each season included urea (153.0 kg hm⁻²), P₂O₅ (84.0 kg hm⁻²), and K₂O (129.0 kg hm⁻²). A compound fertilizer [urea (84.0 kg hm⁻²), P₂O₅ (84.0 kg hm⁻²), and K₂O (84.0 kg hm⁻²)] was used as a basal fertilizer, and urea (69.0 kg hm⁻²) and K₂O (45.0 kg hm⁻²) were used as topdressing. The basal fertilizer was applied before rice transplanting, and the topdressing was applied 6–10 days after rice transplanting. Green manure was sown in the winter, 10–15 days before the late rice was harvested. Rape, milk vetch and ryegrass seeds were spread onto the plot at 37.5 kg hm⁻², 7.5 kg hm⁻² and 15.0 kg hm⁻², respectively, which are the recommended local seeding quantities for each type of grass. The fresh green manure biomass was returned to the same plot 15 days before the early rice was transplanted. All straw (except the rice stubble) was removed from the plots after each seasonal rice harvest. No fertilizer was applied to the green manure planted in winter (Gao et al., 2011, 2013, 2015; Yang et al., 2012, 2014; Zhang et al., 2013). In October of 2013 (at the rice heading stage), three rice root samples were randomly collected in each plot and pooled, and the rice roots were shaken vigorously to remove loosely adhered soil (bulk soil). The soil that tightly adhered to the roots was regarded as rhizosphere soil, which was isolated by washing the roots with sterile water and then centrifuging at high speed (10,000 × g, 10 min) to collect the sediment. The bulk soil was designated as SWF, SRP, SMV and SRG, and the rhizosphere soil was designated as RHWF, RHRP, RHMV and RRHGR for the WF, RP, MV and RG treatments, respectively. All soil was stored at −80 °C. The bulk soil was used for soil characterization and nitrogenase activity analysis.

2.2 Soil characterization

Soil chemical properties were determined according to the methods described by Bao (2000). Briefly, the soil pH was determined with a glass electrode using a soil-to-water ratio of 1:2.5. The soil organic matter (SOM) was determined by the potassium dichromate method. The total N was determined by Kjeldahl digestion. Available N was determined by the alkaline hydrolysis.
diffusion method. Nitrate nitrogen and ammonium nitrogen were measured using the Calcium chloride method. The total P was measured by sodium hydroxide fusion followed colorimetric analysis, and the available P was extracted with 0.5 mol L⁻¹ NaHCO₃ (pH 8.5) and determined colorimetrically as described above. The total K was measured by flame photometry after sodium hydroxide fusion, and the available K was extracted with NH₄OAc and determined by flame photometry. Nitrogenase activity was determined by the acetylene-reduction method in a short-term incubation assay (Wakelin et al., 2010).

2.3. DNA extraction, PCR-amplification, and high-throughput sequencing of barcoded 16S rRNA genes

Total genomic DNA from the rice rhizosphere and bulk soil was extracted using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer’s instructions. The DNA concentration was measured by Pico Green using a FLUOStar OPTIMA fluorescence plate reader (BMG LABTECH, Jena, Germany). The V4 region of the 16S rRNA genes was amplified with the primer pair 515F (5’-GTCTACGGGCTGTGCTCG-3’) and 806R (5’-GCTACHVGGGTWTCTAAAT-3’) (Caporaso et al., 2012) in two steps. Both the forward and reverse primers were tagged with adapter, pad and linker sequences. The first-round PCR (universal primer without barcodes: 515F; 806R) was performed in triplicate using a Gene Amp PCR-System ™ 9700 (Applied Biosystems, Foster City, CA, USA) using 25 μl reactions that contained 2.5 μl of 10 × PCR buffer and 0.5 units of AccuPrimeTM Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 0.4 μM of each primer and 10 ng of template DNA. The thermal cycling conditions were initial denaturation at 94 °C for 1 min; 12 cycles of 94 °C for 20 s, 53 °C for 25 s and 68 °C for 45 s; with a final extension at 68 °C for 10 min. The 3 PCR replicates were pooled and purified by beads. A second round of PCR was performed with the reverse primer mixed with the barcode sequence (12 mer) and the DNA template using 15 μl of the purified products from the first round of PCR and was run for 22 cycles under the same conditions as the first round. The triplicate batches of PCR products were pooled and quantified with PicoGreen. For each sample, 200 ng of PCR product was combined with other purified samples as a library, and re-quantified with PicoGreen. Sample libraries for sequencing were prepared according to the MiSeq™ Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA) and a previously described protocol (Caporaso et al., 2012, 2011). The combined samples were run on a MiSeq at the Institute for Environmental Genomics of the University of Oklahoma.

2.4. Bioinformatic analysis of barcoded 16S ribosomal RNA gene libraries

For processing the sequencing data, raw sequences with perfect matches to barcodes were assigned to different sample libraries and were trimmed using BTRIM with a threshold average quality score higher than 25 over a 5 bp window size and a minimum length of 150 bp (Kong, 2011). Forward and reverse reads with a 50 bp overlap and less than 5% mismatches were combined to obtain longer sequences using FLASH (Magoc and Salzberg, 2011).

After unqualified sequences were trimmed because they were too short or contained an undetermined base ‘N’, combined sequences with a length of 251–254 bp were checked by UCHIME (Edgar et al., 2011) to remove potential chimeric sequences. OTU clustering was carried out with UCLUST at a 97% similarity level (Edgar, 2010), and taxonomic assignment was performed with an RDP classifier (Wang et al., 2007) with a minimal 50% confidence estimate. Singletons were removed for downstream analyses, and random resampling was performed for each sample, with 21,060 sequences due to the difference in the sequencing depth for these samples. The above steps were performed using the in-lab Galaxy pipeline. Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP070941.

Subsequent statistical analyses were performed in R (R Core Team, 2014). Permutational Multivariate Analysis of Variance (PERMANOVA) from the ‘adonis’ function in VEGAN package (v.2.0–3) was used to test the differences in community composition (Oksanen et al., 2012). The Bray-Curtis β diversity was used in this test. To do a principle coordinate analysis (PCoA) of the UniFrac distances, OTU-representative sequences related to archaea and chloroplasts were first removed. The leftover sequences were aligned using the Greengenes public 16S rRNA database (http://greengenes.lbl.gov) with PYNAST (Caporaso et al., 2010), followed by tree computation with FASTTREE (Caporaso et al., 2010). The tree-based UniFrac distance calculation and PCoA analysis were performed in R with “phyloseq” package (McMurdie and Holmes, 2013). The alpha diversity, canonical correspondence analysis (CCA), and Mantel test were implemented using the R VEGAN package (Oksanen et al., 2012). Analysis of variance was performed using SPSS statistical software (IBM, Armonk, New York, USA). To discern the relative contributions of both soil and plant variables, the Partial CCA-based variation partitioning analysis (VPA) was carried out. The highest correlated soil and plant variables from Mantel tests were selected for partial CCA in R VEGAN, and the related proportions were then calculated as described in the online tutorial (http://ordination.okstate.edu/varpar.html) using our own R script, which is available by request.

2.5. Gene copy number quantification using qPCR targeting

Quantitative-PCR (qPCR) assays targeting the total bacteria were conducted with the primer sets of the Eub338 forward primer (5’-CCT ACC GGA GGC ACC AG-3’) and the Eub518 reverse primer (5’-ACC GGG CGT GGT G-3’). PCR was performed according to Fierer and Jackson (2005). Briefly, each analysis included a set of standards, positive and negative controls, and samples, each with three analytical replicates on a 96-well plate. Melting curve analyses of the PCR products were performed after each assay to confirm the PCR amplification quality. The PCR products were confirmed on an agarose gel and then cloned into a pMD18-T Easy vector (Takara Biotech Co., Ltd., Dalian) according to the manufacturer’s protocol. Positive clones were isolated, and plasmid DNA was extracted using a TaKaRa MiniBEST Plasmid Purification Kit (Takara Biotech Co., Ltd., Dalian). Plasmid DNA concentrations ranging from 5.0E-03 to 5.0E-07 ng/μl DNA were used to generate the qPCR standard curves. The relative abundance was estimated by calculating the ratio of the gene copy numbers for each microbial population to the total community gene copy number (i.e., the sum of gene copy numbers for bacteria).

3. Results

3.1. Physicochemical properties of the paddy soil

A total of 36 soil samples and 36 rice root samples were collected from the experimental site under 31 years of rice-rice-green manure rotations. The soil properties of the various treatments with one winter fallow and three other green manure plants are shown in Table 1. Compared with the rice-rice-winter fallow (WF), the green manure rotations (GMs) significantly changed most soil properties. All types of green manure significantly improved (p < 0.05) the soil organic matter (SOM) contents and increased the pH values. Compared to WF, the SOM content of the GMs was
The total nitrogenase activity was found in the rice-rice-milk vetch, with the differences indicated that the bacteria in the RHWF were far more diverse than in any green manure treatments. The trends of Shannon indexes, Simpson indexes and Chao values were consistent with the OTU numbers, which showed that the bacteria in the RHWF were far more diverse than in the RHRH, RHMV and RHRG (Table 2).

PERMANOVA was used to examine the differences in the rhizosphere microbial community among the four treatments (Table S1). The results revealed that the communities of RHRP and RHRG were significantly different from RW, whereas RHRM was marginally different from RHWF (P = 0.057). However, there was no significant difference between each pair of treatments under green manure management (P values are 0.192–0.782). The PCoA profile by weighted Unifrac distances also illustrated that RHWF was clearly separated from RHRH, RHMH and RHRD, and these three green manure treatments were gathered (Fig. 1A).

### Table 2

Effect of long-term winter planting of green manure on soil and plant properties.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Treatment (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.98 ± 0.03a</td>
</tr>
<tr>
<td>SOM%</td>
<td>2.92 ± 0.05a</td>
</tr>
<tr>
<td>TN%</td>
<td>0.19 ± 0.00a</td>
</tr>
<tr>
<td>TK%</td>
<td>0.12 ± 0.00a</td>
</tr>
<tr>
<td>AK (mg/kg)</td>
<td>95.67 ± 7.74a</td>
</tr>
<tr>
<td>NH4–N (mg/kg)</td>
<td>29.67 ± 2.67a</td>
</tr>
<tr>
<td>NO3–N (mg/kg)</td>
<td>3.60 ± 0.10a</td>
</tr>
<tr>
<td>AN (mg/kg)</td>
<td>190.67 ± 5.55a</td>
</tr>
<tr>
<td>AK (nmolC2H4/g-h)</td>
<td>18.67 ± 0.09a</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>87.67 ± 2.20a</td>
</tr>
<tr>
<td>Tiller number</td>
<td>14.67 ± 1.20a</td>
</tr>
<tr>
<td>Straw fresh weight (g)</td>
<td>99.70 ± 0.00a</td>
</tr>
<tr>
<td>Straw dry weight (g)</td>
<td>35.80 ± 0.00a</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>22.83 ± 1.17a</td>
</tr>
<tr>
<td>Root fresh weight (g)</td>
<td>12.41 ± 0.00a</td>
</tr>
<tr>
<td>Root dry weight (cm)</td>
<td>5.29 ± 0.00a</td>
</tr>
<tr>
<td>Rice yield (kg/hm²)</td>
<td>5475.6 ± 108.1a</td>
</tr>
</tbody>
</table>

RWF, rice-rice-fallow; RP, rice-rice-rapeseed treatment; MV, rice-rice-Chinese milk vetch; RG, rice-rice-ryegrass. Values (mean ± standard deviation) indicate the absolute amount of each characteristic. Data within the same row followed by the same lowercase letters are not significantly different at P < 0.05. Different letters in a row indicate a significant difference (P < 0.05).

Analysis of variance (ANOVA) was performed and each treatment had three replicates. SOM: soil organic matter; TN: total nitrogen; TK: total potassium; TP: total phosphorus; AN: alkali-hydrolysable nitrogen; NH4–N: ammonium nitrogen; NO3–N: Nitrate Nitrogen; AK: available potassium.

removing the ambiguous bases and chimaera, the total number of qualified reads was 751,541. Each sample was normalized to 21,060 reads to conduct downstream analyses at the same sequencing depth. On the basis of the OTUs at 97% similarity, RHWF had the greatest richness and was significantly higher (p < 0.05) than RHGMs. Moreover, there were no significant differences among these three RHGMs. The trends of Shannon indexes, Simpson indexes and Chao values were consistent with the OTU numbers, which showed that the bacteria in the RHWF were far more diverse than in the RHRH, RHMV and RHRG (Table 2).

### 3.2. Quantity and diversity of the rice rhizosphere microbial community

The microbial quantity in the various treatments was estimated by the 16S rRNA gene copy number from qPCR (Table 2). The gene copy number in the rice rhizosphere of green manure treatments (RHGMs) was significantly higher than that of RHWF (p < 0.05), indicating that a long-term green manure rotation increased the number of microbes in the rice rhizosphere.

The diversity of the microbial community was assessed by the high-throughput sequencing of the 16S rRNA gene. After filtering the low quality reads, trimming the barcodes and primers, and removing the ambiguous bases and chimaera, the total number of qualified reads was 751,541. Each sample was normalized to 21,060 reads to conduct downstream analyses at the same sequencing depth. On the basis of the OTUs at 97% similarity, RHWF had the greatest richness and was significantly higher (p < 0.05) than RHGMs. Moreover, there were no significant differences among these three RHGMs. The trends of Shannon indexes, Simpson indexes and Chao values were consistent with the OTU numbers, which showed that the bacteria in the RHWF were far more diverse than in the RHRH, RHMV and RHRG (Table 2).
In all treatments, Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria and Bacteroidetes were the dominant phyla and accounted for 81–98% of the total OTUs, followed by a few other minor phyla with an average abundance <1%, including Verrucomicrobia (3.89%), Chloroflexi (1.36%), Planctomycetes (1.70%) and Gemmatimonadetes (1.29%) (Fig. 2). Proteobacteria was the most abundant phylum of the three green manure treatments. The percentages of this phylum in RHRP, RHMV and RHRG were 2.1, 2.5 and 2.9 times higher than RHWF, respectively. However, the most abundant phylum in RHWF was Firmicutes, with percentages of 1.2, 1.8 and 2.8 times higher than RHRP, RHMV and RHRG, respectively (Fig. 2A). At the genus level (Table S2; Fig. S1A), the genus Clostridium was found in all treatments; it was the most dominant group in RHWF (37.5%) and the second or third most dominant group in RHGMs (29.6%, 16.3%, and 10.7% in RHRP, RHMV and RHRG, respectively). Several genera, such as Acidobacteria Gp6, Gp7, and Gp16, were greater than 1% in RHWF but lower than 1% in all of the RHGMs. Acinetobacter (31%–41%) and Pseudomonas (14%–28%) were the preponderant groups in RHGMs but accounted for only 4.4% and 2.5% in RHWF, respectively. At the 97% OTU level, the overlapped and unique OTUs were illustrated by a Venn diagram (Fig. 3). The four communities shared 1655 OTUs, which accounted for 15.1% of the total OTUs. The OTUs in the genera Clostridium, Pseudomonas and Acinetobacter were the major common groups. RHWF had the highest number of unique OTUs (2624; 36.2%), followed by RHMV (1029; 20.7%), RHRP (1002; 20.1%), and RHRG (685; 16.7%) Overall, RHWF contained more bacterial species, and the evenness index was higher than in the RHGMs (Table 2).

3.4. Diversity and composition of the microbial community in bulk soil

In contrast to the rice rhizosphere soils, the microbial
community in bulk soils showed no significant difference between rice-rice-winter fallow treatment (SWF) and the three green manure treatments (SGMs, including SRP, SMV and SRG). The 16S rRNA gene copy number in the SGMs (2.89–7.69 × 10^6) was higher than SWF (2.5 × 10^6) as measured by qPCR (Table S3). SRP (rape) had the highest copy number and was significantly different from SWF, SMV and SRG. The Shannon and Chao diversity indexes indicated that SMV and SRG increased the bacterial diversity in the bulk soil, and SRP decreased the diversity, but no significant differences were observed among all four treatments (Table S3). PCoA analysis also revealed that the SGMs and SWF were not clearly separated (Fig. 1B). Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes and Bacteroidetes were the major groups in the bulk soil bacterial communities at the phylum level and showed no obvious difference in all treatments (Table S4; Fig. 2B, S1B). PERMANOVA revealed no significant differences in the bacterial community among SWF and SRP, SMV (P value is 0.317), but between SWF and SRV, SRG showed some differences (P values are 0.085 and 0.001). There was also no significant difference among the three green manure soil samples (Table S1).

3.5. Environmental variables shaped the rhizosphere bacterial community

A Mantel test was performed to examine the associations between the environmental variables and microbial community in both rhizosphere and bulk soil. No significant associations were detected in the bulk soil microbial communities between the soil and plant variables (Table 3), indicating that they were not impacted by these environmental variables. However, the pH, total potassium (TK), rice yield and root length were significantly associated with the rhizosphere microbial communities (Table 3). Rice yield had the highest correlation (r_M = 0.558, P = 0.001), followed by pH (r_M = 0.401, P = 0.001). These results indicated that some small changes in soil properties (e.g., pH increased 0.26–0.30) and plant variables (e.g., yield increased 10%) had a more significant impact on the rhizosphere microbial community compared to those in the bulk soils. The dual impact factors from soil and plants made the rhizosphere bacteria change more obviously than that in the bulk soils.

To further determine the relative contribution of environmental variables on shaping the rhizosphere microbial community, the three highest correlated soil variables in the Mantel results, including pH, soil organic matter (SOM) and total potassium (TK), and the three plant variables, including rice yield, plant height and root length, were selected for a Canonical correspondence analysis (CCA)-based Variation Partitioning Analysis (VPA) (Fig. 4). The soil and plant variables could explain the 26.8% and 26.2% variation in the rhizosphere bacterial community, respectively, suggesting that both these variables were nearly equally important in shaping the rhizosphere microbial community. Their interaction could explain 11.2% of the variation, leaving 35.8% of the variation unexplained.

4. Discussion

Green manure application to paddy soil is now recognized as a highly valuable management practice in organic agriculture. However, the characteristics of underground microbial community in response to its long-term application are largely unclear. Our previous studies using cultivable and clone library methods indicated that a long-term milk vetch green manure rotation significantly increased the number of endophytic bacteria and altered the soil composition (Zhang et al., 2013). However, using these low throughput methods and a single green manure plant, it was hard to discern the overall changes in the microbial community after general green manure management and to distinguish the real enriched species in the rhizosphere. By using high throughput technology, 16S rDNA sequencing, and another quantitative method, qPCR, this study largely extended the previous studies in green manure applications and characterized the effects of three different green manure plants on the diversity of the microbial community in both paddy soils and rice rhizosphere.

Significant changes occurred in the microbial community around rice roots after 31-years of rice-rice green manure rotations. We found that the application of all three green manures significantly increased the bacterial abundance in the rhizosphere according to the qPCR results (Table 2). In addition, the bacterial richness and compositions in the rhizosphere were also dramatically changed (Table 2; Fig. 2). Proteobacteria was the most abundant phylum in three green manure treatments, whereas Firmicutes was the most abundant phylum in the winter fallow control samples. At the genus level, Acinetobacter and Pseudomonas were enriched in the rice rhizosphere of green manure rotation treatments, and the genus Clostridium was the most dominant group in the rice rhizosphere of the winter fallow. In contrast to the rice rhizosphere, the microbial community in bulk soils showed less obvious differences between the winter fallow samples and the three types of green manure treatments. The 16S rRNA gene copy numbers in the SGMs were higher than the SWF winter-fallow, but only one of the SGM treatments (SRP) was significantly different from the other treatments and the control (Table S3). Moreover, the bacterial richness, compositions and structures of all the bulk soil samples were not obviously distinguishable (Table S4; Fig. 2B, S1B). Lundberg et al. (2012) and Mendes et al. (2014) already found that the taxonomic and functional diversity of microbial communities in the rhizosphere and bulk soils could be quite different, and both plant and soil properties had very strong impacts on shaping the microbial community in the rhizosphere. This suggested that the rhizosphere microbial community could be more sensitive to both plant and soil nutrient changes. Comparing the different responses of the bulk and rhizosphere microbial communities in this study, the effects of long-term rice-rice-green manure rotations on the microbial community in paddy and rice rhizospheres was substantially different.

The distinct rhizosphere microbial community under the green
manure treatments with the winter fallow control was probably caused by the combination of soil and plant impacts (Fig. 5). The large inputs of clipped grass biomass in spring dramatically increased the paddy nutrients (e.g., SOM, NH₄⁻N, NO₃⁻N, TK et al.) and changed the soil physical and chemical properties (e.g., reduced soil density, increased soil pH et al.) (Gao et al., 2013; Yang et al., 2012, 2014). After many years of green manure rotations, some of these changes could be accumulated over a long time, even in the rice growing seasons, such as pH and TK (Table 1). These accumulated changes directly shaped the microbial community in the rhizosphere (Fig. 5), which were identified by Mantel tests (Table 3). Furthermore, green manure application significantly increased the rice yield. Compared to the WF, the rice yield increase of MV, RP and RG was 28.8%, 21.4% and 18.8%, respectively, from 1982 to 2011 (Gao et al., 2013). In our sampling year, the rice yields in MV, RP and PG were significantly greater than WF as well (Table 1). The changes in rice growth, such as the yield and root length, might have altered the root exudates, soil oxygen content and habitat physicochemical properties (Dennis et al., 2010; Ryan et al., 2001), and these biotic and abiotic factors, in turn, may have further altered the composition and diversity of the rhizosphere microbial community (Fig. 5). In addition, the CCA and VPA tests confirmed that the soil and plant properties contributed almost equally to the rhizosphere community (26.8% and 26.2%), and their co-influence was also considerable (35.8%) (Fig. 4). Notably, there was a large amount of variation still unexplained (35.8%) that could be contributed by unmeasured soil and plant variables or stochastic process (Zhou et al., 2014). Interestingly, the richness and alpha-diversity indexes of the rhizosphere community under green manure treatments were significantly lower than the WF control (Table 1). This indicated the number of specific bacterial species was reduced even when the total bacterial population number increased after long-term green manure rotations. One possible reason for this result could be explained by the impact of stronger roots in green manure treatments. The long-term use of green manure might cause the accumulation of root exudates such

**Fig. 4.** (A) Canonical correspondence analysis (CCA) on rhizosphere microbial communities with the selected environmental variables. The environmental variables were chosen by Mantel tests (Table 3) with three highest correlated soil properties, including total potassium (TK), organic matter (OM) and pH, and three highest plant variables, including current year yield (Yield), plant average height (Plant Height) and root average length (Root Length). The percentage of variation explained by each axis is shown, and the relationship is significant ($P = 0.021$). (B) Partial CCA-based variation partitioning analysis (VPA) of microbial communities explained by soil and plant properties.

**Fig. 5.** A conceptual model of the impact of long-term green manure on ecosystem processes in the rice paddy.
as rhizodeposits (Dennis et al., 2010; Ryan et al., 2001), and the better growing rice could also produce more root exudates, which together may enhance the root selection process, resulting in the survival of fewer bacteria in rhizosphere.

Additionally, we also found that the bulk soil bacterial quantity and diversity in the green manure treatments were not significantly greater than the winter fallow control (Table S1, S3). This result was distinct from other studies of crop rotations (Altieri, 1999; Cavagnaro and Martin, 2011), organic farming (Bengtsson et al., 2005; Birkhofer et al., 2008; Mäder et al., 2002), reduced soil tillage (van Capelle et al., 2012) and plant productivity (Hector et al., 1999), which have consistently shown the positive impacts on the diversity of the whole microbial community or specific groups of microorganisms in bulk soils. One potential reason for this discrepancy could be caused by the difference in sampling times. We sampled the paddy soils in October, which was already nine months after the input of the green manure. Most of the soil nutrients from the fresh straw had been absorbed by rice; therefore, the nutrients in the soil between the green manure treatments and the winter fallow control might be less different. As a result, the composition and diversity of the microbial community in paddy soils also showed less difference.

We also found the abundance of Acinetobacter and Pseudomonas was higher in RHMGs than RHWF, and Clostridia in RHMGs was lower than RHWF. Beneficial bacteria in most plants, Azospirillum, Azotobacter, Bacillus, Burkholderia, Pseudomonas, Paenibacillus, Herbaspirillum and some members of the Enterobacteriaceae family, are known for their ability to promote rice growth (Jha et al., 2009; Hayat et al., 2010). Among them, the genus Pseudomonas contains well-known plant-promoting and biocontrol bacteria (Combes-Meynet et al., 2011; Duifff et al., 1999; Leveau and Lindow, 2005; Loper et al., 2012; Weller, 2007). After the long-term application and rotation of green manure, the percentage of Pseudomonas in the rice rhizosphere was 5.7- to 10-times higher than that in the winter fallow, indicating that this well-known plant-associated bacterium more easily accumulated in the rice rhizosphere of green manure rotations. Some species of Pseudomonas are conditional pathogens. However, in this study, the root samples taken from the rice were healthy; therefore, the Pseudomonas had no harmful effects and instead proved to be beneficial to rice growth. Another major group, Acinetobacter, was 31%–41% in the RHMGs rice rhizosphere and 4.4% in RHF. Acinetobacter is commonly found in the wheat rhizosphere (Sachdev et al., 2010). The cultivation of Acinetobacter strains demonstrated that the majority of these bacteria exhibited plant-growth-promoting traits such as nitrogen fixation, siderophore production and mineral solubilization (Gandhi and Muralidharan, 2016; Sachdev et al., 2010; Suzuki et al., 2014; Trotel-aziz et al., 2008). Furthermore, Acinetobacter can produce high-levels of xylanase and aagarase to hydrolyse hemicellulose and structural polysaccharides in the cell wall (Lakshmikanth et al., 2009; Lo et al., 2010), indicating that Acinetobacter spp. probably played an important role in degrading green manure residue. The three dominant groups in green manure treatments, Acinetobacter, Pseudomonas and Clostridia, have been found to form a lignin-degrading bacterial consortium (Wang et al., 2013). Studies have demonstrated that Acinetobacter might play a desirable role in the degradation of plant straw, providing nutrients and promoting plant growth in the rhizosphere of rice. Interestingly, a high percentage of Clostridium was found in RHWF. Plant-associated Clostridium has been seldom reported; only Hong et al. (2015) found that Clostridium is one of the predominant groups in the rhizoplane of Spartina alterniflora using Illumina amplicon sequencing. Clostridium is an anaerobic bacterium. In our studies, Clostridium may have been enriched in RHWF because of high soil densities and smaller soil porosity in the winter fallow treatment compared with green manure application (Yang et al., 2012), conditions that are more likely to generate anaerobic environments.

In summary, our results highlighted the effects of long-term green manure rotation on the soil and microbial community and the feedback of the accumulated bacteria on the growth of rice. Green manure rotation increased the abundance but reduced the richness of the microbial community, and the community structure was significantly changed around the rice rhizosphere. The presence of key groups (Acinetobacter and Pseudomonas in this study) rather than diversity per se may determine the agro-ecosystem function (van der Heijden and Wagg, 2013). To understand the relationship of microbial diversity and its function in the sustain-ability of agricultural systems, future studies should broaden the geographic scope of inquiry and use species-specific assays to investigate the activity of these key genera.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.10.023.

References


