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Variations in soil and plant-microbiome composition with different quality irrigation waters and biochar supplementation

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ABSTRACT

To reduce water scarcity in China, the use of reclaimed water or anaerobically treated piggery wastewater, either alone or coupled with biochar supplementation, is attracting increasing attention. However, little information is available regarding their effects on the soil and plant microbiomes receiving irrigation. The objective of this study was to evaluate different water quality irrigation (distilled water, reclaimed water, and piggery wastewater), biochar supplementation, and their interactions on the microbiomes of rhizosphere and bulk soil, and the root endosphere of maize using high-throughput 16S rRNA amplicon sequencing. The experiments were conducted in greenhouse rhizoboxes. The microbiome functional potentials were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). After a 60-day cultivation period, the bacterial communities and potential functions of rhizosphere, bulk soil, and root endosphere displayed distinct differences between irrigation water sources. Irrigation water quality and biochar supplementation influenced bacterial diversity in rhizosphere soil, and bacterial composition was more sensitive to irrigation water quality than to biochar supplementation in soil and root samples. Reclaimed water and piggery wastewater irrigation decreased the abundance of putative plant growth-promoting rhizobacteria (PGPR) and increased the abundance of known pathogenic bacteria. Biochar supplementation elicited the same behaviour. Mantel tests indicated that soil pH and available P exerted strong influences on the structure of the bacterial community in rhizosphere and bulk soil, but total N significantly influenced the bacterial community structure within the root. The current study implies the potential ecological effects (*e.g.* PGPR and pathogenic bacteria) of the irrigation with different quality water should be considered with biochar supplementation.

1. Introduction

The agriculture sector in China is responsible for approximately 60–70% of the country's water consumption to irrigate crops (Jiang, 2015), abstraction that is contributing to increasingly severe water scarcity. To alleviate this, the government is encouraging the development of irrigation practices using unconventional water resources (*e.g.* reclaimed water and anaerobically treated piggery wastewater, which contain elevated levels of nutrients) in ways which do not pose threats to environmental, animal or human health. The consequences of using alternative water irrigation systems have been studied regarding soil properties, the environmental fate of pollutants (*e.g.* heavy metals, antibiotics, antibiotic resistance genes, *etc.*), and plant productivity (Kiziloglu et al., 2008; Kunhikrishnan et al., 2012; Christou et al., 2017). Though the effects of reclaimed water and piggery wastewater irrigation on soil microbiomes have been assessed in several studies (Bastida et al., 2017; Starke et al., 2017; Iyyemperumal and Shi, 2007), there has been no direct comparison of the water sources upon soil microbiomes in the same soil and under the same cropping regimes. This lack of knowledge constitutes a critical issue because irrigation can influence soil fertility both directly and indirectly, through the bacterial community.

Furthermore, pathogenic bacteria have been detected in soil following wastewater irrigation, posing potential human health hazards associated with the food chain (Benami et al., 2013; Ibekwe et al., 2018). The possible enrichment of pathogenic bacteria in soil and plant material is attracting increasing attention. At the same time, plant growth-promoting rhizobacteria (PGPR), which may synthesize

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compounds that support plant growth, facilitate the uptake of certain nutrients from soil, and prevent or lessen plant from diseases, are beneficial to plant growth (Kloepper and Schroth, 1978; Sharma et al., 2017), and they may also be enriched when soil is irrigated with nutrient-rich wastewater. Some studies have found that PGPR are more abundant in the rhizosphere soil of maize than bulk soil (Yang et al., 2017), and biochar supplementation may increase particular PGPR in rhizosphere soil (Wang et al., 2017). However, the effects of combining irrigation with different water quality with biochar supplementation on the behaviour of pathogenic bacteria and PGPR in soil and plants require considerable investigation.

Biochar is an organic soil amendment which is commonly used in certain soils because it has benefits for soil properties and plant growth. Numerous studies have explored the effects of biochar addition upon soil bacterial communities, but there are no consistent effects. There is evidence that rhizosphere microbial communities are more sensitive to biochar additions than bulk soil (Liu et al., 2017), however, there is also contrary evidences suggesting that communities in both soil compartments are equally sensitive to biochar (Chen et al., 2018). This suggests that the effects of biochar additions to soil vary greatly, possibly dependent upon both biochar and soil physicochemical properties. Studies of combined biochar supplementation with different irrigation water qualities (*e.g.* groundwater, tap water, sewage water, and saline water) have mostly focused on plant growth, pollutant behaviour and soil properties (Sudipta et al., 2013; Subhan et al., 2015; Abid et al., 2017; Almaroai et al., 2014; Pressler et al., 2017). Little is known regarding the effects of biochar upon the microbiomes of rhizosphere and bulk soil under different qualities of water irrigation.

In this study, 1% (w/w) wheat straw biochar was added to soil planted with maize, and irrigated using distilled, reclaimed, or piggery wastewater. The microbiomes associated with the rhizosphere, bulk soil, and the root endosphere were compared after irrigation. The objectives were to: (1) evaluate the effect of biochar supplementation and different types of water irrigation on bacterial community structure in the different soil and plant compartments; and (2) determine any relationships between the microbiome taxonomic distribution and physicochemical properties of the soil/plant system. Potential functions of the bacterial assemblages were predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), and the change of predicted KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways at different levels was observed.

2. Materials and methods

2.1. Experimental design

The experimental site was at Xinxiang, Henan Province, China (35°19′ N, 113°53′ E at an altitude of 73 m). We collected the surface layer (0–20 cm) of fluvo-aquic soil (Chinese Soil System) which had no previous history of agricultural management. The measured properties of the soil were as follows: pH 8.73; electrical conductivity 297.00 μS cm^{-1}; total carbon (TC) 7.17 g kg^{-1}; total nitrogen (TN) 1.42 g kg^{-1}; total phosphorus (TP) 1.18 g kg^{-1}; total potassium (TK) 18.30 g kg^{-1}; Cu 32.00 mg kg^{-1}; Zn 88.80 mg kg^{-1}; and Pb 20.70 mg kg^{-1}. The commercial wheat-straw biochar was purchased from Shangqiu Sanli New Energy Co. Ltd., Henan Province. The pH was 9.80, and it contained 625.84 g kg^{-1} total C, 5.24 g kg^{-1} total N, 0.89 g kg^{-1} total P, 44.24 g kg^{-1} total K, 26.51 mg kg^{-1} Cu, 42.50 mg kg^{-1} Zn, and 9.25 mg kg^{-1} Pb. The surface area and total pore volume of the biochar were 8.52 m^2 g^{-1} and 0.025 cm^3 g^{-1}, respectively.

A rhizobox (120 × 140 × 170 mm, $L \times W \times H$) was used to grow maize plants with a 48-μm nylon mesh dividing the soil into three compartments: rhizosphere soil, non-rhizosphere soil, and bulk soil (Cui et al., 2018). After the soil was air-dried and passed through a 2-mm sieve, it was supplemented with base fertilizers consisting of N (200 mg kg^{-1}), P (100 mg kg^{-1}), and K (200 mg kg^{-1}). Then it was divided into two parts: one part was mixed homogeneously with 1% (w/w) biochar, the other was left unamended. Each rhizobox was packed with 3 kg of soil, and the rhizoboxes were placed in a greenhouse with natural light and humidity at a daily average temperature of 25 ± 2 °C. The treatments are as follows: S: distilled water irrigation; SR: reclaimed water irrigation; SP: piggery wastewater irrigation; SB: 1.0% (w/w) biochar + distilled water irrigation; SRB: 1.0% (w/w) biochar + reclaimed water irrigation; SPB: 1.0% (w/w) biochar + piggery wastewater irrigation. Each treatment had three replicates arranged in a completely randomized design.

Prior to planting, the soil was thoroughly wetted with distilled water and pre-incubated overnight. Maize seeds (Jundan 20) were sown into the rhizosphere soil compartment the following day, and irrigated with distilled, reclaimed, or piggery wastewater. The reclaimed water was a secondary effluent from the Camel Bay sewage treatment plant in Henan Province. Piggery wastewater was obtained from Xinxiang Shengda Animal Husbandry Co. Ltd., Henan Province, following an anaerobic fermentation process. Table S1 presents the properties of these two water types. To obtain a chemical oxygen demand (COD) within the local irrigation water quality standards (Department of Rural and Urban Construction and Environmental Protection, 2005), piggery wastewater was diluted five-fold before use. During the cultivation period, soils were irrigated regularly to maintain the field capacity of the soils, using the same irrigation time, frequency, and quantity of the different water sources.

2.2. Sample collection and measurement of soil and plant physicochemical properties

After a cultivation period of 60 days, a customized soil auger (15 mm diameter, 200 mm length) was sterilized with 70% ethanol and used to collect rhizosphere and bulk soil. Plant roots were also harvested and carefully washed with tap water and sterile water to remove adhered rhizosphere soil and debris. An aliquot of soil and root samples were stored at −80 °C prior to bacterial community analysis. The other portion was stored at room temperature before determination of physicochemical properties, including pH, total N (TN), total P (TP), total K (TK), available N (AN), available P (AP), available K (AK), organic matter (OM), Ca, and Mg. Soil pH was estimated on a 2.5:1 water/soil suspension using a digital pH meter. Soil OM was determined by the potassium dichromate method. TN was determined by Kjeldahl digestion. TP and TK were determined by digestion with NaOH, and measured by Mo-Sb colorimetry and flame spectrometry, respectively. AN was determined using a micro-diffusion technique after alkaline hydrolysis. AP was extracted with sodium bicarbonate ($NaHCO_3$) and measured by Mo-Sb colorimetry. AK was extracted with ammonium acetate (NH_4Ac) with detection by flame spectrometry. In addition, Ca and Mg were measured with atomic absorption spectrometer after microwave digestion (Yang et al., 2013).

2.3. DNA extraction and high-throughput 16S rRNA sequencing

After surface sterilization with H_2O_2, ethanol, NaOCl and sterile H_2O (Gottel et al., 2011), root samples were ground to powder in liquid nitrogen. Soil samples were freeze-dried with filtering through a 2 mm mesh. DNA was extracted from all samples using FastDNA SPIN Kits for soil (MP Biomedicals, USA) according to the manufacturer's instructions. The concentration of extracted DNA was quantified by spectrophotometer (NanoDrop ND-2000c, Thermo Fisher Scientific, USA), and stored at −80 °C before further analysis.

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the universal 338F forward primer (5′-ACTCCT ACGGGAGGCAGCAG-3′) and the reverse 806R primer (5′-GGACTAC-HVGGGTWTCTAAT-3′). The resulting PCR products were pooled and paired-end sequenced (2 × 300 base pairs) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to standard protocols by

Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Raw FASTQ files were screened for high-quality sequences (Phred Quality Score > 20) by removing low-quality reads, unrecognized reverse primers, and any ambiguous base calls. Operational taxonomic units (OTUs) were clustered at a 97% similarity cut-off using UCLUST. Chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was predicted using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) using a minimum bootstrap confidence of 60 (Huang et al., 2015).

Alpha-diversity indices (observed OTUs, ACE, Chao, and Shannon), representing the within-sample microbial diversity, and beta-diversity, representing the similarities and differences in microbiome OTU assemblages in different treatments were analyzed. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software. Pathways relating to Eukaryotes were removed prior to analysis. The relationship among levels 1, 2, and 3 in KEGG pathways is represented in Table S2. The abundance of pathogenic bacteria was obtained by comparing sequencing results of each sample with KEGG and categorizing OTUs by function relating to human diseases (Zheng et al., 2017). The putative PGPR was identified following the descriptions of Wang et al. (2017) and Yang et al. (2017).

2.4. Analytical methods

Microbiome data was processed using Microsoft Excel 2010, and the results were visualized using Origin 8.5 (OriginLab, USA). Analysis of variance (ANOVA) was conducted in SPSS 17.5 to determine treatment effects upon soil and root physicochemical properties, and the composition of bacterial communities. Two-factor ANOVA and permutational multivariate ANOVA (PERMANOVA) were conducted to determine the effects of irrigation water quality and biochar supplementation on alpha-diversity indices and microbiome OTU assemblages among different treatments. Using unweighted-UniFrac distance matrix, changes in beta-diversity and the statistical significances of differences between treatments were tested by principal co-ordinate analysis (PCoA) and analysis of similarity (ANOSIM). *R*-statistic values for ANOSIM analysis range from -1 to 1, where $R = 1$ indicates that communities from different treatments are completely dissimilar. Pearson's correlation analysis was conducted to assess the relationships between the dominant phyla and physicochemical properties of soil and plants. A Mantel test was performed to identify any relationships between the unweighted-UniFrac distance matrix of bacterial communities and the Euclidean distance matrix of the physicochemical properties of soil and plant. Mantel similarity indices (R_M) having $P < 0.05$ were considered statistically significant. In addition, linear discriminant analysis (LDA) effect size (LEfSe) was implemented to reveal differentially abundant OTUs between the different assemblages, identifying bacterial biomarkers that most likely explain the differences between samples (Segata et al., 2011). An alpha of 0.05 and an effect size threshold of 3.5 were used for all biomarkers discussed in this study. STAMP (Statistical Analysis of Metagenomic Profiles) was used to reveal statistically significantly enriched level 3 KEGG pathways.

3. Results

3.1. Overall structural variance in alpha-diversity

Rarefaction curves indicated that the amount of sequence data generated was sufficient to capture the total microbial diversity present in all samples, allowing meaningful comparison between treatments (Fig. S1). Table 1 shows that for each treatment, bacterial richness and diversity was significantly lower for the root endosphere than either rhizosphere or bulk soil, and rhizosphere soil had reduced richness and diversity compared to bulk soil ($P < 0.05$). Compared with distilled water irrigation, reclaimed water or piggery wastewater irrigation increased bacterial richness and diversity in rhizosphere and bulk soil. Biochar supplementation increased bacterial richness in soil and root samples irrigated with distilled water, bulk soil irrigated with piggery wastewater, and root endosphere irrigated with reclaimed water, although not all increases were significant. Biochar supplementation didn't significantly increase bacterial diversity in all the samples. Two-factor ANOVA revealed that irrigation water quality and biochar supplementation had the greatest effects on bacterial richness and diversity in rhizosphere soil, and irrigation water quality only influenced bacterial richness in rhizosphere soil and the root endosphere. Conversely, neither had a significant effect on bacterial richness and diversity in bulk soil ($P > 0.05$; Table 1).

Table 1
The average value of bacterial community richness and diversity in rhizosphere, bulk soil, and root endosphere. Columns marked with the same letter do not differ statistically from each other at $P < 0.05$. Rh represents the rhizosphere soil, Bk represents the bulk soil, Root represents the root endosphere. S: distilled water irrigation, SR: reclaimed water irrigation, SP: piggery wastewater irrigation, SB: 1.0% (w/w) biochar + distilled water irrigation, SRB: 1.0% (w/w) biochar + reclaimed water irrigation, SPB: 1.0% (w/w) biochar + piggery wastewater irrigation.

	OTU	ACE	Chao	Shannon
Rhizosphere				
Rh_S	2347	2935 d	2948 d	6.50 b
Rh_SB	2529	3040 cd	3074 c	6.53 b
Rh_SR	2630	3248 ab	3280 a	6.75 a
Rh_SRB	2660	3273 a	3279 a	6.74 a
Rh_SP	2661	3244 ab	3268 ab	6.78 a
Rh_SPB	2457	3112 bc	3144 bc	6.51 b
Significance based on two-factor ANOVA (F value)				
Biochar supplementation		0.00	0.00	6.68*
Irrigation water quality		17.53**	23.83**	17.01**
Biochar supplementation × Irrigation water quality		3.23	4.76**	8.43**
Bulk soil				
Bk_S	2508	3077 b	3092 a	6.67 a
Bk_SB	2601	3147 ab	3151 a	6.66 a
Bk_SR	2691	3184 ab	3181 a	6.74 a
Bk_SRB	2586	3175 ab	3199 a	6.71 a
Bk_SP	2669	3288 ab	3315 a	6.71 a
Bk_SPB	2775	3337 a	3342 a	6.67 a
Significance based on two-factor ANOVA (F value)				
Biochar supplementation		0.36	0.30	0.42
Irrigation water quality		3.61	3.72	0.78
Biochar supplementation × Irrigation water quality		0.15	0.04	0.06
Root endosphere				
Root_S	965	1487 b	1402 a	3.94 a
Root_SB	1034	1739 a	1507 a	3.20 a
Root_SR	698	1169 b	1033 b	3.24 a
Root_SRB	716	1266 b	1075 b	2.79 a
Root_SP	950	1736 a	1479 a	3.50 a
Root_SPB	616	1257 b	1029 b	3.02 a
Significance based on two-factor ANOVA (F value)				
Biochar supplementation		0.25	1.51	3.62
Irrigation water quality		7.16**	7.90**	1.23
Biochar supplementation × Irrigation water quality		6.42*	4.55*	0.10

Significance levels.
* $P < 0.05$.
** $P < 0.01$.

3.2. Characterization of beta-diversity

OTU-level PCoA and ANOSIM, employing unweighted-UniFrac distances, revealed clustering of OTU assemblages according to sample type, and significant dissimilarity (Fig. 1, Table 2). Assemblages

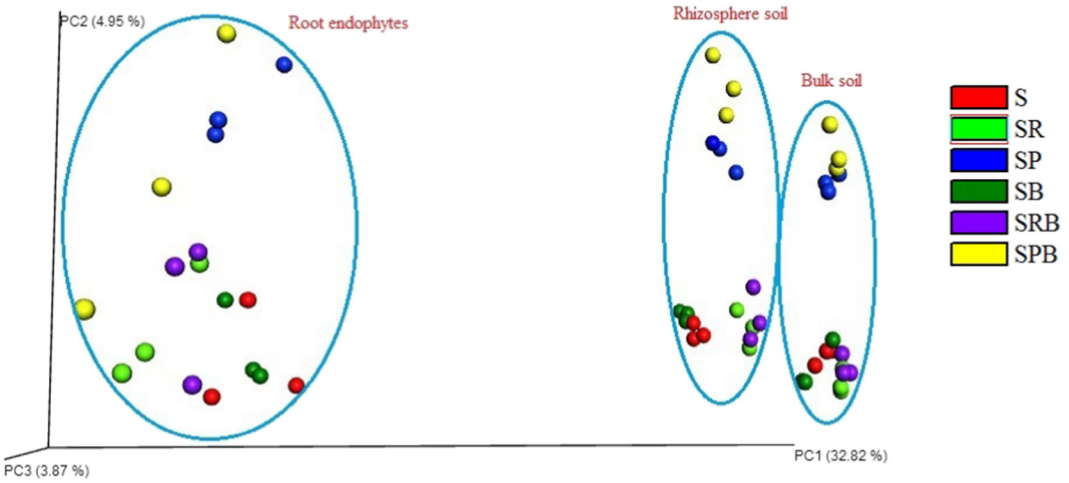

Fig. 1. Principal Co-ordinates Analysis (PCoA) of all kinds of samples based on unweighted-UniFrac distance.

Table 2
Analysis of similarity (ANOSIM) at OTU level.

	R	P
Rhizosphere *vs* bulk soil	0.50	0.001**
Rhizosphere *vs* root endosphere	0.98	0.001**
Bulk soil *vs* root endosphere	0.99	0.001**
Distilled water irrigation *vs* reclaimed water irrigation	0.10	0.024*
Distilled water irrigation *vs* piggery wastewater irrigation	0.21	0.001**
Reclaimed water irrigation *vs* piggery wastewater irrigation	0.22	0.001**
Biochar addition *vs* no addition	−0.02	0.781

Significance levels: *$P < 0.05$; **$P < 0.01$. *R*, ANOSIM test statistic.

associated with the root endosphere were markedly dissimilar from those of rhizosphere and bulk soils. Venn analysis supported this conclusion (Fig. S2). Common OTUs were mainly shared between rhizosphere and bulk soil. Moreover, bacterial assemblages could be separated according to irrigation water source (Table 2). Our previous work identified that irrigation water quality and interaction between water quality and biochar supplementation could explain the differences in microbiome composition in rhizosphere soil and root samples (Cui et al., 2018), consistent with alpha-diversity analysis.

Our previous study revealed that the dominant bacterial phyla in rhizosphere and bulk soils were similar (Fig. 2) (Cui et al., 2018). However, *Proteobacteria*, *Saccharibacteria*, *Verrucomicrobia*, and *Bacteroidetes* were more abundant in rhizosphere soil compared to bulk soil ($P < 0.05$), while *Acidobacteria*, *Chloroflexi*, *Gemmatimonadetes*, and *Nitrospirae* were more abundant in bulk soil compared with rhizosphere soil across the surveyed treatments ($P < 0.01$). There were also clear differences in the dominant bacteria between soil samples and root endosphere samples (Fig. 2). Generally, both reclaimed water and piggery wastewater irrigation significantly increased the abundance of *Bacteroidetes* in rhizosphere soil and decreased the abundance of *Saccharibacteria* in the root endosphere. Biochar supplementation had little influence on soil and plant microbiomes when applied in combination with reclaimed or distilled water irrigation. However, it did have a significant influence on some bacteria in the soil and endosphere when used in combination with piggery wastewater irrigation (Table S3).

To identify bacterial biomarkers of rhizosphere, bulk soil, and root endosphere following irrigation with different quality waters, microbiome variation from the domain to genus levels was examined by LEfSe analysis (LDA score > 3.5, $P < 0.05$). The results identified several OTUs with significantly different abundances following the irrigation treatments, especially between distilled water irrigation and piggery wastewater irrigation (Fig. 3). LEfSe identified 41, 28 and 17 differentially abundant OTUs in rhizosphere, bulk soil, and the root endosphere, respectively. LDA scores were used to visualize the shifts in bacterial community in Fig. S3. Of the three compartments studied here, the rhizosphere was the only one for which any OTUs were more abundant following reclaimed water irrigation. In this case, *Cellvibrio* (from family to genus) were most strongly associated with reclaimed water irrigation. Seven groups of bacteria were most strongly associated with distilled water irrigation, namely *Massilia* (from class to genus), *Sphingomonas* (from order to genus), *Sphingobium* (from order to genus), *Rhizobiaceae* (the family), *Cyanobacteria* (from phylum to class), *Micrococcaceae_g_unclassified* (from family to genus), and *Saccharibacteria_g_norank* (from phylum to genus). Seven groups were most strongly associated with piggery wastewater irrigation, namely *Erythrobacteraceae_g_unclassified* (from family to genus), *Clostridium_sensu_stricto_1* (from phylum to genus), *MWH_CFBK5_g_norank* (from phylum to genus), *Micrococcales* (the order), *Lysobacter* (from family to genus), *Pseudomonas* (from class to genus), and *Cellvibrionales* (the order) (Fig. 3). The majority of OTUs showing increased abundance in bulk soil were again associated with piggery wastewater irrigation. Eight groups of bacteria were most strongly associated with piggery wastewater irrigation, namely, *Lysobacter* (from phylum to genus), *Methylophaga* (from phylum to genus), *Pseudomonadales* (the order), *Marinobacter* (from phylum to genus), *Erythrobacteraceae_g_unclassified* (from family to genus), *Rhizobiales* (the order), *Clostridium_sensu_stricto_1* (from phylum to genus), and *Flavobacteriales* (from class to family). Two groups were most strongly associated with distilled water irrigation, namely *Gemmatimonadetes_g_norank* (from order to genus) and *Family I_Subsection III* (from order to family) (Fig. 3). In contrast to the two soil compartments, differences in OTU abundance in the root endosphere were limited but were greatest following irrigation with distilled water. Seven groups were most strongly associated with distilled water irrigation, namely *Massilia* (from family to genus), *Chloroflexi* (the phylum), *Propionibacteriales* (the order), *Nocardioides* (from family to genus), *Glycomyces* (from order to genus), *Luteolibacter* (the genus), and *Saccharibacteria_g_norank* (from phylum to genus). Only *Clostridiales* (from class to order) were most strongly associated with piggery wastewater irrigation (Fig. 3).

For bacterial biomarkers at genus level, LEfSe identified 11, 6 and 5 OTUs in rhizosphere, bulk soil, and root endosphere respectively, and most of these belonged to the *Proteobacteria* (Table S4). Irrespective of irrigation water quality, in rhizosphere soil, biochar supplementation increased the abundance of *Erythrobacteraceae_g_unclassified* and *MWH-CFBk5_g_norank* but decreased the abundance of *Saccharibacteria_g_norank* and *Micromonosporaceae_g_unclassfied*. In bulk soil, biochar supplementation increased the abundance of *Clostridium_sensu_stricto_1*, but decreased the abundance of *Gemmatimonadetes_g_norank*. In root endosphere, biochar

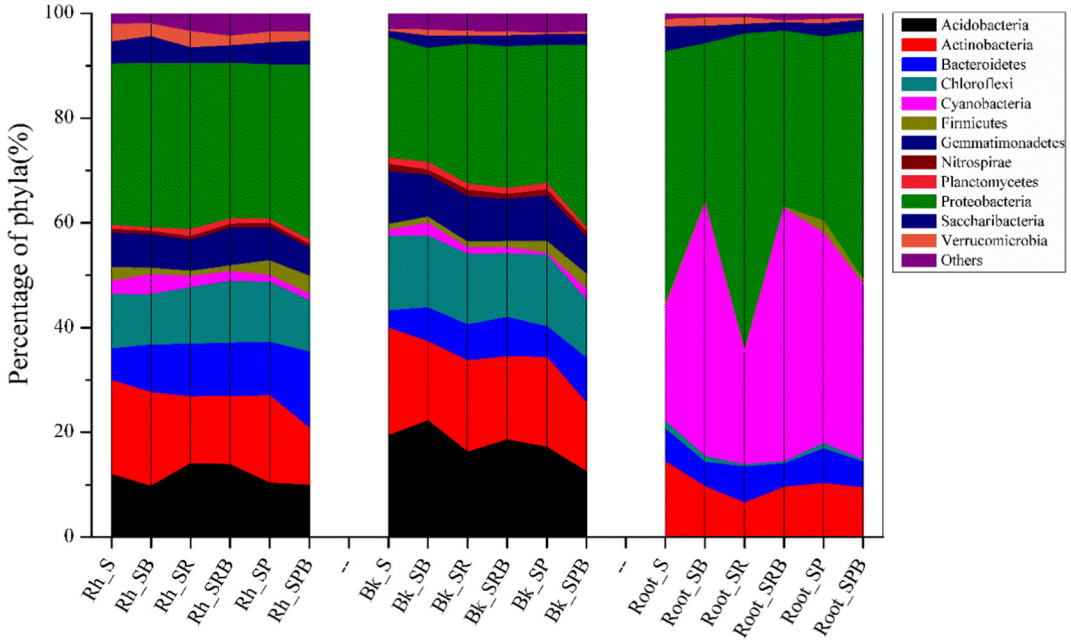

Fig. 2. Bacterial community at phylum level in rhizosphere, bulk soil, and root endosphere.

supplementation did not increase the abundance of any bacteria, but decreased the abundance of *Glycomyces* and *Luteolibacter* (Table S4).

3.3. PGPR variation in rhizosphere, bulk soil, and root endosphere

The known PGPR in different soil and plant compartments were highest in the root endosphere, followed by rhizosphere and bulk soil (Table 3). In the absence of biochar supplementation, reclaimed water irrigation and piggery wastewater irrigation increased the abundance of *Bradyrhizobium* and *Phenylobacterium* in soil but decreased their abundance in the root endosphere. *Pseudomonas* abundance increased in soil and plant compartments following reclaimed water irrigation but decreased following piggery wastewater irrigation. For *Gemmatimonas*, *Streptomyces*, and *Nonomuraea*, either reclaimed water irrigation or piggery wastewater irrigation decreased their abundance in soil and plant compartments. When combined with biochar, these PGPR decreased in soil compartments, and their abundance also decreased in the root endosphere except for *Pseudomonas*, *Streptomyces* and *Nonomuraea*.

3.4. KEGG pathways variation in the predicted metagenomes

PICRUSt was used to predict potential KEGG pathways associated with the predicted OTUs. The main level 1 pathways were Metabolism, Genetic Information Processing, and Environmental Information Processing (Fig. S4). As with PCoA ordination, predicted KEGG pathways in the root endosphere samples clustered separately from rhizosphere and bulk soil samples, and rhizosphere and bulk soil samples also displayed evident differences. This suggests contrasting functional potentials associated with OTU assemblages in the different soil and plant compartments. The relative abundance of level 2 pathways is displayed in Table S5. For total predicted pathogenic bacteria, the results indicated that reclaimed water and piggery wastewater irrigation increased the abundance of pathogenic bacteria slightly when compared with distilled water irrigation, and biochar supplementation could significantly increase their abundance in rhizosphere soil and root endosphere irrigated with reclaimed water and piggery wastewater (Table S5).

STAMP analysis was used to identify significant differences in abundance of KEGG pathways at level 3 between different treatments (Fig. 4). Irrigation and biochar supplementation had significant influences on the predicted functional pathways in the rhizosphere, bulk soil and root endosphere. In rhizosphere soil, irrigation with piggery wastewater was associated with an increased abundance of “glycine, serine and threonine metabolism” and “tropane, piperidine and pyridine alkaloid biosynthesis” – both N-associated processes - compared to distilled water irrigation, whereas irrigation with reclaimed water resulted in an increase in pathogenic pathways associated with *Vibrio cholerae* infection, *Vibrio cholerae* pathogenic cycle, pathogenic *Escherichia coli* infection, shigellosis and pertussis. When receiving reclaimed wastewater, the addition of biochar resulted in a significant decrease in a limited number of predicted functions including “atrazine degradation” associated with herbicide degradation and “*V. cholera* infection” but a significant increase in “indole alkaloid biosynthesis”. In contrast, the addition of biochar in rhizospheres irrigated with piggery wastewater resulted in a much greater number of significant changes in predicted microbiome function; including decreases in “butanoate metabolism”; “nitrotoluene degradation”; “fatty acid metabolism”; “xylene degradation” and “dioxin degradation”; possibly suggesting that biochar addition resulted in reduced concentrations of these compounds in rhizosphere pore water. There were also significant increases in functions associated with N-metabolism including “arginine and proline metabolism”, “glycine, serine and threonine metabolism” “pyrimidine metabolism”, “nitrogen metabolism”, “purine metabolism” and “D-glutamine and D-glutamate metabolism”, but also “*Staphylococcus aureus* infection”, “*Vibrio cholerae* infection”, “*Vibrio cholerae* pathogenic cycle”, and “pertussis”.

For bulk soil, there were limited numbers of significantly different functions associated with wastewater irrigation (compared to distilled water irrigation). There was an increase in “biosynthesis of ansamycins” under reclaimed wastewater irrigation, “glycine, serine and threonine metabolism” and “bacterial secretions systems” under piggery wastewater irrigation and a decrease in “cyanoamino acid metabolism” and “biosynthesis of 12-, 14- and 16-membered macrolides” under piggery wastewater irrigation. Addition of biochar in bulk soil again resulted in greater differences in predicted function, particularly under piggery wastewater irrigation. In this instance, the addition of biochar was associated with significant decreases in “citrate cycle” and “biosynthesis of type II polyketide backbone” but associated with a much greater number of increased functions, including “bacterial

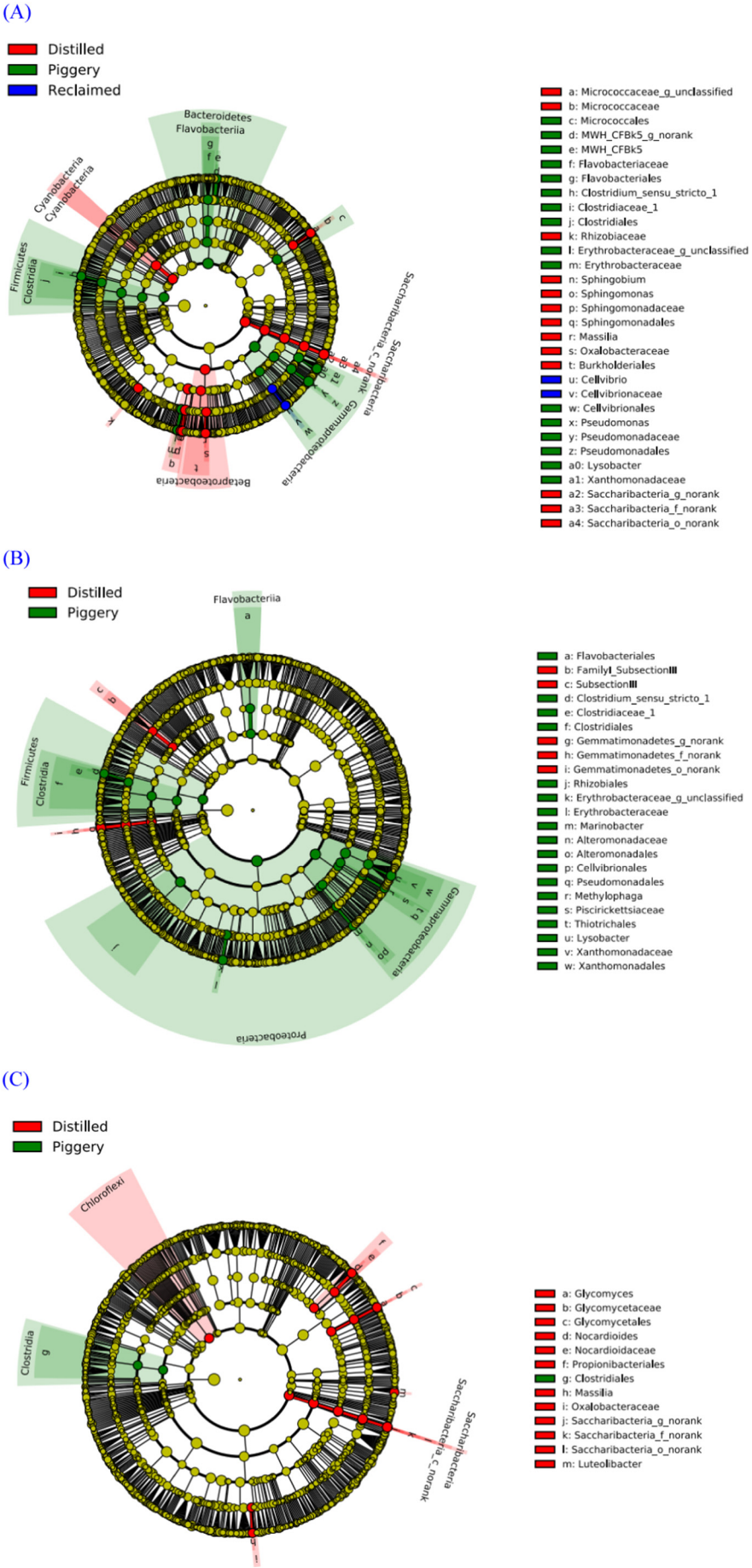

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Fig. 3. Bacterial biomarkers with LDA scores of 3.5 or greater in bacterial communities of rhizosphere soil (A), bulk soil (B), and root endosphere (C).

invasion of epithelial cells", "folate biosynthesis", "nicotinate and nicotinamide metabolism", "tetracycline biosynthesis", "*V. cholera* pathogenic cycle", and "ubiquinone and other terpinoid-quinone biosynthesis". It is clear that the addition of biochar increased pathways associated with *Vibrio cholerae* pathogenic cycle under piggery wastewater irrigation both in rhizosphere and bulk soil.

Predicted responses of bacterial communities within the root endosphere were qualitatively different from those predicted in the soil compartments. The effect of using piggery wastewater irrigation (compared to distilled water irrigation) in the maize endosphere was to significantly increase "pyrimidine metabolism". There were no differences predicted under reclaimed water irrigation. The addition of biochar resulted in an increased number of predicted differences. Under reclaimed wastewater irrigation, the addition of biochar resulted in reductions in "D-alanine metabolism", "selenocompound metabolism" and "glycosaminoglycan degradation", while under piggery wastewater irrigation biochar addition resulted in the reduction of "sphingolipid metabolism", "glycosaminoglycan degradation", "other glycan degradation", "glycosphingolipid biosynthesis" and "selenocompound metabolism". There were no pathways predicted to be significantly increased by the addition of biochar in the maize root endosphere.

In summary, the number of predicted pathways significantly affected by biochar addition were consistently higher under piggery wastewater irrigation than reclaimed water irrigation in all three soil and plant compartments. The addition of biochar resulted in contrasting effects upon the abundance of potential pathogens, which increased under piggery wastewater irrigation but reduced under reclaimed water irrigation.

3.5. The correlation between bacterial community and environmental factors

The physicochemical properties of soil and plant in the rhizosphere, bulk soil, and roots are presented in Table S6 (Cui et al., 2018). Some bacterial phyla had significant relationships with soil and plant properties (Table S7). The abundance of *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, and *Saccharibacteria* in rhizosphere and bulk soil were correlated with various edaphic factors (pH, TN, AN, AP, AK, OM, and Mg). In the root endopshere, *Actinobacteria* and *Firmicutes* were correlated with Mg and Ca, respectively. Mantel tests were also performed to examine the relationship between bacterial OTU assemblage and physicochemical dissimilarities of soil and plant in rhizosphere, bulk soil, and root endosphere (Table 4). The results demonstrated that the bacterial community structure of rhizosphere soil was significantly correlated with pH ($R_M = 0.629$, $P = 0.001$), AP ($R_M = 0.711$, $P = 0.001$), AK ($R_M = 0.265$, $P = 0.012$), and Mg ($R_M = 0.568$, $P = 0.001$); the bacterial community structure of bulk soil also was significantly correlated with pH ($R_M = 0.581$, $P = 0.001$), TN ($R_M = 0.253$, $P = 0.018$), and AP ($R_M = 0.742$, $P = 0.001$); but the bacterial community structure of the root endosphere was only significantly correlated with TN ($R_M = 0.323$, $P = 0.015$).

4. Discussion

4.1. Effects of coupled biochar supplementation and irrigation water quality on alpha-diversity

In this study, we characterized microbiomes associated with rhizosphere and bulk soil, and maize root endospheres in systems which received biochar supplementation and irrigation with different quality waters. Bastida et al. (2017) did not observe any differences in microbial diversity in soils irrigated with reclaimed wastewater, but our study suggests that irrigation with reclaimed water significantly increases the Shannon diversity index of microbiomes in rhizosphere soil. This may be related to the background of soil, the properties of reclaimed water, and the irrigation regime. Our results found that biochar supplementation increased slightly or decreased bacterial diversity in soil, which was in accordance with previous studies. Supplementation with biochar derived from rice stalks was found to increase bacterial diversity slightly in pot experiments (Chen et al., 2018), and supplementation with biochar derived from peanut shells was found to decrease bacterial diversity in a field experiment (Wu et al., 2014). However, in contrast to our results, bacterial diversity increased significantly in layered soil columns following supplementation with biochar derived from corn straw supplementation (Xu et al., 2016). These inconsistent results could be due to different experimental methods (*i.e.* pot trials, the experimental period), the experimental treatments, biochar type and its different physicochemical properties (*i.e.* surface area). Compared with rhizosphere and bulk soil, OTU richness and diversity indices were lower in the root endosphere, and this is consistent with previous studies (Fonseca-García et al., 2016; Estendorfer et al., 2017). The rhizosphere bacterial alpha-diversity of 27 modern maize in-bred lines grown under field conditions has previously been observed to be significantly reduced, compared to the bulk soil (Peiffer et al., 2013), and is consistent with our results. This phenomenon has been ascribed to a root "filtration effect" where bacterial diversity decreases with increasing proximity to the root (Fan et al., 2017).

4.2. Effects of coupled biochar supplementation and irrigation water quality on microbiome composition

Consistent with studies with crops such as soybean (Liu et al., 2017) and wheat (Fan et al., 2017), *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* dominated microbial communities in our maize experiment. OTU assemblages associated with the root endosphere clustered separately from rhizosphere and bulk soils, and the two soil compartments themselves clustered separately. There were marked differences in

Table 3
Relative abundance of PGPR (> 0.1%) in rhizosphere, bulk soil, and root endospheres under different treatments. S: distilled water irrigation, SR: reclaimed water irrigation, SP: piggery wastewater irrigation, SB: 1.0% (w/w) biochar + distilled water irrigation, SRB: 1.0% (w/w) biochar + reclaimed water irrigation, SPB: 1.0% (w/w) biochar + piggery wastewater irrigation.

Genus	Rhizosphere						Bulk soil						Root endosphere					
	S	SB	SR	SRB	SP	SPB	S	SB	SR	SRB	SP	SPB	S	SB	SR	SRB	SP	SPB
Bradyrhizobium	0.10	0.12	0.10	0.10	0.13	0.08	0.10	0.06	0.10	0.08	0.10	0.10	0.21	0.10	0.13	0.08	0.09	0.05
Gemmatimonas	0.34	0.31	0.29	0.24	0.32	0.16	0.34	0.40	0.44	0.42	0.38	0.22	0.03	0.01	0.01	0.00	0.00	0.00
Phenylobacterium	0.08	0.08	0.17	0.10	0.12	0.05	0.08	0.04	0.11	0.07	0.08	0.05	0.06	0.06	0.05	0.02	0.04	0.04
Pseudomonas	1.19	0.67	4.18	2.17	0.80	6.09	1.19	0.07	0.28	0.56	0.04	0.94	1.11	2.86	4.88	14.32	3.12	10.99
Streptomyces	0.78	0.92	0.29	0.34	0.43	0.39	0.78	0.20	0.30	0.29	0.34	0.19	2.43	2.55	0.52	5.89	5.83	8.50
Nonomuraea	0.17	0.14	0.12	0.18	0.09	0.07	0.17	0.07	0.12	0.10	0.15	0.09	0.08	0.10	0.03	0.11	0.14	0.04

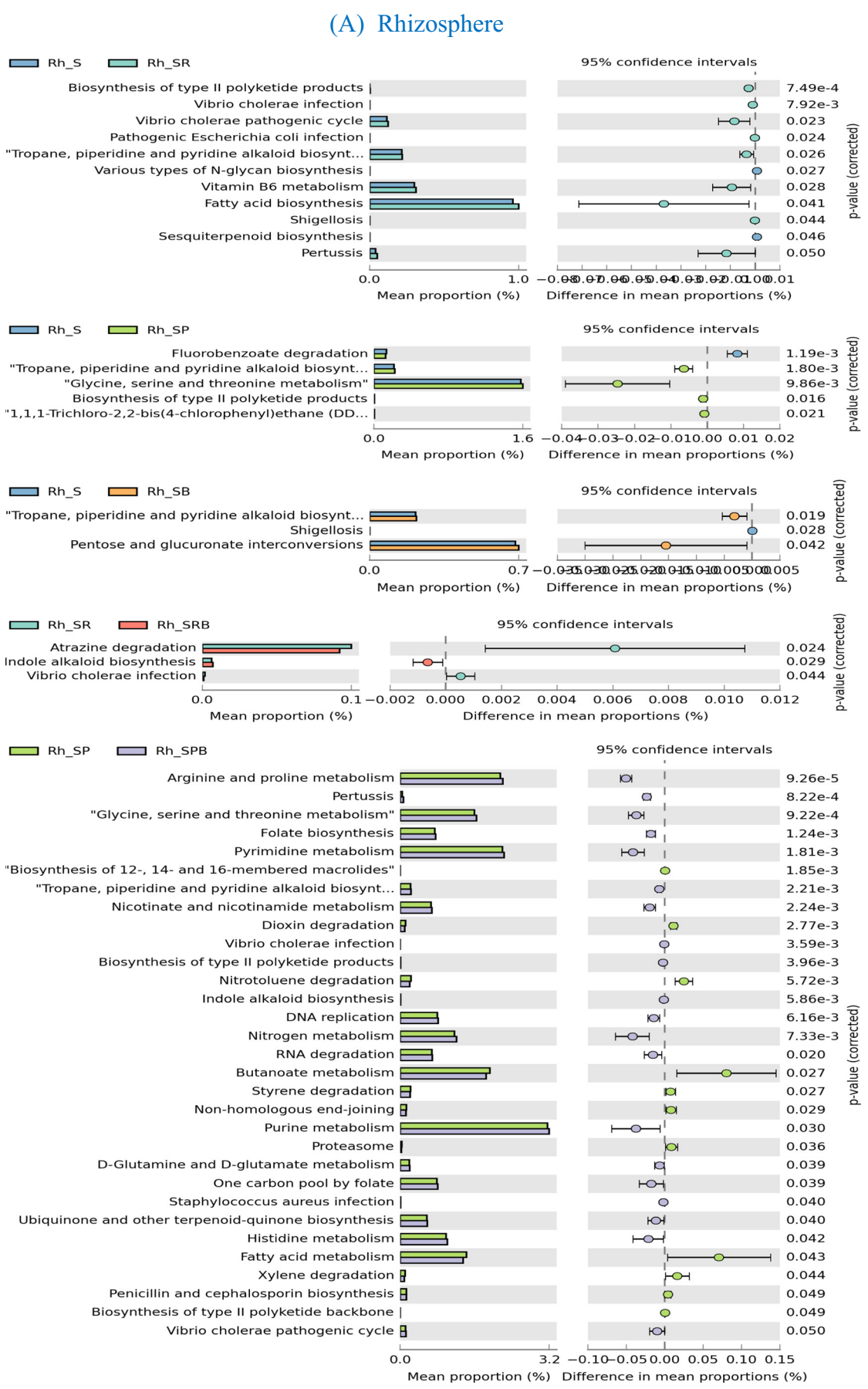

Fig. 4. STAMP analysis on the KEGG pathways at level 3 that differed between different treatments in rhizosphere (A), bulk soil (B) and root endosphere (C).

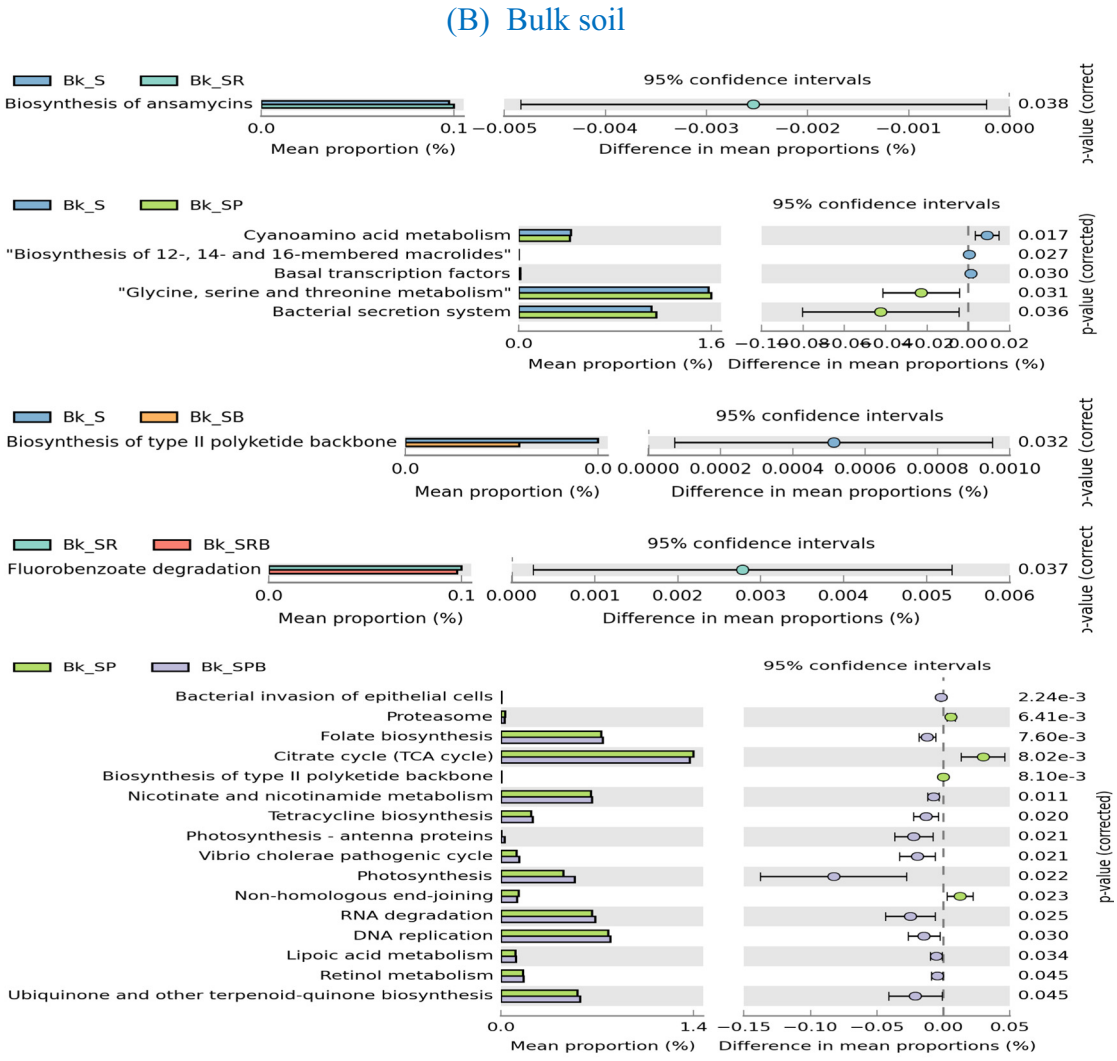

Fig. 4. (*continued*)

Table 4
Mantel correlation statistic (R_M) relating soil and plant physicochemical properties to bacterial communities associated with the rhizosphere, bulk soil, and root endosphere. Significant correlations are shown in bold.

Sample	Item	pH	TN	TP	TK	AN	AP	AK	OM	Ca	Mg
Rhizosphere	R_M	**0.629**	0.164	−0.060	0.044	−0.041	**0.711**	**0.265**	0.177	−0.104	0.568
	P	0.001	0.056	0.485	0.629	0.676	0.001	0.012	0.054	0.265	0.001
Bulk soil	R_M	**0.581**	**0.253**	−0.015	0.068	0.062	**0.742**	0.077	0.045	−0.663	−0.002
	P	0.001	0.018	0.870	0.528	0.556	0.001	0.263	0.622	0.572	0.990
Root endosphere	R_M	–	**0.323**	−0.030	−0.151	–	–	–	–	−0.051	−0.105
	P	–	0.015	0.773	0.213	–	–	–	–	0.723	0.449

microbiome composition between the rhizosphere and bulk soils: *Acidobacteria* were less abundant, and *Proteobacteria* and *Bacteroidetes* more abundant in rhizosphere soil than bulk soil. This may be associated with the nutrient-rich nature of rhizosphere soil (Fierer et al., 2007).

Given their contrasting OTU compositions, it is perhaps not surprising that the bacterial communities in different soil compartments displayed varied responses to irrigation water quality and biochar supplementation. The trends observed in our study were in accordance with the results of Suleiman et al. (2016) and Bastida et al. (2017), who demonstrated that reclaimed water or piggery wastewater irrigation increased the abundance of *Bacteroidetes*, and decreased the abundance of *Acidobacteria*. In addition, biochar supplementation had the same trend (Xu et al., 2016). This trend is related to the pH and nutrient status change, since *Bacteroidetes* and *Acidobacteria* are more abundant in neutral/alkaline and acidophilic environments respectively, and are classified as copiotrophs and oligotrophs, respectively (Fierer et al., 2007; Sheng and Zhu, 2018). Moreover, the use of different water quality for irrigation, with or without biochar supplementation, also significantly affected root endosphere communities. This may be due to external factors (irrigation water quality and biochar supplementation) influencing the nitrogen status of the plant, which has been shown to shape the plant-soil interface and thus indirectly influences root endosphere (García-Salamanca et al., 2013; Estendorfer et al., 2017). Pearson's correlation and Mantel tests identified that the properties of soil showed various significant correlations with bacterial community composition at different taxonomic levels. Similarly, Wang et al. (2018) observed that pH and AP were important predictors of bacterial community composition both in rhizosphere and bulk soil.

LEfSe analysis was performed to identify bacterial biomarkers (*i.e.* OTUs having significantly increased abundance) associated with the different water quality irrigation strategies. Bacterial biomarkers in rhizosphere soil were more numerous than those in bulk soil, indicating that the effects of irrigation water quality on rhizosphere bacterial community was more evident than those on bulk soil. Phyla typically considered to be copiotrophs (*Bacteroidetes*, *Firmicutes*, and *Proteobacteria*) (Ferrari et al., 2015), were all enriched following piggery wastewater irrigation.

4.3. Effects of coupled biochar supplementation and irrigation water quality on KEGG pathways and functional bacteria

In light of the changes in OTU assemblage, it is important to understand any associated variation in potential microbiome function. This is not straightforward when using 16S rRNA amplicon approaches, however, we attempted to predict the potential functional genes associated with rhizosphere, bulk soil, and root endosphere microbiomes based on the presence of bacterial OTUs. The major KEGG pathways in our study, Metabolism, Genetic Information Processing, and Environmental Information Processing, were also the major pathways in flooded paddy soil and sediment (Xiao et al., 2017; Roberto et al., 2018). Under different irrigation water sources, lipid metabolism and carbohydrate metabolisms decreased in rhizosphere and bulk soil with biochar supplementation (Table S5), consistent with the results of Sun et al. (2016) who investigated the metabolic functions in biochar pellets aged in soil after 34 months.

The selectivity of roots for the rhizosphere soil microbiome resulted in higher abundance of putative PGPR in rhizosphere soil (Yang et al., 2017), but different water quality irrigation and biochar supplementation had various effects upon specific PGPR. In contrast to a previous study (Chen et al., 2016), our results demonstrated that *Pseudomonas* and *Streptomyces* decreased in soil samples with biochar supplementation, which maybe related with the differences in biochar and soil properties. In addition, compared with distilled water irrigation, reclaimed water irrigation and treated piggery wastewater irrigation did not contribute to an increase in pathogenic bacteria, suggesting that the use of appropriately treated wastewater for agriculture irrigation is reasonable (Velho et al., 2012; Benami et al., 2016). However, coupled irrigation and biochar supplementation should be used with caution in long-term soil irrigation.

5. Conclusions

Our results suggested that supplementing soil with biochar followed by irrigation with water of different quality (distilled water, reclaimed water, or piggery wastewater) had varied effects on the bacterial diversity and composition in different compartments (rhizosphere, bulk soil, and root). Roots of maize exhibited a distinct bacterial assemblage characterized as having reduced diversity compared with soil samples. Irrigation water quality rather than biochar supplementation changed the bacterial composition, and piggery wastewater irrigation resulted in the most significant changes in bacterial biomarkers abundance and predicted functional pathways. For PGPR and pathogenic bacteria, different water quality irrigation should consider the effect of biochar supplementation. Specifically, in rhizosphere and bulk soil, the importance of soil properties for bacterial community structure differed, but pH and available P were the most important factors shaping bacterial assemblages. These findings shed new light on the soil and plant microbiomes affected by biochar supplementation and irrigation water quality, which contribute for better soil-management strategies. Further, metagenomes and metatranscriptomics are needed to ascertain how irrigation and biochar supplementation influence plant growth and microbiome function.

Competing interests

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2019.04.026.

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