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Biochar alters soil microbial communities and potential functions 3–4 years after amendment in a double rice cropping system

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ABSTRACT

The effects of biochar application on soil microbial communities and functional characteristics and their correlations with soil fertility properties were explored in a double rice cropping system three to four years after a single biochar amendment. Three treatments including a control, a low (24 t ha^{-1}), and a high (48 t ha^{-1}) application rate of straw-derived biochar were constructed. Biochar amendment significantly increased the abundance of bacteria and fungi by up to 102 % and 178 %, respectively, which might be probably caused by the increases in soil total organic carbon (TOC), total nitrogen, and rice biomass as compared with the control. However, the abundance of archaea was only slightly elevated after biochar amendments. Bacteria/fungi ratios were significantly decreased by up to 61.4 % in the biochar treatments, probably because fungi were the dominant decomposers of increased recalcitrant carbon from biochar and rice biomass. Biochar stimulated the relative abundance of Acidobacteria, which favours soil organic carbon accumulation. Biochar increased the relative abundances of *Mortierella* and *Westerdykella*, which are more beneficial to plant growth and TOC degradation. Furthermore, potential phytopathogens of *Athelia* and *Penicillium* were decreased with biochar amendment. The results demonstrate that biochar application should be sustained as an effective measure for improving the microbial characteristics of paddy field by ameliorating its soil properties.

1. Introduction

Biochar, a recalcitrant, carbon-rich material produced via biomass pyrolysis under oxygen-limited conditions, can store carbon in soils on a millennium scale and is porous and alkaline (Lehmann and Joseph, 2015). It has extensive potential application in improving soil nutrient cycling (Haider et al., 2017; Lehmann and Joseph, 2015). Biochar contains available nutrients and can retain soil nutrients by its functional categories, pore structure, and high cation exchange capacity, as well as change microbial communities and increase microbial activity and abundance (Lehmann and Joseph, 2015; Ye et al., 2017). In a 140 d field experiment, Yang et al. (2019) observed that biochar addition enhanced soil organic carbon, ammonium (NH_4^+-N), dissolved organic carbon (DOC), total nitrogen (TN), and rice yields in paddy fields. Other studies have reported increased soil mineral N content (NH_4^+-N and nitrate (NO_3^--N)), and plant biomass in one year field experiments (Nelissen et al., 2015). Haider et al. (2017) found that the pH value and cation exchange capacity for biochar treatments decreased with biochar aging in the soil in a four-year field experiment. Similarly, Wang et al. (2018a) reported that biochar amendment increased soil DOC, NH_4^+-N, NO_3^--N, microbial biomass N, and C in the first year after biochar addition, but there was no significant effect after three years. Biochar application has also been shown to increase soil total organic C (TOC), TN, and total phosphorus (TP) content in a four year field experiment, owing to the accumulation of native organic matter in the paddy soil by stabilizing rhizodeposits (Weng et al., 2017). Consequently, the impacts of biochar application on soil fertility change with biochar ageing in soils. Because the labile components in aged biochar are exhausted, the

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long-term biochar effects on soil fertility can be investigated by studying field-aged biochar.

Microorganisms play a critical role in maintaining and improving soil fertility through biogeochemical processes (Sahu et al., 2017). Biochar can affect the soil microbial community by changing soil physiochemical properties, using biochar as a source of mineral nutrients or energy, and changing the soil-plant-microbe feedback loop (Ameloot et al., 2013). For example, the addition of anthropogenic or wildfire-produced biochar enhanced the microbial abundance and activity in a loamy sand soil in an incubation experiment, which might be due to the increases in microbial habitat and available carbon (Kolb et al., 2009). As reported by Chen et al. (2013), the addition of biochar to a paddy field for 1.5 years increased the bacterial abundance by 28 %–64 %, but decreased the fungal abundance by 35 %–46 %, thus strongly affecting bacterial and fungal community structures; this might be due to the stimulation of bacterial growth and inhibition of fungal growth by neutral or slightly alkaline soil conditions. Furthermore, biochar increased the native soil organic carbon in paddy soils, which might be due to the decreases in microbial mineralization rates of native soil organic carbon caused by stabilized rhizodeposits from microstructures (Weng et al., 2017). Based on the 16S rRNA gene sequencing, Nan et al. (2020) found that the low rate application (8 t ha^{-1} yr^{-1}) of biochar annually enhanced the complexity and stability of the soil bacterial community structure by increasing soil TOC content.

The impact of biochar amendment on the functional characteristics of microbial community have been studied. Zhou et al. (2019) showed that biochar addition increased the amino acid, carbohydrate, and energy metabolism, and changed the bacterial community by increasing the pH value and TN content in a 45 d incubation experiment, which accelerated the decomposition and transformation of pig manure and rice straw. Dai et al. (2018) found that the easily mineralizable C in biochar increased soil saprotroph abundance in a 150 d incubation experiment, leading to a relative decrease in soil fungal pathogens. The bacterial and fungal communities and functional characteristics affected by biochar remain poorly understood in durations longer than two years after a single application.

Paddy fields account for approximately 20 % (165 million ha) of the global irrigated croplands and provide the main source of food for more than 50 % of the population on earth (FAO, 2016). Paddy soils have been experiencing degradation due to long-term application of synthetic fertilizers during recent decades, thus becoming a serious threat to food security (Godfray, 2015). Biochar was tentatively applied into paddy fields and was effective in increasing soil fertility and improving soil health in field experiments (Nan et al., 2020; Wang et al., 2018a). Biochar properties (e.g. aromatic moieties, labile fractions, and physical structures) and its effects on soil properties vary with time, which may further induce microbial community change over time (Jones et al., 2011; Wang et al., 2019). For example, Jones et al. (2011) revealed that the rate of microbial respiration was increased by the easily degradable materials in fresh biochar but was decreased by 3 y aged biochar at 15, 20, and 25 °C in incubation experiments, which might be due to the increase in the soil aggregate stability (Chen et al., 2018b). Chen et al. (2013) found decreased fungal abundance because of the increase in soil pH 1.5 years after a single biochar amendment. In contrast, Yao et al. (2017) observed that aged biochar enhanced soil fungal abundance in the third year after a single biochar addition, which might be caused by the increased soil total C, TN, NO_3^--N, available potassium, and water content. However, most recent studies were obtained using a few samples limited only to the first two years after a single biochar application. Therefore, the impacts of biochar aging on the bacterial, archaeal, and fungal communities and on their potential functions are still not fully known (Ladygina and Rineau, 2013; Lehmann and Joseph, 2015).

In the current study, it was hypothesized that aged biochar could alter the abundance, community composition, and potential functions of soil bacteria, archaea, and fungi by changing soil fertility properties after a single application in paddy fields. To test this, the impacts of biochar application on the soil fertility properties, abundance, community composition, and functional categories of bacteria and fungi three to four years after a single application (2014–2016) in a typical double rice paddy were investigated.

2. Materials and methods

2.1. Site description

A typical paddy field with double rice cropping for more than 50 years was chosen as the experimental field (28.55 °N, 113.33 °E; 80 m asl) in Changsha County, Hunan Province, China. The study region has a subtropical monsoon climate. The annual average temperature is 17.5 °C, and the annual mean precipitation is 1330 mm. The soil of the paddy field was classified as a Hydragric Anthrosol (Table S1; FAO, 2015) with the parent material of granite red soil.

2.2. Field experiment

The field experiment was started in 2012. Details of the experimental design were described by Shen et al. (2014) and Wang et al. (2018a). Briefly, the experiment consisted of three treatments with different biochar application rates: 0 (CK; control), 24 (LB; low biochar) and 48 (HB; high biochar) t ha^{-1}. The field plot for each treatment had an area of 7 m × 5 m, with three replicates. The biochar used in this study had a feedstock of wheat straw, which was pyrolyzed to biochar at 500 °C, and was applied once before rice transplanting in April 2012. Its basic properties are listed in Table S1. The N fertilizer application rates were 120 and 150 kg N ha^{-1}, respectively, in the early and late rice seasons, while phosphorus and potassium fertilizers application rates were 40 kg P_2O_5 ha^{-1} and 100 kg K_2O ha^{-1} respectively in every rice season. The early rice was transplanted in late April or early May and harvested in the middle of July, while the late rice was transplanted in late July and harvested in late October. The period from November to the middle of the following April is the fallow season.

2.3. Sampling and physicochemical analysis

Soil and plant samples were collected in the third and fourth annual cycles of the field experiment from April 2014 to April 2016. The collection frequency and analytical parameters for the soil samples are shown in Table S2. Five soil cores (0–20 cm) were collected randomly in each plot using a disposable sterilized soil auger (square-hole auger was used during the flooding period), and were mixed thoroughly to make one sample after pouring out the water. After removed visible stones or plant residues manually, the sample for each plot was evenly divided according to the measured properties. The soil samples were stored at 4 °C for mineral nitrogen and DOC analyses, naturally air-dried (~25 °C) to measure TOC, TN, TP and pH, and stored at −80 °C for DNA extraction. The soil bulk density (BD) was determined using the cutting ring method after late rice harvesting in the 2014–2016 annual cycles (Chinese Meteorology Agency, 1993).

Soil NH_4^+-N and NO_3^--N were determined with a flow-injection autoanalyser (FIA Star 5000 Analyser, Foss Tecator, Denmark). DOC was determined using a TOC analyser (TOC-VWP, Shimadzu Corporation, Japan). The pH, TOC, TN, and TP were measured as described previously (Wang et al., 2018a). At harvest, rice biomass from each plot was determined by removing rice grains, straw, stubble, and roots from five 1 m^2 areas.

2.4. DNA extraction and quantitative polymerase chain reaction (qPCR)

The DNA was isolated from each soil sample collected at tillering, flowering, filling, ripening, and the fallow season as described by Chen et al. (2010) (Table S2). The quality and concentration of the DNA were analysed by a Nanodrop spectrophotometer (ND-1000, Thermo

Scientific, USA). The abundance of the bacterial 16S ribosomal RNA (rRNA), archaeal 16S rRNA, and 18S rRNA genes was measured in triplicate by a quantitative real-time PCR (qPCR) detection system (ABI prism 7900, Applied Biosystem, USA), with the primer sets 1369 F/1492R (Suzuki et al., 2000), 109 F/934R (Großkopf et al., 1998), and NS1/Fung (Hoshino and Morimoto, 2008), respectively.

Each reaction solution (10 μL) contained 5 μL of 2 × SYBR Green PCR Master Mix (TaKaRa Bio, Japan), 0.2 μL of Rox Reference DyeII, 0.4 μL of 10 μM each primer, and 5 ng DNA. The cycling conditions of bacterial 16S rRNA were used: 94 °C for 31 s; 5 cycles of 92 °C for 5 s, 57 °C for 28 s, and 70 °C for 25 s; 31 cycles of 96 °C for 5 s, 58 °C for 25 s, and 68 °C for 25 s. The amplification conditions for archaeal 16S rRNA were as follows: 96 °C for 31 s; 39 cycles of 96 °C for 5 s, 65 °C for 25 s and 70 °C for 20 s. The amplification conditions for 18S rRNA were used: 94 °C for 31 s; 42 cycles of 94 °C for 6 s and 62 °C for 25 s. Serial tenfold stepwise dilutions of linearized plasmids were made to generate standard curves. The qPCR efficiencies were in the range of 97 %–105 %.

2.5. Illumina Hiseq sequencing

The primer pairs 341 F/806R and 1737 F/2043R were utilized to amplify the V3–V4 region of the 16S rRNA gene (Caporaso et al., 2011; Muyzer et al., 1993) and the ITS region 1 of the fungal rRNA gene (Degnan and Ochman, 2012), respectively. A unique barcode was joined to the 5' end to identify the PCR products. The amplification conditions for the 16S rRNA and the fungal ITS were as follows: 96 °C for 2 min, followed by 31 cycles of 96 °C for 9 s, 55 °C for 28 s, and 70 °C for 35 s, and a final extension at 70 °C for 12 min. The triplicate PCR products were mixed together. They were purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA). Sequencing was performed on the Illumina HiSeq PE250 platform (Novogene Co. Ltd., Beijing, China). The raw sequences were deposited in the National Center for Biotechnology Information under the accession number PRJNA605641.

2.6. Bioinformatics

Raw sequences of the 16S rRNA and ITS genes were demultiplexed and quality-filtered using QIIME (version 1.91; Caporaso et al., 2010). Details of the quality-filtered methods were detailed in our previous study (Wang et al., 2019). Any sequence $\leq$ 250 bp for 16S and $\leq$ 50 bp for ITS was discarded. ITSx1.0.11 was used to extract the ITS1 region and to remove non-target sequences (Nilsson et al., 2010). Operational taxonomic units (OTUs) were clustered using Usearch (version 7.0.1001) with 97 % similarity cutoff for the 16S rRNA gene (Edgar, 2010). The reads of the ITS gene were processed using the program CROP's unsupervised Bayesian clustering algorithm to cluster the sequences into OTUs at a 95 % similarity cutoff (version 1.33; Hao et al., 2011). After singleton OTUs were removed, the resulting OTU tables were rarefied to 34917 and 3664 sequences per sample for the 16S and ITS genes, respectively, to ensure equal sampling depth. OTU representative sequences were aligned by PyNAST for 16S (Caporaso et al., 2010) and MUSCLE for ITS (Edgar, 2010). The representative reads of the 16S and ITS genes were assigned using Ribosomal Database Project classifier (Wang et al., 2007), based on the Silva (QIIME_release_123) and UNITE (QIIME _12_11_ITS) reference databases, respectively.

For the prediction of bacterial and fungal community function, PICRUSt2 (version 2.2.0 beta) and FUNGuild (version 1.1) were used (Douglas et al., 2020; Nguyen et al., 2016). The OTU table generated from the 16S rRNA gene data was input into PICRUSt2 for metagenome prediction using the Kyoto Encyclopedia of Genes and Genomes database. The final output table included the predicted functional abundance at various levels. Any bacterial OTUs with the nearest-sequenced taxon index > 2 was excluded from the output table by default (Douglas et al., 2020). However, the limitation of PICRUSt2 is that the predictions are biased toward existing reference genomes, which means that multiple functions of the same taxon are less likely to be identified. The OTU table generated from the ITS gene data was imported into FUNGuild for functional guild assignment using the FUNGuild database (> 13,000 fungal taxa). FUNGuild assigns functional characteristics of fungi based on the matches at the fungal taxonomic levels along with a confidence level ("highly probable", "probable", and "possible") (Nguyen et al., 2016). Any fungal taxon with "unassigned" was removed. One limitation is that some fungi do not assign in a single guild. In the cases where split ecologies are known, FUNGuild is set to assign "possible".

2.7. Statistical analyses

FastTree (Version 2.1.7) software was used to build phylogenetic trees based on the Jukes-Cantor model (1000 bootstrap replicates; Price et al., 2010). OTU richness, Shannon index, and Faith's phylogenetic diversity (PD) index were analysed in QIIME (Faith, 1992). Analysis of similarities (ANOSIM) and Bray-Curtis based principal coordinates analysis (PCoA) were conducted to compare microbial community structure between the treatments in R (Version 3.3.1) with the package "vegan". Significant impacts of biochar application, time, and their interactions on microbial abundance were examined by repeated-measures ANOVA. ANOVA was performed to determine the significant differences between treatments in R ($p < 0.05$). Redundancy analysis (RDA) was carried out to analyse the correlations among soil fertility property, rice biomass, and microbial abundance and community structures, and the base-10 logarithmic transformation was performed on the measured bacterial, archaeal, and fungal abundance. RDA was conducted in the packages "vegan", and ANOVA was carried out to evaluate the significance of the RDA model in the R software (999 permutations).

3. Results

3.1. Soil fertility properties

During the two annual cycles, soil fertility was improved by the biochar amendment over time (Fig. 1). No significant difference was detected in soil NH_4^+-N ($P = 0.21$–0.89) content among all treatments. In 2014–2015, no significant differences were detected in soil NO_3^--N and DOC ($P = 0.24$–0.96). In 2015–2016, the soil NO_3^--N contents in the biochar treatments were significantly increased; however, biochar significantly decreased DOC content ($P < 0.05$). No significant differences in DOC contents were detected between the LB and HB treatments ($P = 0.63$), but NO_3^--N content in LB was obviously higher than that in HB ($P < 0.05$). No significant differences in soil BD were observed among the three treatments ($P = 0.08$–0.32) in the 2014–2016 annual cycles, except that the LB was significantly higher than CK in 2015–2016 ($P < 0.05$). Biochar application significantly and consistently enhanced soil pH value, TOC, TN, and TP content during 2014–2016 ($P < 0.05$), except for TP in 2014–2015 ($P = 0.09$–0.87). These parameters also increased with biochar application rate.

3.2. Soil bacterial and fungal abundance

Biochar amendment significantly increased the abundance of the bacterial 16S rRNA and the 18S rRNA gene copies in rice paddy soil ($P < 0.05$), but did not significantly affect the archaeal 16S rRNA gene copies ($P = 0.08$–0.28). Significant differences were not observed in the bacterial ($p = 0.05$–0.36) and archaeal ($P = 0.31$–0.35) 16S rRNA and 18S rRNA ($P = 0.72$–0.91) gene copies between the LB and HB treatments (Figs. 2 and 3). The abundance of the bacterial 16S rRNA gene for LB and HB treatments (except in the fallow season) increased by 11.6 %–101.8 % and 4.2 %–53.9 %, respectively, compared with the CK (Fig. 2a). The 18S rRNA gene copy numbers from the LB and HB treatments were measured to be 16.1 %–177.8 % and 36.9 %–139.7 % greater than in the CK treatment, respectively ($P < 0.05$) (Fig. 2c).

Biochar application significantly decreased the bacteria/fungi ratio

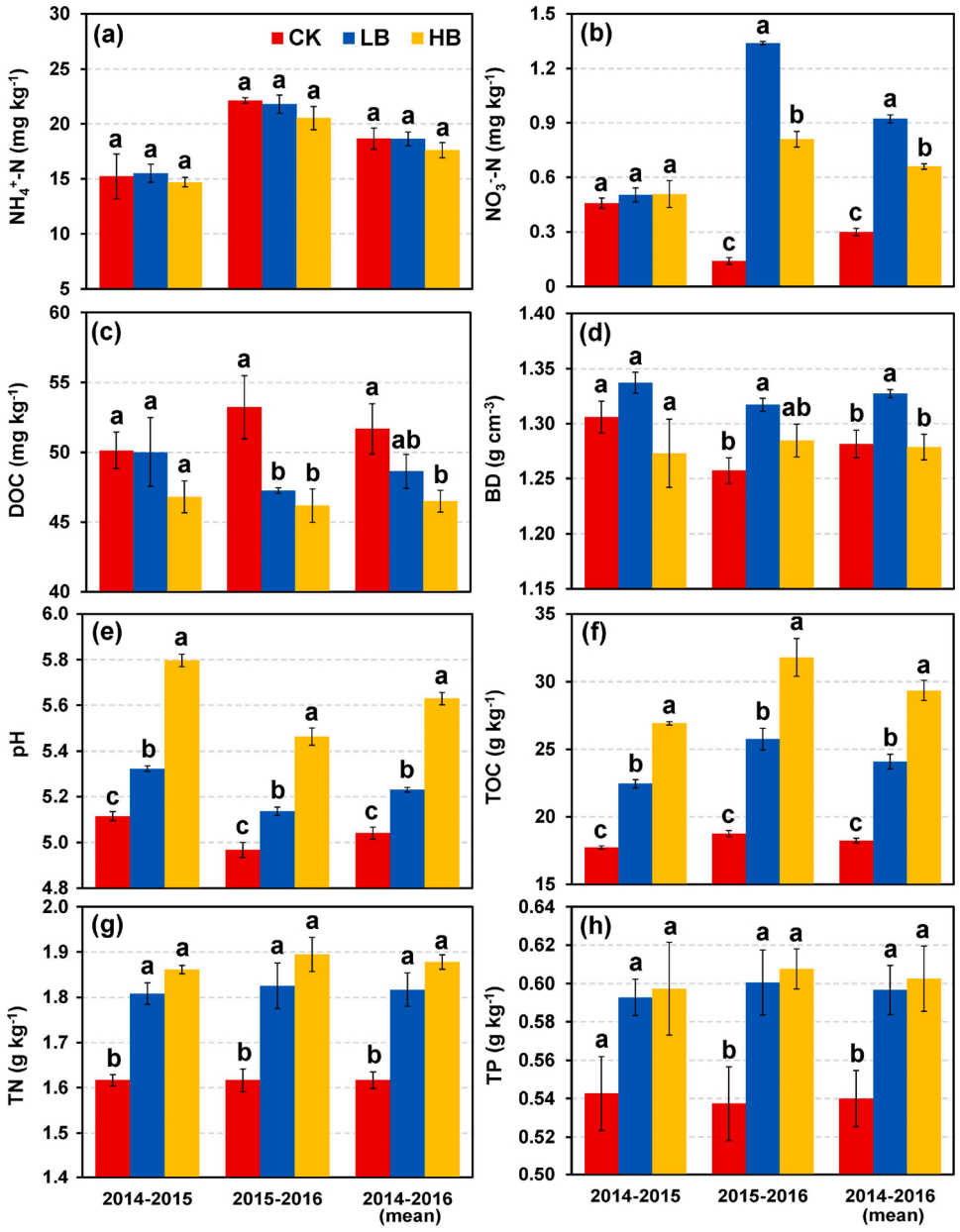

Fig. 1. Soil fertility properties for the three treatments in 2014–2016. Different letters represent significant differences across the treatments ($p < 0.05$). Vertical bars indicate standard errors (n = 3).

($P < 0.05$), but no obvious differences were found in the archaea/fungi ($P = 0.41$–0.60) and bacteria/archaea ($P = 0.10$–0.67) ratios between the biochar and CK treatments. No obvious differences were detected in bacteria/fungi ($P = 0.11$–0.71), archaea/fungi ($P = 0.47$–0.55), and bacteria/archaea ($P = 0.08$–0.40) ratios between the LB and HB treatments (Figs. 3 and 4). Compared with CK, the bacteria/fungi ratios of the LB and HB treatments were reduced by 2.2 %–43.2 % and 18.9 %–61.4 %, respectively, except that the ratios for the LB and HB treatments increased at tillering in the late rice season of 2014–2015 and at ripening in the early rice season of 2015–2016 ($P = 0.29$–0.89; Fig. 4a).

RDA explained 84.8 % of the total variation in bacterial, archaeal, and fungal abundance, 83.5 % of which is explained in the first two axes (Fig. 5a). TN, TOC, and rice biomass were significantly and positively correlated with bacterial, archaeal, and fungal abundance, accounting for 20.5 %, 15.9 %, and 34.8 % of the total variation, respectively ($P < 0.05$; Fig. 5a).

3.3. Bacterial and fungal diversity and community structure

Alpha diversity indices were determined using OTU number, Shannon index, and phylogenetic diversity (Table S3). There were no significant differences in the alpha diversity indices of bacteria between the CK and biochar treatments in 2014–2016 ($P = 0.06$–0.94). Biochar application increased fungal alpha diversity indices, except that the Shannon index from LB treatment was significantly lower than that from CK treatment in 2014–2015 ($P < 0.05$).

The PCoA analysis showed that biochar application greatly influenced the soil bacterial and fungal community structures (Figs. 6a and 7a). Approximately 51.2 %–67.1 % and 83.1 %–90.7 % of the total variation were explained by the first and second axes for bacterial and fungal communities, respectively, in 2014–2016. These results were confirmed by ANOSIM (Table S4).

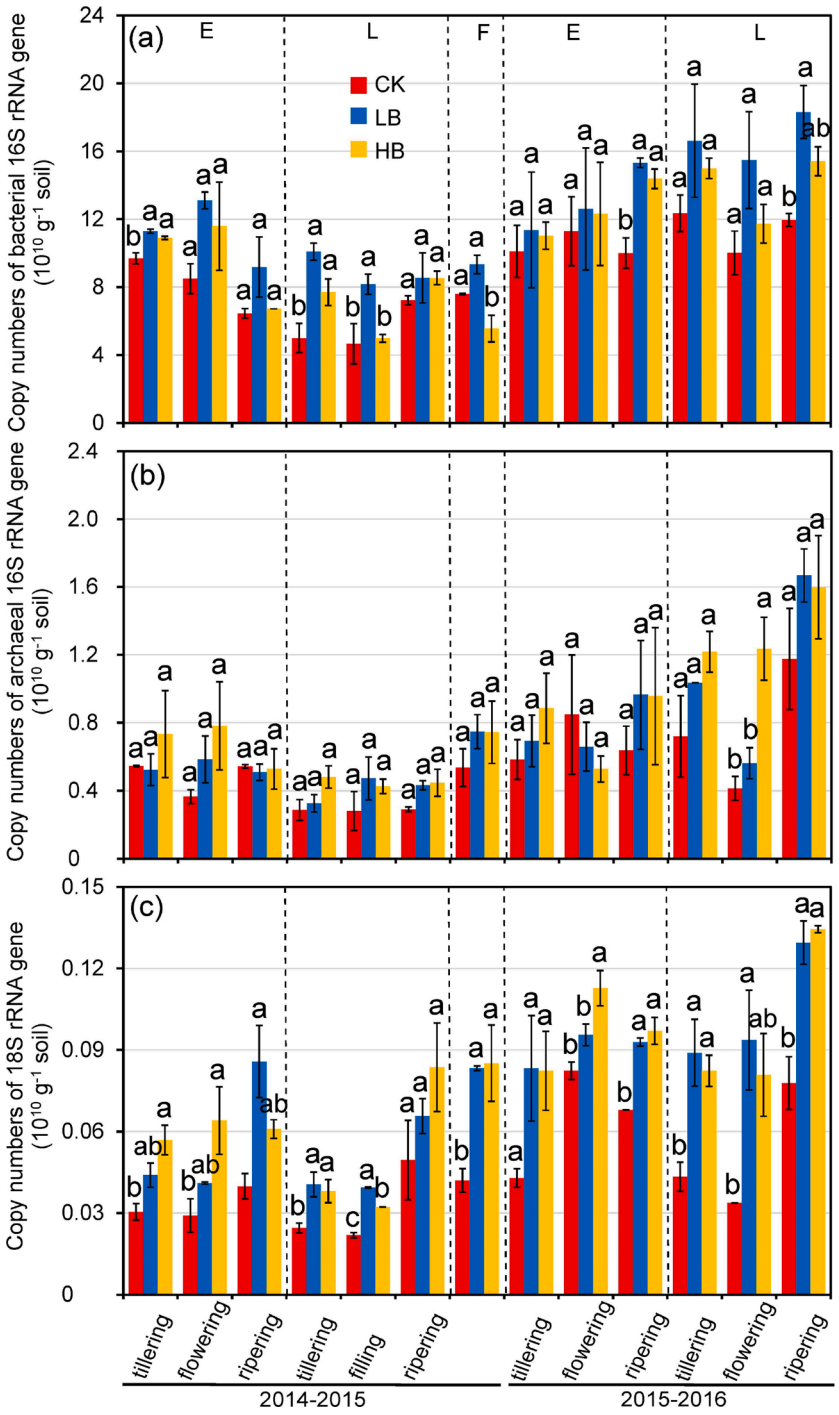

Fig. 2. Abundance of the bacterial (a) and archaeal (b) 16S rRNA and 18S rRNA (c) genes during the 2014–2016 annual cycles. E, L, and F: early rice season, late rice season, and fallow season, respectively. Different letters represent significant differences across the three treatments ($p < 0.05$). The vertical bars indicate standard errors (n = 3).

3.4. Bacterial community composition

The most abundant bacterial phyla included Proteobacteria (39.7 %–51.8 %), Acidobacteria (13.9 %–20.3 %), Nitrospirae (7.3 %–8.6 %), Chloroflexi (4.7 %–9.0 %), Actinobacteria (3.7 %–6.1 %), Verrucomicrobia (1.2 %–5.0 %), and Firmicutes (1.6 %–2.6 %), which overall accounted for 81.6 %–89.6 % of the sequences in different treatments (Fig. 6b). The relative abundance of Acidobacteria was consistently higher in biochar application treatments than CK. In contrast, the percentages of Proteobacteria, Actinobacteria, Verrucomicrobia, and Firmicutes were consistently decreased in the soils amended with biochar, except that those of Actinobacteria for LB and of Verrucomicrobia for HB were increased in 2015–2016 and 2014–2015, respectively, compared to CK. The other dominant bacterial phyla were not persistently influenced by biochar application. At the genus level, biochar treatments consistently enhanced the abundance of *Geobacter, Candidatus* Koribacter, *Candidatus* Solibacter, *Bryobacter,* and *Candidatus* Nitrosotalea (Fig. 6c) and decreased the relative abundance of *Anaeromyxobacter, Desulfobacca,* and *Syntrophorhabdus*, compared with CK.

RDA explained 79.9 % of the total variability in the dominant

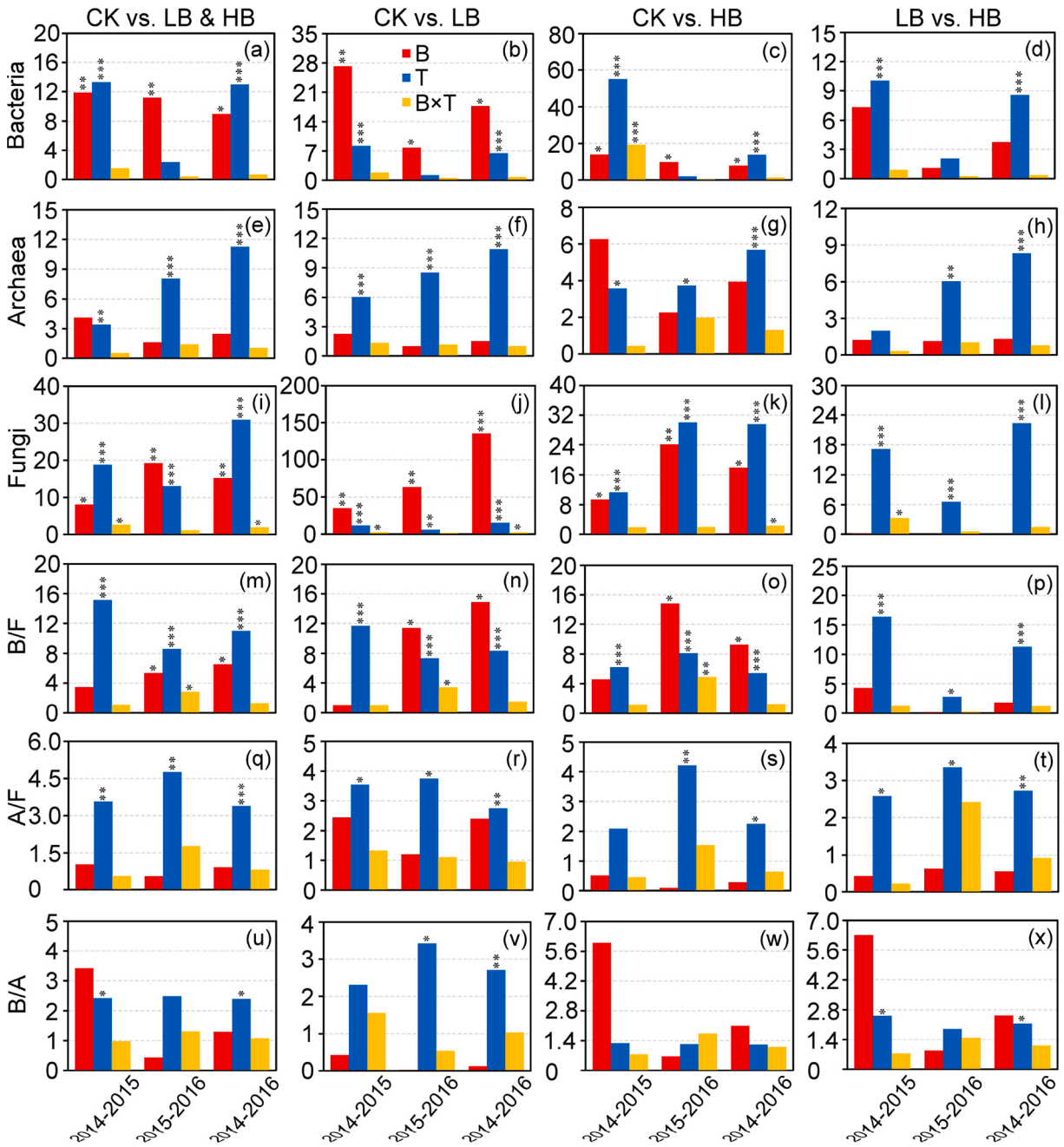

Fig. 3. Repeated measures analysis for the effects of biochar application rate (B), time series (T), and their interactions (B × T) on the abundance of bacterial, archaeal and fungal, and the ratios of abundance of bacteria to fungi, archaea to fungi and bacteria to archaea in the 2014–2016 annual cycles. Bacteria, archaea, and fungi: bacteria, archaea, and fungi abundance, respectively. B/F, A/F and B/A: ratios of abundance of bacteria to fungi, archaea to fungi and bacteria to archaea, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

bacterial genera, of which 63.9 % is explained by the first two axes (Fig. 5b). The dominant bacterial genera in LB and HB were separated from those in the control. TN, TOC, and rice biomass were significantly correlated with the dominant bacterial genera, accounting for 18.1 %, 22.7 %, and 16.0 % of the total variability, respectively ($p < 0.01$). TN and TOC were positively correlated with the percentages of *Candidatus* Koribacter, *Geobacter,* and *Haliangium* but were negatively correlated with those of *Syntrophorhabdus, Desulfobacca, Anaeromyxobacter*, and *Sphingomonas*. Rice biomass was positively correlated to the percentages of *Candidatus* Koribacter and *Geobacter* but were negatively correlated with those of *Bryobacter, Candidatus* Nitrosotalea, *Candidatus* Solibacter, *Sphingomonas, Anaeromyxobacter*, and *Desulfobacca*.

3.5. Fungal community composition

The dominant fungal phyla among all treatments were Ascomycota (33.5 %–57.4 %), Basidiomycota (15.1 %–62.0 %), and Zygomycota (4.3 %–48.2 %), representing more than 96.9 % of the fungal reads (Fig. 7b). The relative abundance of Basidiomycota was consistently decreased, and that of Zygomycota was increased in the LB and HB treatments compared with CK. At the genus level, the relative abundance of *Mortierella, Westerdykella,* and *Pyrenochaetopsis* were consistently higher, whereas those of *Psilocybe, Athelia, Arachnion, Penicillium*, and *Dimorphospora* were consistently lower in the LB and HB treatments as compared with CK (Fig. 7c).

RDA explained 69.8 % of the total variation in the main fungal

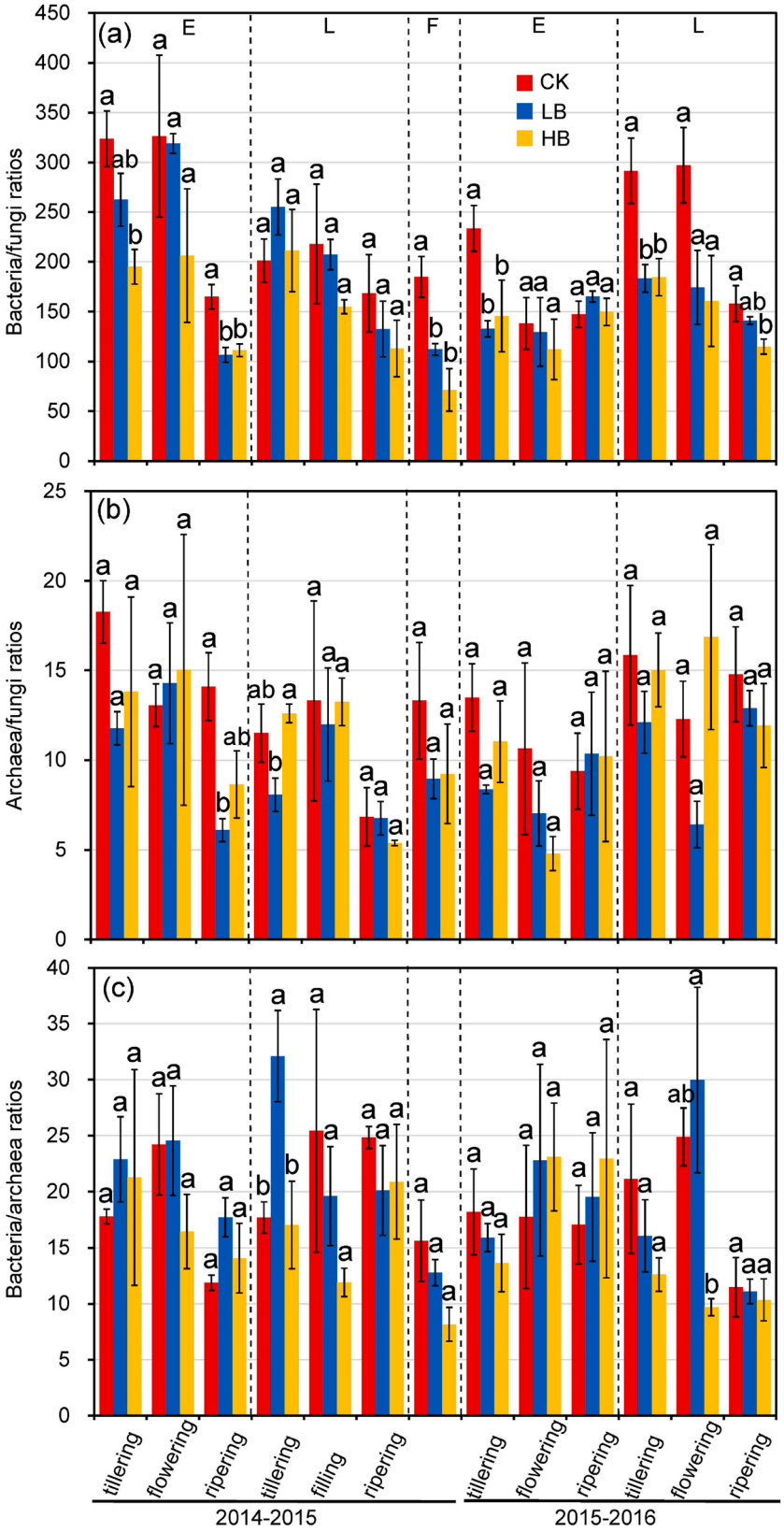

Fig. 4. Bacterial 16S rRNA: 18S rRNA (Bacteria/fungi ratios) (a), archaeal 16S rRNA: 18S rRNA (Archaea/fungi ratios) (b) and bacterial 16S rRNA: archaeal 16S rRNA (Bacteria/archaea ratios) (c) copy number ratios during the 2014–2016 annual cycles. E, L, and F: early rice season, late rice season, and fallow season, respectively. Different letters represent significant differences across the three treatments ($p < 0.05$). The vertical bars indicate standard errors (n = 3).

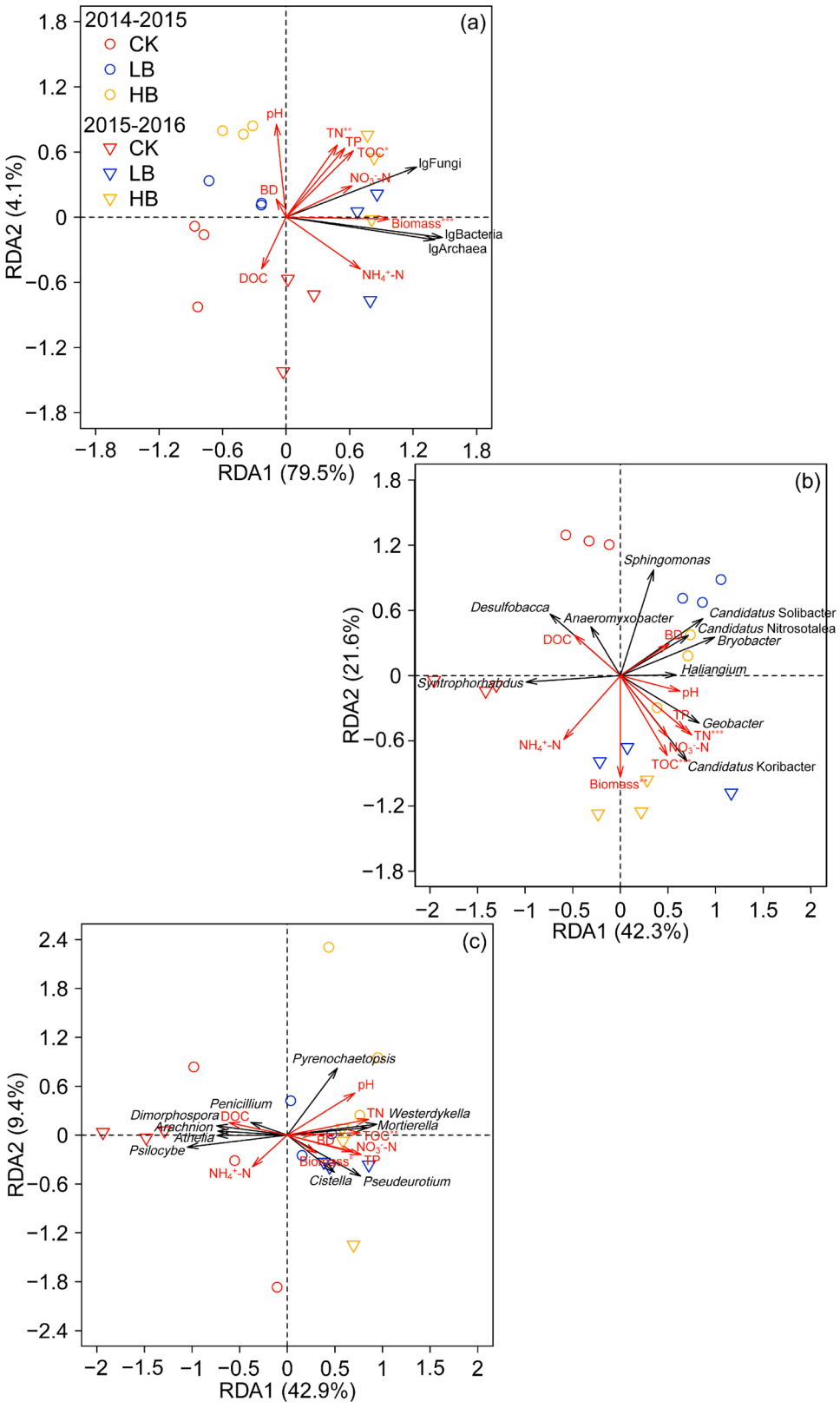

Fig. 5. Redundancy analysis (RDA) estimated the relationships among soil fertility property, rice biomass, abundance of bacteria, archaea, and fungi (a), and relative abundance of the dominant bacterial (b) and fungal (c) genera in the 2014–2016 annual cycles. 2014–2015 and 2015–2016: the 2014–2015 and 2015–2016 annual cycles, respectively. lg: base-10 logarithm of microbial abundance. Biomass: dry matter of rice biomass (t ha^{-1}). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

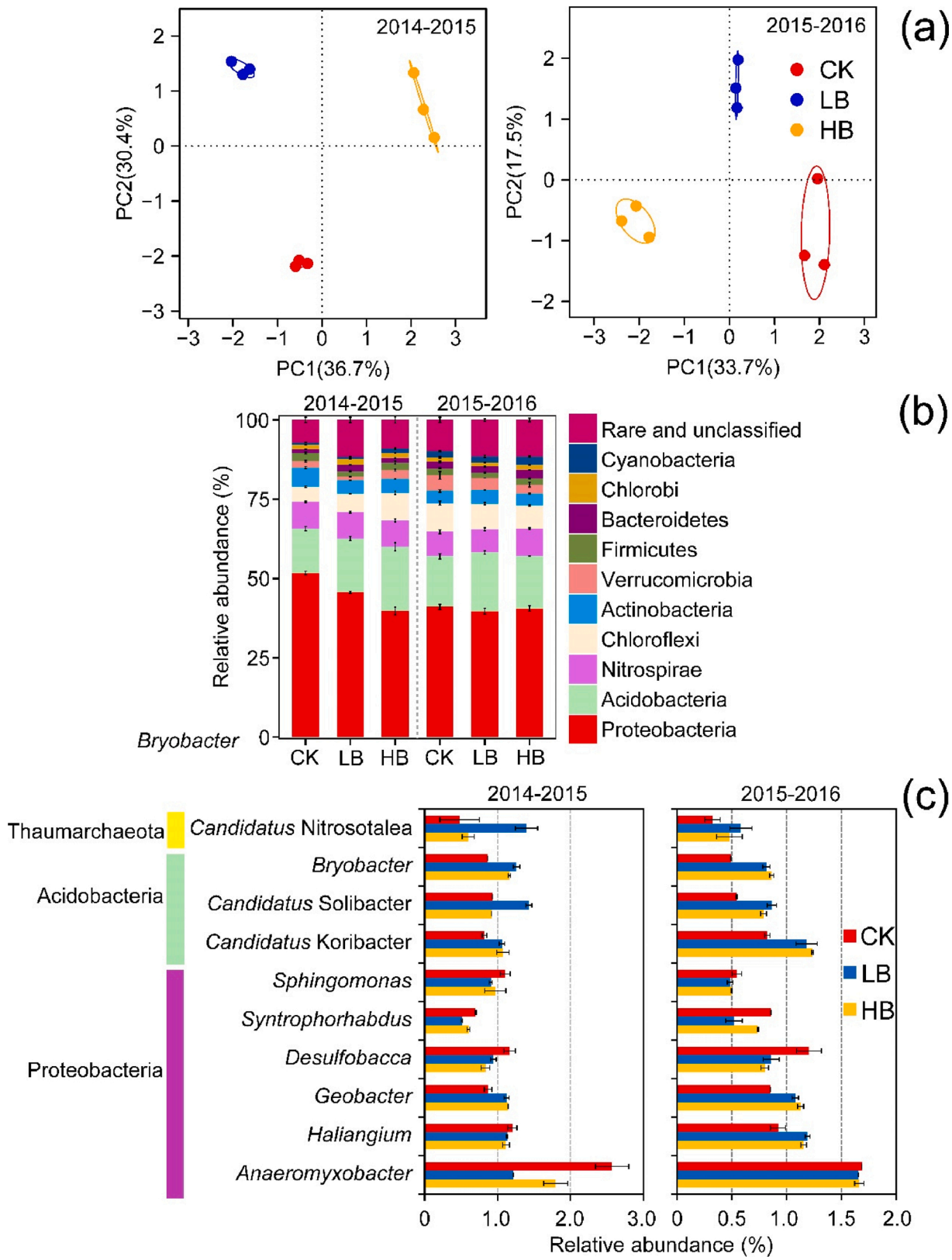

Fig. 6. Principal coordinates analysis (PCoA) based on Bray-Curtis similarities of bacterial communities (a), relative abundance (%) of the 10 most abundant bacterial phyla (b), and genera (c) under the three treatment regimes in 2014–2016. The ellipses in the plot indicate the confidence areas (0.95).

genera, of which 52.3 % was explained by the first two axes (Fig. 5c). The main fungal genera for the LB and HB treatments were separated from those in the control. Rice biomass and TOC were significantly correlated with the dominant fungal genera, accounting for 10.1 % and 24.9 % of the total variation, respectively ($p < 0.05$). TOC was positively correlated with the percentages of *Pyrenochaetopsis, Westerdykella, Mortierella, Pseudeurotium,* and *Cistella* but were negatively correlated with those of *Penicillium, Dimorphospora, Arachnion, Athelia,* and

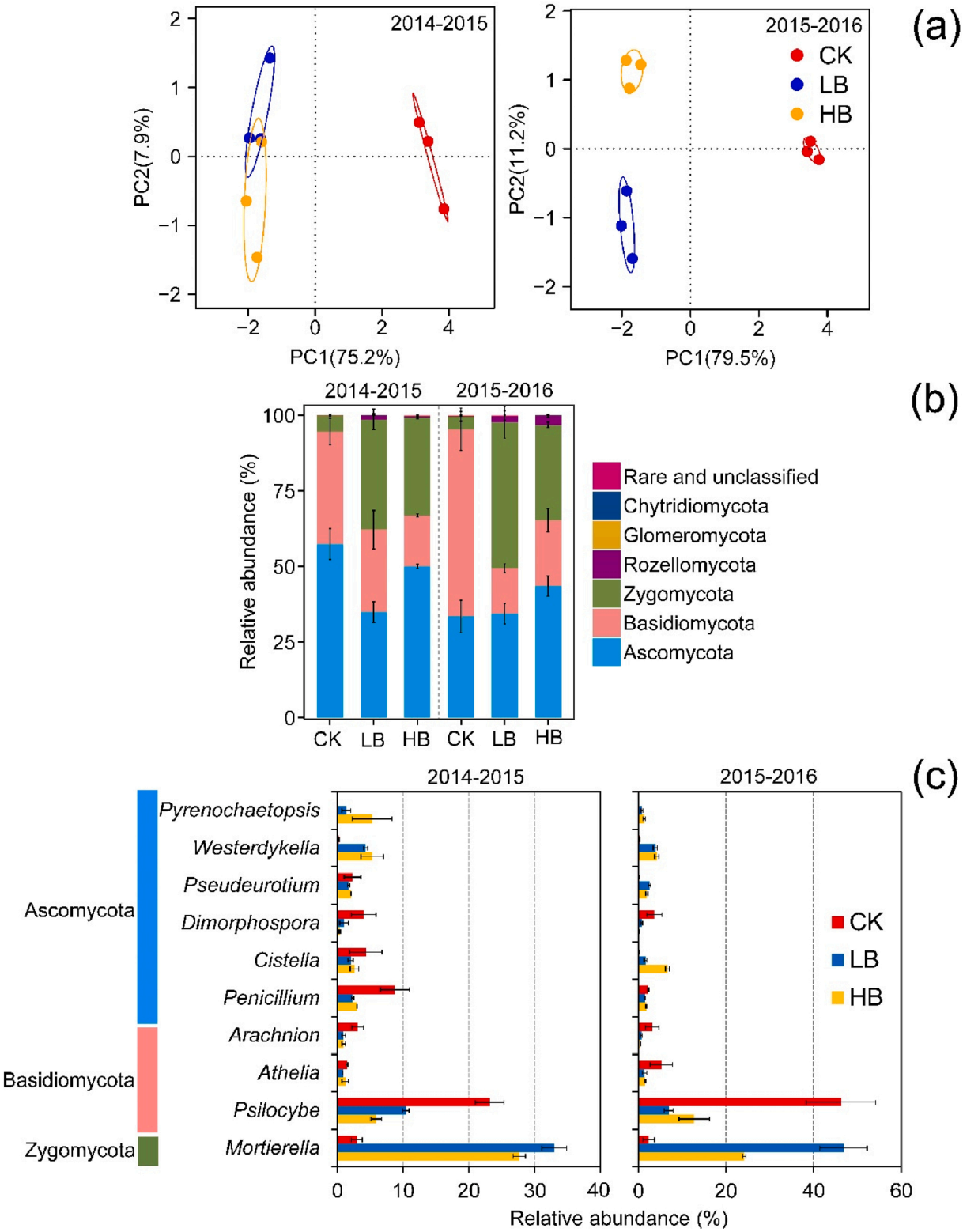

Fig. 7. Principal coordinates analysis (PCoA) based on Bray-Curtis similarities of fungal communities (a), relative abundance (%) of the dominant fungal phyla (b), and the 10 most abundant genera (c) under the three treatment regimes in 2014–2016. The ellipses in the plot indicate the confidence areas (0.95) of the treatments.

Psilocybe. Rice biomass was positively correlated with the percentages of *Westerdykella, Mortierella*, *Pseudeurotium,* and *Cistella* but were negatively correlated with those of *Penicillium, Dimorphospora, Arachnion*, *Athelia,* and *Psilocybe*.

3.6. Bacterial and fungal functional characteristics

The relative abundance of the bacterial functional groups, such as those involved in the metabolism of amino acids, energy, cofactors and vitamins, nucleotides, glycan biosynthesis and metabolism, enzyme families, replication and repair, translation, transcription, and folding,

sorting and degradation were consistently higher in the biochar treatments, except that transcription for HB treatment was lower than that for CK in 2015–2016 ($P = 0.31$). In contrast, the relative abundance of those associated with lipid metabolism, membrane transport, and xenobiotic biodegradation and metabolism for the LB and HB were consistently lower when compared with CK (Fig. 8a).

Overall, the dominant fungal functional categories in biochar treatments had consistently higher relative abundance of soil saprotrophs, litter saprotrophs, leaf saprotrophs, and endophytes than CK (Fig. 8b). In contrast, the relative abundance of undefined saprotrophs, wood saprotrophs, epiphytes, and plant pathogens were consistently reduced in biochar treatments compared with CK. Biochar treatments enhanced the relative abundance of lichenized and lichen parasites in 2014–2015 but suppressed their relative abundance in 2015–2016.

4. Discussion

4.1. Biochar stimulated microbial growth in paddy soils

Numerous researchers have shown that biochar application can strongly influence soil microbial abundance by changing soil chemical and physical properties. However, their results were mainly obtained using a few samples involving fresh biochar application during the period less than two years (Ladygina and Rineau, 2013; Lehmann and Joseph, 2015). Biochar enhances soil bacterial abundance and the bacteria/fungi ratio (Chen et al., 2013) but decreases soil fungal abundance in field experiments (Chen et al., 2013) one to two years after biochar addition, owing to the stimulation of bacterial growth and inhibition of fungal growth by the neutral or slightly alkaline soil environment (Chen et al., 2013; Rousk et al., 2009). In contrast, the current study found that biochar addition significantly increased the gene copy numbers of both

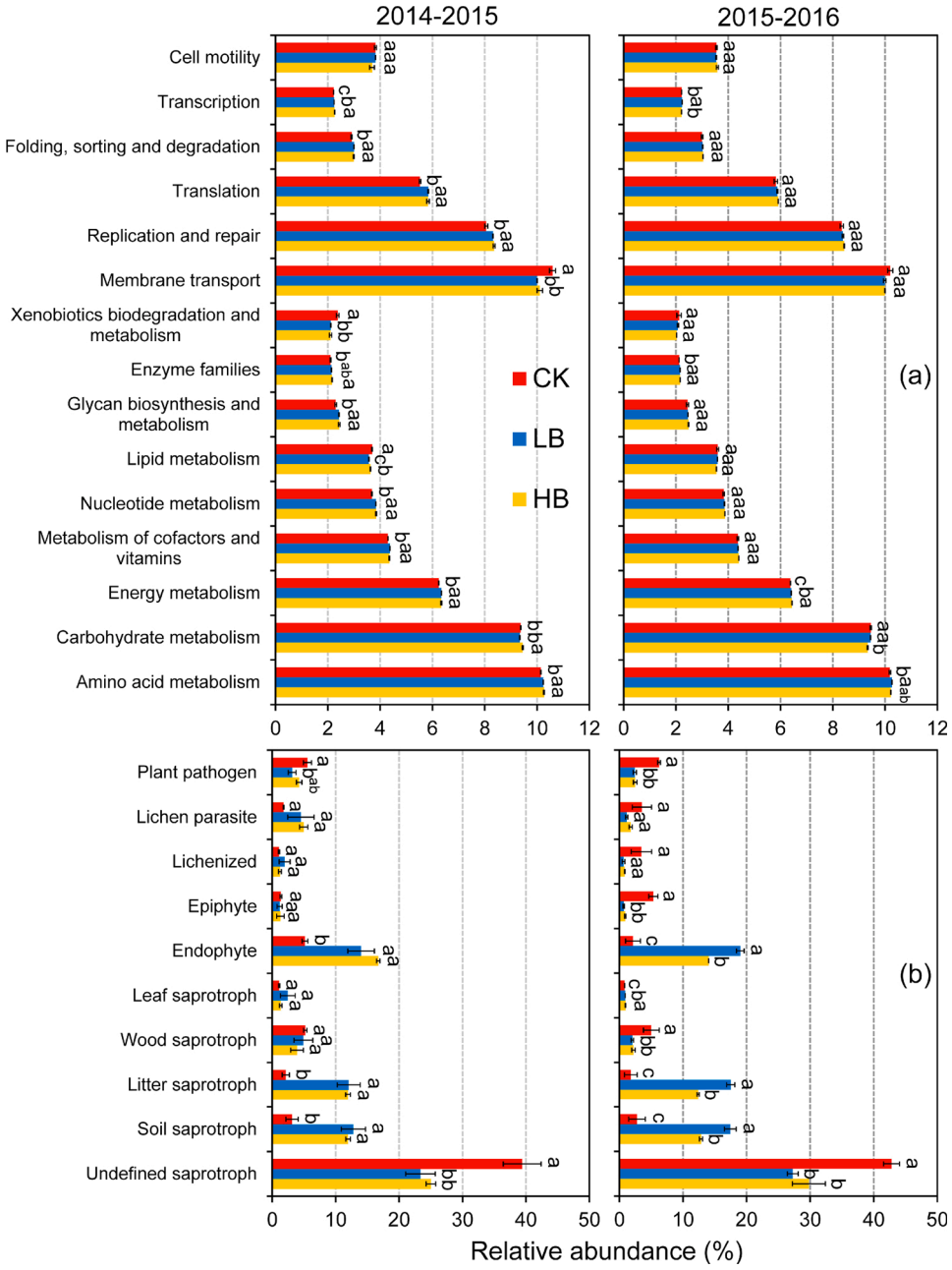

Fig. 8. Relative abundance (%) of the 15 most dominant bacterial functional categories (a), and the 10 most abundant fungal functional groups (b), under the three treatments in the 2014–2016 annual cycles. Different letters represent significant differences across the treatments ($p < 0.05$).

bacterial 16S rRNA and 18S rRNA, and significantly decreased the bacteria/fungi ratio in the third to fourth year after biochar amendment ($P < 0.05$; Figs. 2–4). In the third to fourth year after biochar amendment, soil alkaline substances were removed by rice harvest (Zhu et al., 2018), and the resulting acidic soil environment (pH 5.0–5.8) does not inhibit fungal growth (Fig. 1; Rousk et al., 2009). Moreover, the easily degradable materials (e.g., NH_4^+-N and DOC) of biochar were exhausted, resulting in the increases of the proportions of recalcitrant materials (Fig. 1; Dong et al., 2017; Wang et al., 2018a). Fungi are the major decomposers of recalcitrant materials, e.g., wood, lignins, and tannins (Wiednera and Glaser, 2013). The increase in plant residues, e.g., stubble, root, and root exudates, in the treatments was probably due to the increased rice biomass (Fig. S1; Wang et al., 2018a). Thus, the biochar-induced increases in plant residues, TN content, and the degradation of aged biochar might have increased bacterial and fungal abundance by providing more substrates, especially from the latter (Figs. 2 and S1; Brenzinger et al., 2018). This result was confirmed by RDA, which showed that TN, TOC, and rice biomass were positively correlated with bacterial and fungal abundance (Fig. 5a).

4.2. Biochar induced changes in bacterial community and potential functions

At the bacterial phylum level, biochar treatments increased the relative abundance of Acidobacteria in 2014–2016 (Fig. 6b). Similarly, Mackie et al. (2015) found that vineyard soil amended with biochar increased the relative abundance of Acidobacteria in a two year field experiment. In contrast, decreases were observed in the percentages of Proteobacteria (R-categories) and gram-positive bacteria (Actinobacteria and Firmicutes) in the current study three and four years after biochar application. The difference might be because Acidobacteria (K-categories) can outcompete copiotrophs in the condition of decreased DOC content (Fig. 1c; Meyer, 1994; Yu et al., 2018). The biochar-induced increases in Acidobacteria might have contributed to TOC accumulation and decreased DOC (Fig. 1c, f) because Acidobacteria has been identified as the major bacterial phylum in soils with low mineralization rates, which could enhance TOC accumulation, but decrease soil low molecular weight (LMW) compounds of DOC (Zhang et al., 2018). Proteobacteria (R-categories) and gram-positive bacteria (Actinobacteria and Firmicutes) generally utilize easily degradable substances from biochar, and they were decreased, when easily degradable substances (e.g., LMW DOC) from biochar were exhausted. The hardly degradable biochar-C (e.g., fused aromatic rings) might be decomposed by other microorganisms e.g., fungi (Mao et al., 2012; Yu et al., 2018).

Compared to CK, *Geobacter* was significantly increased three to four years after biochar amendment based on the 16S rRNA gene sequencing (Fig. 6c). *Geobacter* fixes nitrogen, harvests electricity from organic matter, and uses carbon compounds (Coates and Lovley, 2015). Thus, the increase in *Geobacter* might be because biochar is electrically conductive, and soil application with biochar could increase TOC content because of high recalcitrant carbon content of biochar and increased crop residues (Table S1; Figs. 1f and S1; Wang et al., 2018a; Xu et al., 2013). This is supported also by the correlation between *Geobacter* and TOC ($p < 0.001$) (Fig. 5b). The increased TN might contribute to more crop residues, which could result in the increase in the *Geobacter*. Biochar increased the abundance of *Candidatus* Koribacter, *Candidatus* Solibacter, and *Bryobacter* (belonging to K-categories Acidobacteria), which can outcompete copiotrophs in conditions of low nutrient availability (Fig. 6b, c; Catão et al., 2014; Kulichevskaya et al., 2010; Zhang et al., 2018). This is probably because biochar amendment decreased soil easily degradable substances (e.g., DOC) (Fig. 1; Chen et al., 2018a).

From the PICRUSt2 analysis, the major Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways in this study were also the major pathways in agricultural soils (Fig. 8a) (Xiao et al., 2017). Compared to CK, higher TOC, TN, TP, bacterial and fungal abundance, and rice biomass were observed in the biochar treatments (Figs. 1–3, and S1), which supported the results of PICRUSt2, i.e., the relative abundance of genes for the metabolism of amino acids, cofactors and vitamins, energy, nucleotides, enzyme families, replication and repair, glycan biosynthesis and metabolism, translation, transcription, and folding, sorting and degradation were increased by biochar application, except that the HB treatment had a lower transcription in 2015–2016 compared with CK ($p = 0.31$). This indicates that the biochar amendment enhanced the activity and turnover rate of the microbial community and plant residue degradation (Zhu et al., 2019). Compared with CK, the increase in Acidobacteria abundance and decrease in Proteobacteria and gram-positive bacteria (Actinobacteria and Firmicutes) abundance in the biochar treatments are consistent with the decrease in the lipid metabolism and xenobiotic biodegradation and metabolism abundance as predicted by PICRUSt2 (Figs. 6b and 7 a). In contrast, biochar increased the percentages of lipid metabolism and xenobiotic biodegradation and metabolism after incubation for 30 and 200 days, respectively (Liang et al., 2020; Zhu et al., 2019). This could have resulted from the fact that biochar-C was mainly decomposed by bacteria in the early phase and degraded by fungi in the late phase (Yu et al., 2018). The current findings contrast with the results of Liang et al. (2020), in which a high biochar amendment rate increased the relative abundance of membrane transport as compared with CK after 30 days of incubation. The contrasting responses of membrane transport to biochar application might be correlated with biochar residence time in soils. The microorganisms then counteract these disadvantages through membrane transporters (Fei et al., 2020). However, the soil microbial community compositions amended with biochar may be more stable than those of the CK once the soil microorganisms have adapted to the new soil environment (Nan et al., 2020). Thus, a decrease in the percentage of membrane transport three to four years after a single biochar application was found.

4.3. Biochar induced changes in fungal community and potential functions

The current study showed that Zygomycota, Basidiomycota, and Ascomycota were the major fungal phyla, and this was consistent with previous studies in paddy fields (Fig. 7b; Zhang et al., 2019). The relative abundance of Basidiomycota and Zygomycota became lower and higher after biochar amendment in the 2014–2016 annual cycles, respectively (Fig. 6b). In general, the abundance of the fungal phyla Zygomycota was higher in soils with rich organic matter, e.g., forest soil, than those in other soils (Li et al., 2017). A possible reason for the phenomenon is that the microorganisms grew rapidly in response to the increases in TOC (Fig. 1f). Biochar decreased the relative abundance of Basidiomycota possibly due to the niche competition between Basidiomycota and Zygomycota (Dai et al., 2018).

At the genus level, the paddy fungal community was dominated by *Mortierella, Psilocybe, Athelia, Arachnion, Penicillium, Cistella, Dimorphospora, Pseudeurotium, Westerdykella*, and *Pyrenochaetopsis*. The results corroborate those reported from the fungal communities in the soils (Yao et al., 2017). Biochar amendment increased the relative abundance of *Mortierella* ($p < 0.05$) (Fig. 7c). *Mortierella* is a kind of saprotroph that thrives in soil with abundant TOC, which was confirmed by RDA (Fig. 5c; Clemmensen et al., 2015). *Mortierella* assist mycorrhizal fungi and crops in phosphorus acquisition (Osorio, 2011). Furthermore, *Mortierella* have shown great capacity to decompose plant residues and to degrade aromatic hydrocarbons (Ellegaard-Jensen et al., 2013; Osono, 2005). Gkarmiri et al. (2017) found that the genus *Mortierella* primarily assimilated older structural pools through a stable isotope tracer technique; thus, biochar might be decomposed by *Mortierella*. Here, the relative abundance of *Mortierella* increased as plant litter increased from the increased rice biomass and higher soil aromatic compound content from the added biochar (Fig. S1; Wang et al., 2018a), which is supported by the RDA (Fig. 5c). Similar to *Mortierella*, biochar

increased the percentage of the saprotrophic fungi *Westerdykella* ($P < 0.05$) possibly by increasing the TOC content and rice biomass (Tedersoo et al., 2014). Similar results were obtained using RDA, which showed that TOC was positively correlated with the *Westerdykella* (Fig. 5c). A greenhouse-based pot experiment by Srivastava et al. (2012) showed that *Westerdykella* enhanced rice and pea growth, increased soil TOC, available P, and enzyme activities. On the other hand, biochar application decreased the relative abundance of *Psilocybe, Athelia, Arachnion, Penicillium*, and *Dimorphospora* belonging to the class Basidiomycota or Ascomycota, possibly through competition between Basidiomycota, Ascomycota, and Zygomycota (Fig. 6c). The fungal genera *Athelia* and *Penicillium* are potential plant pathogens in soil, which can cause necrosis by attacking plant cell walls and cause extensive decay by blue mould (Baminger et al., 2001; Gauthier, 2015; Neri et al., 2006). However, the potential mechanisms of the changes in the fungal community caused by biochar treatments remain unclear and more studies are needed.

The paddy fungal functional guilds obtained in this study corroborate the FUNGuild analyses in agricultural soils (Fig. 8b; Wang et al., 2018c). So far, only a few studies have investigated the impacts of biochar addition on the fungal functional categories three to four years after biochar amendment. Increased relative abundance of soil saprotrophs (e.g., rhizosphere fungus), litter saprotrophs, and leaf saprotrophs were found after biochar application, which might be due to enhanced soil recalcitrant carbon content and rice biomass, thus increasing soil TOC content and biomass of plant residues and exudates (e.g., stubble, roots, root exudates, and litter) (Figs. 1f and S1; Nguyen et al., 2016). Biochar further improved plant growth, soil fertility, and microbial community, which might have resulted in the increase in the percentage of endophytes in this study (Figs. 1 and 8b; Dahlman et al., 1991; Rodriguez et al., 2009). Symbiotic endophytic fungi can obtain nutrients from their plant hosts while improving host growth and enhancing host tolerance for abiotic and biotic stress, such as drought, insect herbivores, and pathogens (Dahlman et al., 1991; Omacini et al., 2001; Rodriguez et al., 2009). Biochar application decreased the relative abundance of undefined saprotrophs, wood saprotrophs, epiphytes, and plant pathogens, which might be due to the enhanced microbial and plant growth resisting epiphyte and pathogenic fungus and competing with undefined saprotrophs and wood saprotrophs (Dai et al., 2018). Dai et al. (2018) also found that biochar addition caused saprotrophic fungus to enhance competitive capacity, which could result in the relative decrease in fungal pathogens.

5. Conclusions

Our study demonstrated that biochar altered the abundance, community composition, and potential functions of soil bacteria and fungus by changing the soil fertility properties three to four years after biochar application in a double rice paddy. Biochar increased TN, TOC, and rice biomass, which significantly increased bacterial and fungal abundance. However, bacteria/fungi ratios were significantly decreased. There were no significant differences in the abundance of bacteria and fungus and in bacteria/fungi ratios between LB and HB treatments. Biochar application increased beneficial soil microorganisms due to the increases in TOC and rice biomass. However, potential phytopathogens were decreased probably due to the competitive capacity of other microbial groups and the enhancement of the plant host to resist pathogens, which was good for soil organic carbon accumulation and plant growth. These findings clarify the soil microbial mechanisms on biochar amendment that improve soil fertility and productivity a few years after its application. Further research is necessary to study the microbial mechanisms associated with the long-term (> five years) impact of biochar application on soil fertility and productivity.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agee.2020.107291.

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