Modification of Rhizosphere Bacterial Community Structure and Functional Potentials to Control *Pseudostellaria heterophylla* Replant Disease

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Abstract

Replant disease caused by negative plant-soil feedback commonly occurs in a *Pseudostellaria heterophylla* monoculture regime. Here, barcoded pyrosequencing of 16S ribosomal DNA amplicons combined with phylogenetic investigation of communities by reconstruction of unobserved states (PICRUST) analysis was applied to study the shifts in soil bacterial community structure and functional potentials in the rhizosphere of *P. heterophylla* under consecutive monoculture and different soil amendments (i.e., bio-organic fertilizer application [MF] and paddy-upland rotation [PR]). The results showed that the yield of tuberous roots decreased under *P. heterophylla* consecutive monoculture and then increased after MF and PR treatments, which was consistent with the changes in soil bacterial diversity. Both principal coordinate analysis and the unweighted pair-group method with arithmetic means cluster analysis showed the distinct difference in bacterial community structure between the consecutively monocultured soil (relatively unhealthy soil) and other relatively healthy soils (i.e., newly planted soil, MF, and PR). Furthermore, taxonomic analysis showed that consecutive monoculture of *P. heterophylla* significantly decreased the relative abundances

of the families Burkholderiaceae and Acidobacteriaceae (subgroup 1), whereas it increased the population density of families Xanthomonadaceae, Phyllobacteriaceae, Sphingobacteriaceae, and Alcaligenaceae, and *Fusarium oxysporum*. In contrast, the MF and PR treatments recovered the soil microbiome and decreased *F. oxysporum* abundance through the different ways; for example, the introduction of beneficial microorganisms (in MF) or the switching between anaerobic and aerobic conditions (in PR). In addition, PICRUST analysis revealed the higher abundances of membrane transport, cell motility, and DNA repair in the consecutively monocultured soil, which might contribute to the root colonization and survival for certain bacterial pathogens under monoculture. These findings highlight the close association between replant disease of *P. heterophylla* and the variations in structure and potential functions of rhizosphere bacterial community.

Keywords: cultural and biological practices, disease management, fungi, herbaceous/flowering plants, ornamentals, pathogen diversity, prokaryotes

Plant-soil feedbacks (PSF) refer to the changes to soil properties that are caused by plants which, in turn, influence the performance of plants (van der Putten et al. 2013). Up to 20% of plant photosynthates may be released into the rhizosphere by plant roots, which shape the root-associated microbiome (Lareen et al. 2016; Lundberg and Teixeira 2018; Poole 2017). In turn, the root-associated microbiome is a key modulator for soil health and plant performance (Berendsen et al. 2012; Hu et al. 2018). Plant responses to PSF can be negative, neutral, or positive (Kaisermann et al. 2017; van der

Putten et al. 2013). Replant disease or soil sickness, a typical negative PSF, is featured with a decline in crop yield and a prevalence of soil-borne diseases as a result of repeated planting of the same plant species in the same soil for multiple years (Huang et al. 2013). Replant disease caused by negative PSF commonly occurs in the monoculture cultivation of a range of crops in intensive agriculture such as horticultural crops and medicinal plants (Tian et al. 2016; L. K. Wu et al. 2016a; Zhang et al. 2011). In particular, approximately 70% of medicinal plant species with tuberous roots suffer from replant disease when grown in monoculture (Chen et al. 2015; L. K. Wu et al. 2016a). *Pseudostellaria heterophylla*, an important traditional Chinese medicine (TCM), is commonly used for curing inappetence, debility, diabetes, and weakness after illness (Hua et al. 2016). However, consecutive monoculture of *P. heterophylla* results in a severe decline in both quality and yield of tuberous roots, the most useful part in TCM (L. K. Wu et al. 2016b).

Soil microbial community structure and functions are of crucial importance in determining soil quality and ecosystem stability and sustainability (Schloter et al. 2003; Yang et al. 2017; Zhao et al. 2014). Various studies demonstrated the distinct difference in soil bacterial communities between disease-suppressive soils and disease-conducive soils (Carrión et al. 2018; Gómez Expósito et al. 2017; Kyselková et al. 2009; Li et al. 2015). Mendes et al. (2011) attributed soil suppressiveness of *Rhizoctonia solani* to the high abundances of several bacterial taxa such as Pseudomonadaceae, Burkholderiaceae, and Xanthomonadales in the sugar beet rhizosphere. Shen et al. (2015a) pointed out that soils naturally suppressive to banana Fusarium wilt disease harbored unique bacterial communities, with higher abundances of the phylum Acidobacteria and the genera *Chthonomonas*, *Pseudomonas*, and *Tumebacillus*. In recent years, increasing studies using different crops demonstrated that the occurrence of replant disease was closely related to the shifts

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in soil bacterial communities (Dong et al. 2016; Li et al. 2014a; Mazzola and Manici 2012; Tan et al. 2017; Xiong et al. 2015; Zhou and Wu 2018). Likewise, our previous studies using denaturing gradient gel electrophoresis found that consecutive monoculture of *P. heterophylla* greatly altered the structure of rhizosphere bacterial community with a reduction in the abundance of beneficial rhizobacteria and resulted in the declined soil suppressiveness to *Fusarium oxysporum*, which showed a high pathogenicity to *P. heterophylla* (Chen et al. 2017; L. K. Wu et al. 2016b; Zhao et al. 2015). Therefore, restructuring the rhizosphere microbial community has become the key point for improving soil quality and alleviating *P. heterophylla* replant disease.

Currently, some approaches, including biological control by adding bio-organic fertilizer and crop rotation were widely applied in intensive agriculture for the protection of plants against soilborne pathogens (Hou et al. 2018; Li et al. 2018; Ling et al. 2014; Shen et al. 2015b; Wang et al. 2016, 2017). Reestablishment of soil suppressiveness to soilborne diseases by bio-organic fertilizer application was attributed not only to general suppression by increasing soil microbial diversity and activity but also to specific suppression by fostering specific beneficial microorganisms (Ling et al. 2014; Qiu et al. 2012). By contrast, crop rotation was reported to affect soil microbial community structure by increasing temporal plant diversity and increasing microbial diversity by providing a greater concentration and diversity of organic materials in comparison with the monoculture system (Peralta et al. 2018; Yao et al. 2006; Zhou et al. 2017). Zhao et al. (2017) indicated that, in comparison with *Panax notoginseng* monoculture, crop rotation could enhance the soil microenvironment for the growth of more abundant and diverse soil microbial communities, increase the bacterial diversity and the abundance of beneficial microorganisms (i.e., *Bacillus* spp.), and decrease the ratio of *F. oxysporum*/fungi in soil. Furthermore, Latz et al. (2012) demonstrated that the abundance of soil bacteria producing the antifungal compounds 2,4-diacetylphloroglucinol and pyrrolnitrin increased with plant diversity, suggesting that plant diversity contributes to plant community resistance against pathogens by fostering a beneficial bacterial community. In addition, crop rotation between flooded and upland soil conditions could alter soil microbial community structure and functions by regulating the soil physicochemical properties (i.e., soil aggregate, redox potential, and soil pH) (Breidenbach et al. 2017; Gaydon et al. 2012; Hou et al. 2018; Zhou et al. 2014). Breidenbach et al. (2017) demonstrated the different soil microbial communities in flooded rice versus upland maize soil, mainly due to the different soil environmental conditions (i.e., anoxic flooded soil supporting an anaerobic microbial community versus oxic drained soil supporting an aerobic microbial community).

In this study, both microbial fertilizer application and paddy-upland rotation were carried out in *Pseudostellaria heterophylla*

monoculture regimes to assess their efficacy on controlling replant disease. Moreover, deep pyrosequencing of 16S ribosomal DNA (rDNA) amplicons combined with phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis was applied to study the bacterial community structure and functional responses to different soil amendments. We hypothesized that both microbial fertilizer application and paddy-upland rotation could recover the soil microbiome by promoting bacterial diversity and modulating specific microbial composition.

Materials and Methods

Field experiment and sampling. *P. heterophylla* ‘Zheshen 2’ was used as the test plant material in this study. *P. heterophylla* was generally planted in November and harvested in July of the next year. The field experiment was carried out at Guanyang County, Fuding City, Fujian Province, China (27°25′ N, 120°04′ E). This region is a subtropical monsoon climate with the annual mean precipitation of 2,041 mm. Five treatments were carried out in a field previously cultivated with rice (*Oryza sativa*). The timeframe for experiments and sampling is presented in Figure 1. Experiments included a control with no *P. heterophylla* cultivation during the whole experimental period (CK), newly planted plots (NP), 2-year consecutively monocultured plots (CM), 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *P. heterophylla* (MF), and *P. heterophylla*-rice-*P. heterophylla* rotation plots (PR). Among them, microbial fertilizer FAFU consisted of effective microorganisms (consisting of lactic acid bacteria, yeast, actinomycetes, photosynthetic bacteria, and certain fungi) (Xu 2001) and indigenous antagonistic bacteria previously isolated from *P. heterophylla* rhizosphere (*Burkholderia*, *Pseudomonas*, and *Bacillus* spp.). These indigenous antagonistic bacteria show strong antagonistic activities against the soilborne pathogens of *P. heterophylla* (i.e., *F. oxysporum*, *F. moniliforme*, and *Talaromyces helices*) (Chen et al. 2017; H. M. Wu et al. 2017; L. K. Wu et al. 2016a,b). The bio-organic fertilizer was prepared by incubating a mixture of these beneficial microorganisms in an organic mixture of soybean meal and fish meal (2:1, wt/wt) according to the solid fermentation method (L. K. Wu et al. 2016a). The solid-state fermented fertilizer (480 kg per 666.7 m²) was applied in the 1-year *P. heterophylla*-cultivated plots on 14 October 2014 according to L. K. Wu et al. (2016a). All five treatments had three replicate plots and the study plots were closely adjacent to each other to keep the same environmental conditions. Moreover, all treatments were kept under the same field management during the whole experimental period.

At the expansion stage of tuberous roots (15 May 2015), the rhizosphere soils of *P. heterophylla* (NP, CM, MF, and PR) and control soils (CK) were sampled. For rhizosphere soil sampling, the whole plants were carefully dug out of the soil and shaken to remove loosely attached bulk soil. The soil tightly attached to tuberous roots, called

Fig. 1. Timeframe for the field experiment and soil sampling. CK, NP, and CM represent the control with no *Pseudostellaria heterophylla* cultivation, the newly planted plots, and 2-year monocultured plots, respectively. MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *P. heterophylla*. PR represents *P. heterophylla*-rice-*P. heterophylla* rotation plots.

rhizosphere soil, was brushed off and then collected. Each treatment had three biological replicates from the replicate plots. All soil samples were sieved through 2-mm mesh. A portion of soil was stored at -80°C for the extraction of soil total DNA. The rest of the soil for each treatment was air dried and used for soil nutrient determination according to the methods described by L. K. Wu et al. (2016a). On 3 July 2015, the tuberous roots of *P. heterophylla* from all treatments (NP, CM, MF, and PR) were separately dug out, washed, and sun dried, after which the dry weight was measured.

Soil total DNA extraction, PCR amplification, and barcoded pyrosequencing. The extraction of soil total DNA was performed by using a BioFast soil Genomic DNA Extraction kit (BioFlux, Hangzhou, China). The quality of extracted soil DNA was checked on 1% agarose gels and the DNA concentrations from different soil samples were determined at the same time by using a Nanodrop 2000C Spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.). Then, high-quality soil DNA was used for the amplification of variable regions 3 to 4 of bacterial 16S rDNA gene via the primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGG TATCTAAT-3') with the unique barcode for each sample.

The PCR product from each sample was equally mixed and then used to construct the sequencing library via a TruSeqDNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, U.S.A.). The high-quality library was sequenced using a HiSeq2500 platform (Illumina, San Diego, CA, U.S.A.) and 250-bp paired-end reads were obtained.

Processing of pyrosequencing data. The paired-end reads within each sample were separated by the unique barcode and then truncated by removing the barcode and primer sequences. Sequence assembly of the paired-end reads was performed using FLASH software (v1.2.7) (Magoč and Salzberg 2011). The low-quality reads were filtered out using Trimmomatic software (v0.33) (Bolger et al. 2014) and the chimeric sequences were removed using UCHIME software (v4.2) (Edgar et al. 2011) to obtain the effective reads (SRA accession PRJNA554972). The effective reads were binned into operational taxonomic units (OTUs) using QIIME (version 1.8.0) (Caporaso et al. 2010), with 97% similarity. Species annotation was performed by using RDP classifier (version 2.2; <https://sourceforge.net/projects/rdp-classifier/>) (Wang et al. 2007) against the SILVA database (release 128; <https://www.arb-silva.de>) (Quast et al. 2012).

Furthermore, α diversity (OTU number, Pielou's evenness, and Shannon diversity indices) was calculated to analyze species complexity within a sample. The β diversity analysis, including principal coordinate analysis (PCoA) and the unweighted pair-group method with arithmetic means (UPGMA) clustering based on the Bray-Curtis distance, was applied to analyze species complexity differences between samples. In addition, the PICRUST approach (Langille et al. 2013) was applied in this study to predict the functional composition of a metagenome based on a 16S profile.

Quantitative PCR of *F. oxysporum* in different soil samples. The abundance of *F. oxysporum*, serving as an indicator of *P. heterophylla* replant disease in a monoculture regime (Chen et al. 2017; L. K. Wu et al. 2016b; Zhao et al. 2015), in different soil samples, was

determined through quantitative PCR (qPCR) with the species-specific primers ITS1-F (5'-CTTGGTCATTAGAGGAAGTAA-3') and AFP308R (5'-CGAATTAACGCGAGTCCCAA-3') (Lievens et al. 2005). The PCR mixture and amplification conditions were conducted according to L. K. Wu et al. (2015).

Statistical analyses. One-way analysis of variance (ANOVA) followed by a least significant difference test ($P < 0.05$) was carried out to determine the significance of yield of tuberous roots, abundance of specific taxa, soil physicochemical parameters, and α diversity indices among different treatments. Extended errors bars were performed in The Statistical Analysis of Metagenomic Profiles software (Parks et al. 2014) with Welch's *t* test and Welch's inverted confidence interval method to determine significant differences between two groups for taxonomic (at family level) and functional (at KEGG level 2) profiles. *P* values for taxonomic profiles were adjusted with Benjamini-Hochberg false discovery rate (FDR) correction (Pachioni et al. 2014) and denoted *q* values ($q < 0.05$). Statistical differences for KEGG pathways (level 3) were estimated by ANOVA with Tukey-Kramer post hoc test using a Benjamini-Hochberg FDR correction (the parameters for filtering out were as follows: *q* value > 0.05 , effect size < 0.8). Similarity percentage (SIMPER) analysis based on the relative abundances of genera was performed to determine the top taxa contributing to the dissimilarities between samples by using the PRIMER V5 software package (PRIMER-E Ltd., Plymouth, U.K.).

Results

The growth status of *P. heterophylla* under different treatments.

Compared with the NP treatment, the CM treatment seriously affected *P. heterophylla* above- and belowground growth with a significant ($P < 0.05$) decline in the yield of tuberous roots (Fig. 2A and B). The MF and PR treatments significantly ($P < 0.05$) improved the yield of tuberous roots as compared with CM. There was no significant difference in yield between NP and MF but the yield in NP was significantly ($P < 0.05$) higher than that in PR (Fig. 2B). In addition, the abundance of *F. oxysporum*, an indicator of *P. heterophylla* replant disease, significantly ($P < 0.05$) increased in CM and then significantly ($P < 0.05$) decreased in PR and MF (Fig. 2C). Pearson correlation analysis revealed that the yield of tuberous roots was significantly negatively correlated with the abundance of *F. oxysporum* ($r = -0.918$, $P < 0.01$).

The soil physicochemical parameters under different treatments.

Compared with the NP treatment, the CM treatment showed a significant ($P < 0.05$) increase in total and available N, total P, and available K. Compared with the CM treatment, the MF treatment showed a significant ($P < 0.05$) decrease in total and available N and total and available P but a significant ($P < 0.05$) increase in total and available K. The highest available N, total and available P, total and available K, and pH were found in the PR treatment. As for soil pH, the CM treatment significantly ($P < 0.05$) decreased the pH value in the *P. heterophylla* rhizosphere, whereas the MF and PR treatments significantly ($P < 0.05$) increased the pH value (Table 1).

Overview of 16S rDNA genes pyrosequencing. In total, 803,341 effective tags were generated from 15 soil samples; 57,597, 55,225,

Fig. 2. A, Growth status; B, tuberous root yield; and C, rhizosphere *Fusarium oxysporum* abundance of *Pseudostellaria heterophylla* under different treatments. NP and CM represent the newly planted and 2-year monocultured plots, respectively, PR represents *P. heterophylla*-rice-*P. heterophylla* rotation plots, and MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *P. heterophylla*.

51,893, 51,123, and 51,943 effective tags were obtained from CK, NP, CM, MF, and PR, respectively. Rarefaction analysis showed that the observed species number tended to reach a saturation level at 40,000 sequences (Supplementary Fig. S1). An OTU cluster showed that 1,590, 1,603, 1,581, 1,582, and 1,587 OTUs were obtained from CK, NP, CM, MF, and PR, respectively. On average, 89.3, 89.2, 88.5, 87.6, 83.8, and 68.7% of effective tags could be assigned to the phylum, class, order, family, genus, and species levels, respectively.

Bacterial α -diversity analysis. CK showed the highest OTU number, evenness, and diversity indices (Pielou's evenness and Shannon diversity indices), while CM showed the lowest OTU number, evenness, and Shannon diversity indices (Fig. 3). All three indices decreased in CM and then increased after MF and PR treatments (Fig. 3). PR showed a significantly ($P < 0.05$) higher OTU number, evenness, and diversity indices in comparison with CM (Fig. 3).

PCoA and cluster analysis. Both PCoA and UPGMA cluster analysis showed a distinct difference in soil bacterial community structure between the unhealthy soils (i.e., CM) and the relatively healthy soils (i.e., CK, NP, MF, and PR) (Fig. 4). The first two principal components (PC1 and PC2) of PCoA explained 57.43 and 11.78% of the total variations, respectively. CM was separated from the other soil samples across the first axis (Fig. 4A). Cluster analysis showed that CK and PR, and NP and MF, were clustered together, respectively, which were considerably different from the CM treatment (Fig. 4B).

Shifts in rhizosphere bacterial community structure under different treatments. Taxonomy analysis showed that phyla Proteobacteria, Acidobacteria, Thaumarchaeota, and Actinobacteria were dominant in all samples. The relative abundances of phyla Proteobacteria and Bacteroidetes were significantly ($P < 0.05$) higher in CM than in other treatments. However, the relative abundances of phyla Acidobacteria, Chloroflexi, Nitrospirae, and Verrucomicrobia decreased in CM and then increased after MF and PR treatments (Supplementary Fig. S2).

Furthermore, Venn diagram analysis based on the OTUs that were more abundant in all healthy soils (NP, MF, and PR) than in unhealthy soils (CM) showed that 826 OTUs were shared among NP > CM, MF > CM, and PR > CM (Supplementary Fig. S3). The sum of the relative abundances of these 826 OTUs was higher than 60% in all samples except CM (25.93%). These 826 OTUs that were more abundant in healthy soils (NP, MF, and PR) were mainly assigned to the phyla Proteobacteria, Acidobacteria, Thaumarchaeota, and Actinobacteria. In addition, the shifts in the specific bacterial taxa at the family and genus levels under different treatments were given as follows.

Effects of consecutive monoculture on rhizosphere bacterial community structure. At the family level, as compared with CK, *P. heterophylla* cultivation (NP and CM) led to a significant decrease in the relative abundances of the families Haliangiaceae and Nitrospiraceae but a significant increase in the family Alcaligenaceae (Supplementary Fig. S4). With the extended monoculture, the relative abundances of some other families such as Xanthomonadaceae, Phyllobacteriaceae and Sphingobacteriaceae significantly increased (CK versus CM). As compared with NP, the relative abundances of the families Xanthomonadaceae, Chitinophagaceae, and Phyllobacteriaceae significantly increased in CM but the abundances of the families Acidobacteriaceae (subgroup 1), Xanthobacteraceae, and Burkholderiaceae significantly decreased in CM (Fig. 5A). Furthermore, SIMPER analysis based on the relative abundances of the genera showed the top genera that contributed 70% to the dissimilarity between NP and CM.

Effects of microbial fertilizer application on rhizosphere bacterial community structure. As compared with the CM treatment, the MF treatment significantly increased the relative abundance of the family Burkholderiaceae while decreasing the relative abundances of the families Xanthomonadaceae, Chitinophagaceae, and Phyllobacteriaceae (Fig. 5B). Furthermore, several genera belonging to the above-mentioned families contributed to the observed differences between

Table 1. Changes in soil physicochemical parameters under different treatments^z

Treatments	Total N (g·kg ⁻¹)	Available N (mg·kg ⁻¹)	Total P (g·kg ⁻¹)	Available P (mg·kg ⁻¹)	Total K (g·kg ⁻¹)	Available K (mg·kg ⁻¹)	pH
CK	2.19 b	201.50 e	0.24 e	62.10 d	8.00 c	357.97 b	5.21 b
NP	2.16 b	229.35 c	0.33 c	82.78 b	8.21 b	121.47 e	4.49 d
CM	2.50 a	271.09 b	0.37 b	81.97 b	7.39 e	203.77 d	4.41 e
MF	2.16 b	215.50 d	0.30 d	72.48 c	7.81 d	236.10 c	4.52 c
PR	1.98 c	302.28 a	0.54 a	114.03 a	8.97 a	400.99 a	5.30 a

^z Total N, available N, total P, available P, total K, and available K represent total nitrogen, available (alkali-hydrolyzable) nitrogen, total phosphorus, available phosphorus, total potassium, and available potassium, respectively. CK, NP, and CM represent the control with no *Pseudostellaria heterophylla* cultivation, the newly planted plots, and 2-year monocultured plots, respectively. MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *P. heterophylla* and PR represents *P. heterophylla*-rice-*P. heterophylla* rotation plots. Different letters in columns represent significant differences among different treatments (least significant difference test, $P < 0.05$).

Fig. 3. Calculations of **A**, operational taxonomic unit number; **B**, Pielou's evenness; and **C**, Shannon diversity indices of soil bacterial community in different treatments. CK, NP, and CM represent the control with no *Pseudostellaria heterophylla* cultivation, the newly planted plots, and 2-year monocultured plots, respectively. MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *P. heterophylla* and PR represents *P. heterophylla*-rice-*P. heterophylla* rotation plots.

MF and CM, and showed a similar trend after microbial fertilizer application. In particular, the relative abundance of *Burkholderia* spp. (belonging to the family Burkholderiaceae) significantly increased after MF treatment.

Effects of paddy-upland rotation on rhizosphere bacterial community structure. As compared with the CM treatment, the PR treatment significantly increased the relative abundances of the families Acidobacteriaceae (subgroup 1), Solibacteraceae (subgroup 3), and Nitrosomonadaceae while decreasing the relative abundances of the families Xanthomonadaceae, Chitinophagaceae, Alcaligenaceae, and Phyllobacteriaceae (Fig. 5C). The top genera with 70% cumulative contribution to the dissimilarity between CM and PR are listed in Supplementary Table S1.

Shifts in the predicted functional potentials of rhizosphere bacterial community under different treatments. PICRUSt analysis was carried out to predict the functional potentials of the soil bacterial community's metagenome from the 16S rRNA gene information. The results showed the difference in predicted functional potentials of rhizosphere microbial communities between relatively healthy (CK, NP, MF, and PR) and unhealthy (CM) plots (Fig. 6; Supplementary Fig. S5). In total, 23 pathways (at KEGG level 2) were predicted. Among them, 8, 5, and 12 predicted pathways were found to have significant statistical differences ($P < 0.05$) between NP and CM, CM and MF, and CM and PR, respectively (Fig. 6). Several predicted pathways were significantly enriched in CM in comparison with relatively healthy soil (NP, MF, and PR); among them, genes associated with signal transduction, membrane transport, and replication and repair. However, the opposite was true for genes presumptively involved in carbohydrate metabolism (Fig. 6).

Discussion

Replant disease caused by negative PSF commonly occurs in extensive and monocultural agriculture, and plays a significant role in mediating individual plant performance. In this study, as compared with the NP treatment, the CM treatment led to a significant decline in the yield of *P. heterophylla* tuberous roots, along with an increase in the abundance of *F. oxysporum*. Similarly, the relative abundance of *F. oxysporum* significantly increased under consecutive monoculture of other crops such as peanut, cotton, *Rehmannia glutinosa*, and *Panax notoginseng*, which was considered to be the key factor

limiting production in the monoculture regime (Dong et al. 2016; Li et al. 2014b, 2015; Wu et al. 2018a). In recent years, numerous studies have appeared illustrating the mutual relationship between plants and their associated microorganisms (Browne et al. 2018; Lundberg and Teixeira 2018; Sánchez-Cañizares et al. 2017; Wei et al. 2018). Plant-associated microorganisms can be considered to be part of the plant's pan-genome or second genome, which are crucial for plant health and productivity (Berendsen et al. 2012; Tkacz and Poole 2015). Therefore, barcoded pyrosequencing of 16S rDNA amplicons combined with PICRUSt analysis was applied to study the shifts in soil bacterial community composition and functional potentials in the rhizosphere of *Pseudostellaria heterophylla* under consecutive monoculture and different soil amendments.

The findings in this study were in line with many previous studies on other crops that consecutive monoculture decreased the diversity of soil bacterial community (Liu et al. 2014; Tan et al. 2017; Xiong et al. 2015) while the practices of bio-organic fertilizer application or crop rotation increased soil bacterial diversity in the rhizosphere (Ling et al. 2014; Shen et al. 2015b; Yuan et al. 2014; Zhao et al. 2018; Zhou et al. 2017). Moreover, the changes in the soil pH were consistent with the changes in the Shannon diversity index of soil bacterial communities under different treatments. It has been previously reported that the bacterial diversity was influenced by soil pH (Lauber et al. 2009; Rousk et al. 2010). Rousk et al. (2010) indicated that the relative abundance and diversity of bacteria were positively related to soil pH. In addition, soil bacterial diversity is considered to be a critical factor to maintain soil health, and determine soil suppressiveness to soilborne pathogens (Garbeva et al. 2006; Qiu et al. 2012; Wang et al. 2017). Previous studies of *Panax notoginseng* demonstrated that bacterial diversity was higher in the rhizosphere of healthy plants than in the rhizosphere of diseased plants (Tan et al. 2017; Z. X. Wu et al. 2015). Therefore, the reduction of soil bacterial diversity might result in the decline in soil suppressiveness under CM treatment, while improved soil bacterial diversity contributed to the increase in the yield of *Pseudostellaria heterophylla* tuberous roots after MF and PR treatments.

The results of the present study also indicated that consecutive monoculture and different soil amendments significantly influenced bacterial community structure and composition. Similar to our findings, a previous study found that consecutive monoculture of *R. glutinosa* led to a significant increase in the relative abundances of

Fig. 4. A, Principal coordinate analysis (PCoA) for principal components PC1 and PC2 and B, unweighted pair-group method with arithmetic means clustering of soil bacterial communities based on the Bray-Curtis distance. CK, NP, and CM represent the control with no *Pseudostellaria heterophylla* cultivation, the newly planted plots, and 2-year monocultured plots, respectively. MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *P. heterophylla*. Numbers following the treatments in B represent the three replicates.

Xanthomonadaceae, Sphingomonadaceae, and Alcaligenaceae in the rhizosphere or rhizoplane soil (Wu et al. 2018b), implying that these taxa may be associated with replant disease. Jiang et al. (2017) also demonstrated increased populations of typical pathogenic Xanthomonadaceae bacteria under an apple replanting system. In addition, Sanguin et al. (2009) indicated that the outbreak stage of take-all decline in wheat monoculture was mainly characterized by the prevalence of Proteobacteria, including members of Phyllobacteriaceae

and Sphingomonadaceae, which was in line with the findings in this study. However, it should be noted that the families Xanthomonadaceae and Chitinophagaceae were also reported to be associated with soil suppressiveness to wheat head blight (Campos et al. 2016). This discrepancy might be due to the different environmental conditions, host types, pathogen species, and rhizocompartment. Further work is needed to explore the roles of these two families (Xanthomonadaceae and Chitinophagaceae) in rhizosphere of consecutively monocultured

Fig. 5. Mean proportion (%) and difference in mean proportion (%) of significantly (Welch's *t* test, Benjamini-Hochberg false discovery rate correction, $q < 0.05$) different taxa at the family level between different treatments. NP and CM represent the newly planted and 2-year monocultured plots, respectively; MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *Pseudostellaria heterophylla*; and PR represents *P. heterophylla*-rice-*P. heterophylla* rotation plots. Statistical Analysis of Metagenomic Profiles extended error bar plots for **A**, NP versus CM; **B**, CM versus MF; and **C**, CM versus PR. The parameters for filtering out were as follows: q value > 0.05 , difference between proportions $< 0.5\%$ or difference between ratios < 2.0 .

P. heterophylla. By contrast, the relative abundances of families Burkholderiaceae, Xanthobacteraceae, and Acidobacteriaceae significantly decreased under *P. heterophylla* consecutive monoculture. These families were found to be positively correlated with soil suppressiveness on other crops (Campos et al. 2016; Hou et al. 2018; Liu et al. 2016; Sarikhani et al. 2017). In particular, specific members of the

family Burkholderiaceae have proved to harbor antagonistic activities against soilborne pathogenic fungi and contribute to soil suppressiveness (Carrión et al. 2018; Kyselková et al. 2009; Mendes et al. 2011). Indeed, certain *Burkholderia* strains previously isolated from *P. heterophylla* rhizosphere soil showed strong antagonistic activities against *F. oxysporum*, one of the causal agents of root rot disease of

Fig. 6. Mean proportion (%) and difference in mean proportion (%) of predicted and significantly (Welch's *t* test, $P < 0.05$) different functional categories (at KEGG level 2) between different treatments. The pathways were predicted based on 16S sequences. The predicted metabolic pathways belonging to 'Organismal Systems' and 'Human Diseases' on KEGG database were grouped into 'Others' group. NP and CM represent the newly planted and 2-year monocultured plots, respectively; MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *Pseudostellaria heterophylla*; and PR represents *P. heterophylla*-rice-*P. heterophylla* rotation plots. Statistical Analysis of Metagenomic Profiles extended error bar plots for **A**, NP versus CM; **B**, CM versus MF; and **C**, CM versus PR.

P. heterophylla (L. K. Wu et al. 2016a,b). These results suggested that the decline in the soil suppressiveness due to a reduction in the abundance of beneficial rhizobacteria under consecutive monoculture might be another factor contributing to replant disease of *P. heterophylla*.

Bio-organic fertilizer application has been widely reported to control soilborne disease and improve crop yield by manipulating the soil bacterial community structure (Ling et al. 2014; Wang et al. 2017). In this study, the MF treatment significantly decreased the relative abundances of the CM-enriched families such as Xanthomonadaceae, Chitinophagaceae, and Phyllobacteriaceae while increasing the relative abundance of the family Burkholderiaceae. There was no significant difference between NP and MF in terms of the relative abundance of the family Burkholderiaceae. Wang et al. (2016) found that long-term application of bio-organic fertilizers could significantly increase the relative abundance of Burkholderiales in apple orchard soil. Moreover, the formulated bio-organic fertilizer containing *Burkholderia* GanoEB2 was proven to effectively suppress Ganoerma disease in oil palm (Ili Nadhrah et al. 2015). Therefore, the increase in *P. heterophylla* yield after the MF treatment might be due to the enrichment of specific antagonistic bacteria (i.e., *Burkholderia* spp.) in addition to the improvement of soil bacterial diversity.

Increasing plant diversity by intercropping or rotation has also been widely applied as an efficient strategy to reduce soilborne diseases by reshaping the soil microbiome (Latz et al. 2012; Li et al. 2018; Peralta et al. 2018; Zhou et al. 2017). Li et al. (2018) found that intercropping with *Atractylodes lancea* significantly decreased the relative abundances of the families Xanthomonadaceae, Alcaligenaceae, and Chitinophagaceae in the peanut rhizosphere, which was consistent with the findings under paddy-upland rotation in the current study. However, unlike with the MF treatment, the PR treatment did not increase the relative abundance of the family Burkholderiaceae but mainly increased the relative abundances of the families Acidobacteriaceae (subgroup 1), Solibacteraceae (subgroup 1), and Archangiaceae, which might be due to the creation of anaerobic conditions when rice was planted after the preceding *P. heterophylla* harvest (Lopes et al. 2014; Maarastawi et al. 2018). Rice cropping was reported to be positively correlated with the abundance of members of the orders Acidobacteriales and Solibacterales (Lopes et al. 2014). Moreover, Hou et al. (2018) indicated that *Acidobacteria* subgroups GP16 and GP6 played essential roles against pathogens in paddy-upland cropping systems. In addition, it was found that paddy-upland rotation obviously increased more microbial diversity than upland rotation, and improved soil health for sustainable agriculture (Hou et al. 2018). Therefore, the increase in *P. heterophylla* yield after PR treatment might be closely associated with the eminence of paddy-upland rotation in promoting bacterial diversity and modulating specific microbial composition (i.e., Acidobacteriales and Solibacterales) due to the switching between flooding and drying under anaerobic and aerobic conditions.

Furthermore, the PICRUST approach was applied to predict the functional potentials of the microbial communities in the rhizosphere of *P. heterophylla* and the results revealed the difference in predicted functions between relatively healthy (CK, NP, MF, and PR) and unhealthy (CM) plots. Z. X. Wu et al. (2016) found that relative abundance of gene families related to carbohydrate metabolism tended to be higher in the rhizosphere of healthy *P. notoginseng*, while those related to signal transduction, membrane transport, cell motility, and metabolism of other amino acids tended to be higher in the rhizosphere of diseased plants, which was in line with the findings in this study. In addition, a previous study on *R. glutinosa* rhizosphere soil using shotgun metagenomic sequencing showed that the levels of the clusters of membrane transport (i.e., ABC transporters) and DNA repair were higher in consecutively monocultured soil than in newly planted soil for harmful or indicator microorganisms (Wu et al. 2018c). Among them, membrane transport was reported to be associated with defense mechanisms, resistance to toxic or

Fig. 7. Schematic representation of the proposed microbial community responses to *Pseudostellaria heterophylla* consecutive monoculture and different soil amendments. Upward and downward arrows represent the upregulation and downregulation of specific microbial taxa under different treatments, respectively. CM represents the 2-year consecutively monocultured plots (as indicated by red arrows), MF represents the 1-year cultivated plots first treated with the microbial fertilizer FAFU and then replanted with *P. heterophylla* (as indicated by blue arrows), and PR represents *P. heterophylla*-rice-*P. heterophylla* rotation plots (as indicated by green arrows).

antimicrobial compounds, and bacterial rapid growth by regulating the transport function across cell membranes (Wu et al. 2018c; Z. X. Wu et al. 2016). Bacterial motility is involved in root colonization by mediating the movement toward the root in soil and spread in the root after successful infection (Z. X. Wu et al. 2016). Therefore, the higher abundances of membrane transport, cell motility, and DNA repair in CM might contribute to the root colonization and survival for certain bacterial pathogens in the monocultured soil of *P. heterophylla*. It should be noted, however, that the PICRUSt approach is limited by the number of available genomes, although PICRUSt analysis has been shown to replicate metagenomes to a high degree of accuracy, and gene expression of soil microbial community cannot be captured by this approach as well (Jiang et al. 2016; Langille et al. 2013; Suenaga 2012).

In conclusion, the findings in this study revealed that the replant disease of *P. heterophylla* was closely associated with the dysbiosis of rhizosphere microbiota. The MF and PR treatments could increase soil bacterial diversity and reshape the rhizosphere bacterial community to a similar status with NP or CK (Fig. 7). Both practices can be chosen to alleviate *P. heterophylla* replant disease, depending on the water availability in the geo-authentic production areas. We also showed that the MF and PR treatments recovered the soil microbiome through different ways; for example, the introduction of beneficial microorganisms (in MF) or the switching between flooded soil and upland soil conditions (in PR). Our results reinforce the importance of the rhizosphere microbiome in maintaining soil quality and plant health. Further work is needed to investigate functional gene expression of the rhizosphere microbiome with soil metatranscriptomics or metaproteomics and to link the functional genes of uncultured organisms to phylogenetic groups.

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