Biochar and nitrogen fertilizer promote rice yield by altering soil enzyme activity and microbial community structure

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Abstract

Biochar can significantly change soil properties and improve soil quality. However, the effects of long-term combined application of biochar (B) and nitrogen (N) fertilizer on relationships between soil enzyme activity, microbial community structure, and crop yield are still obscure. We characterized these relationships in a long-term (8 years) field experiment with rice, two biochar rates of 0 and 13.5 t ha$^{-1}$ year$^{-1}$ (B0 and B) and two N fertilizer rates of 0 and 300 kg N ha$^{-1}$ year$^{-1}$ (N0 and N). The repeated, long-term combined applications of biochar and N fertilizer significantly increased microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN), but biochar decreased the abundance of total bacteria, fungi, actinomycetes, and gram-positive and gram-negative bacteria as well as the amount of total phospholipid fatty acids. The activity of leucine aminopeptidase (LAP) decreased significantly in the biochar-amended and N fertilized treatment, but the LAP activity either remained unchanged or increased with biochar amendment at N0. The relative abundance of bacterial phylum Chloroflexi was increased in the combined biochar and N fertilizer treatment. The changes in soil organic matter and the activity of α-1,4-xyllosidase were the major properties influencing soil bacterial community composition, whereas the structure of fungal community was governed by MBC, MBN, and LAP activities. In addition, long-term biochar and N fertilizer applied together significantly increased rice yield (more than biochar and nitrogen fertilizer applied alone). Yield was significantly positively correlated with LAP activity, but significantly negatively correlated with the relative abundance of Chloroflexi. In conclusion, long-term biochar and nitrogen fertilizer applications increased rice yield, which was associated with altered soil microbial community and enhanced activity of some enzymes.

Keywords

biochar, enzyme activity, long-term experiment, microbial community, nitrogen fertilizer, phospholipid fatty acids, rice yield
1 | INTRODUCTION

Excessive nitrogen (N) fertilizer input in the areas of intensive agriculture in China has caused many environmental problems (Ju et al., 2009; Zhang et al., 2013). At present, combined application of biochar and N fertilizer has been considered one of the most effective and sustainable agricultural practices for reducing environmental risk and improving soil fertility and crop yield (Ibrahim et al., 2020; Liao et al., 2016; Sekaran et al., 2020). Biochar has the highly aromatic and porous structure that alters soil chemical and physical properties and enzyme activity, improves soil nutrient retention, and provides habitat for soil microorganisms (Lehmann et al., 2011; Liu et al., 2021). Soil microorganisms can mediate the involvement of soil enzymes in nutrient cycling by altering the soil microecological environment, which, in turn, can have an impact on yield (Lehmann et al., 2011). Although there are studies on the changes in soil microbial communities, soil enzyme activities, and crop yield under biochar application (Amoakwah et al., 2022; Kuzyakov et al., 2009; Li et al., 2022; Pokharel et al., 2020), so far none of these studies has elucidated the relationship between soil microorganisms, enzyme activities, and yield increase under long-term biochar application.

The abundance and activity of soil microorganisms are important indicators of soil quality (Li et al., 2021). Extracellular enzymes allow microorganisms to obtain building blocks and energy from complex biomolecules present in the soil, providing the driving forces for biogeochemical cycles in the soil ecosystem (Lopes et al., 2021). Thus, soil enzymes and soil microorganisms have an indispensable role in soil organic matter decomposition, nutrient release, and yield maintenance.

Biochar application could influence microbial communities by altering soil physicochemical properties and nutrient cycling (Anderson et al., 2011; Lehmann et al., 2011; Wagg et al., 2014; Zheng et al., 2019). For example, an increased soil organic carbon (SOC) content and higher abundance of bacteria were observed during 1-year biochar application (Tian et al., 2019). Similarly, meta-analysis showed that biochar consistently increased some physicochemical properties (e.g., soil pH, total nitrogen [TN], and cation exchange capacity) and altered microbial parameters (e.g., microbial abundance and community structure) in many soils in both short-term laboratory incubations (≤90 days) and field studies (1–3 years) (Ameloot, De Neve, et al., 2013; Ameloot, Graber, et al., 2013; Gul et al., 2015; Lehmann et al., 2011; McCormack et al., 2013). However, it remains unclear how repeated biochar additions over long time change the soil microbial community structure, and ultimately crop yield.

Soil enzyme activity can influence soil fertility and crop yield by participating in the decomposition of soil organic matter and the nutrient cycling (Lehmann et al., 2011). Biochar has large specific surface area that helps improve soil properties and soil enzyme activity (Abbas et al., 2018; Zhang et al., 2021). Meta-analysis showed that biochar significantly increased the activities of urease and alkaline phosphatase (Pokharel et al., 2020). However, soil is a multiphase medium, and enzyme activity is directly or indirectly influenced by various soil conditions (Meena & Rao, 2021). Hence, some studies have shown that the effect of biochar application on enzyme activity and crop yield varied greatly depending on soil conditions (Czimczik & Masiello, 2007; Lammirato et al., 2011; Pokharel et al., 2020).

The biochar-mediated changes in soil physicochemical properties and shifts in microbial community structure vary in upland and paddy fields (Gul et al., 2015). Most published studies deal with the effect of biochar application in upland conditions; for example, Yu et al. (2018) showed that biochar and nitrogen fertilizer application increased soil microbial biomass but did not alter microbial community structure in soybean field. Xu et al.'s (2020) meta-analysis found that gram positive bacteria, gram negative bacteria, and total phospholipid fatty acid (PLFA) did not significantly change with biochar. In addition, Ibrahim et al. (2020) found that biochar–fertilizer interactions reduced bacterial diversity in yellow soil. Given large differences in environmental factors in paddy vs upland fields that shape the microbial communities associated with plant roots, more work on characterizing the effects of long-term biochar application is needed in rice paddies. In flooded rice soil, there are aerobic and anoxic zones, associated with the selection of specific microbial groups with aerobic, anaerobic, or facultative metabolism (Breidenbach et al., 2016; Brune et al., 2000). Short-term biochar amendment reduced fungal and increased bacterial abundance in an acid paddy soil (Chen et al., 2016). Other studies confirmed that short-term biochar application increased crop yield by improving microbial abundance, community structure, and enzyme activities (Ali et al., 2020; Kannan et al., 2021). However, none of these studies focused on the impact of long-term, repeated biochar applications either alone or in combination with nitrogen fertilizer on the microbial community structure as well as the relationships among various constituents of microbial community and rice yield.

The main objective of this study was to answer the three questions on how repeated, long-term biochar and nitrogen fertilizer additions: (a) create new habitats and alter the soil environment for microorganisms, which may lead to changes in microbial abundance, community structure, and activity, and (b) change the enzyme activities, and (c)
whether the changes in these soil indicators can be related to rice yield.

2 | MATERIALS AND METHODS

2.1 Site description and experimental design

The biochar and nitrogen (N) amendment study was conducted at Yesheng Town, Qingtongxia City, China (106°11′35″E, 38°07′26″N). The study area has a temperate continental monsoon climate with an average temperature of 8.9°C. The mean annual precipitation is 193 mm. The organic matter in the surface layer (0–20 cm) was 16.1 g kg⁻¹, and TN and total phosphorus (TP) were 1.08 and 0.60 g kg⁻¹, respectively. Soil pHwater was 8.49, and the soil bulk density was 1.56 g cm⁻³.

The experiment was established in 2012 and was designed to test two factors: with or without biochar (0 and 13.5 t ha⁻¹) and with or without N fertilizer (0 and 300 kg N ha⁻¹). The experiment was set up in randomised complete block design with three replications. The plot size was 13 m × 5 m. The rice variety Ningjing 43 was planted on April, transplanted on May, and harvested on September in 2012. The N fertilizer applied was urea (N, 46% w/w) at the rate of 150 kg N ha⁻¹. Double superphosphate (P, 20% w/w) and potassium chloride (K, 50% w/w) were applied as basal fertilizers at the rates of 39.3 kg P ha⁻¹ and 74.5 kg K ha⁻¹ along with biochar before transplanting rice. Later, 90 and 60 kg N ha⁻¹ were applied in the seedling stage and jointing stage, respectively.

Biochar was produced by pyrolysis of wheat straw at 350°C by the Sanli New Energy Company, Shandong Province, China. Biochar had C, N, and P contents (w/w) of 65.7%, 0.5%, and 0.1%, respectively, with a pHwater of 7.78 (Liu et al., 2021). Biochar and fertilizers were broadcast on the soil surface and incorporated into the soil by ploughing to a depth of approximately 20 cm. To maintain consistency, ploughing was also performed in the plots without biochar. Crop management was consistent across plots and years.

2.2 Soil sampling

Soil samples (0–20 cm) were obtained on October in 2019. Five soil cores from two diagonal lines through each plot were collected and homogenized to form a composite sample. Soils were transported to the laboratory in a refrigerator. Each sample was divided into three parts: one was air-dried and passed through a 2 mm sieve for determination of the soil physicochemical properties; second was stored at 4°C for the soil enzyme analyses and PLFA; and the third was stored at −80°C for microbial analysis.

2.3 Soil physicochemical analyses

SOC was measured using the oxidation method (Bao, 2000). Soil TN content was determined using the Kjeldahl method (Bao, 2000). The available phosphorus (AP) was determined by the Olsen-P method. Available potassium (AK) was extracted with ammonium acetate and measured by atomic absorption spectrophotometry. TP content was measured after digestion in HF-HClO₄. Soil pH (1:2.5, soil:water) was measured by the potentiometric method. Nitrate (NO₃⁻-N) and ammonium (NH₄⁺-N) nitrogen were determined using a continuous flow analyzer. Soil microbial biomass carbon (MBC) and soil microbial biomass nitrogen (MBN) were determined by the chloroform fumigation direct extraction method as described in Beck et al. (1997).

2.4 Soil enzyme activity analyses

We measured the activities of six soil hydrolytic enzymes (α-1,4-glucosidase [AG], β-1,4-glucosidase [BG], β-D-cellobiohydrolase [CB], β-1,4-xyllosidase [BX], β-1,4-N-acetyl-glucosaminidase [NAG] and leucine aminopeptidase [LAP]), and two oxidative enzymes (polyphenol oxidase [PPO] and peroxidase [PER]). For all enzymes, we used six analytical replicates for each soil sample and control. We measured enzyme activities following a method modified from Jing et al. (2016) using fluorometry. The detailed process was described by Sun, Li, et al. (2021) and Jing et al. (2016).

2.5 PLFA analysis

PLFA was performed based on the method of Frostegård et al. (1993) and Buyer and Sasser (2012). Briefly, 2 g (dry weight equivalent) of soil was extracted in a mixture consisting of dichloromethane, methanol, and citrate buffer (1:2:0.8, v/v/v). Lipids were extracted and fractionated by solid-phase extraction. The fatty acids from phospholipids were converted to fatty acid methyl esters (FAME) by transesterification and were qualitatively and quantitatively analyzed using an Agilent 7890A GC equipped with a CP-7693 autosampler and a flame ionization detector. Ten milliliters of methyl nonadecanoate fatty acid (19:0; Sigma-Aldrich) were added as an internal standard. Identification of peaks
was based on comparison of retention times to known standards (MID’s Sherlock software system). The concentration of individual PLFAs was expressed as ng PLFA-C g⁻¹ soil. Amounts were derived from the relative area under specific peaks, as compared with the 19:0 peak value (internal standard), which was calibrated according to the standard curve made from a range of concentrations of the 19:0 FAME standard dissolved in hexane. Individual fatty acids were used as signatures for various functional groups of microorganisms. PLFAs were classified according to the literature (Buyer & Sasser, 2012; Frostegård et al., 1993) and were divided into five microbial groups (Dominchin et al., 2021; Moore-Kucera & Dick, 2008) listed in Table 1.

2.6 | DNA extraction

Soil DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories) following the manual. Purity and quality of the genomic DNA were checked on, respectively, 1% w/v agarose gels and a NanoDrop spectrophotometer (Thermo Scientific).

2.7 | Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA gene hypervariable V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Dennis et al., 2013). For fungi, PCR amplification of the ITS1 region was performed using the forward primer ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and the reverse primer ITS2 (5′-TGCGTTCTTCATCGATGC-3′) (Wang et al., 2020). The PCR was carried out on a Mastercycler Gradient (Eppendorf, Germany) using 25 μl reaction volumes, containing 12.5 μl 2x Taq PCR MasterMix, 3 μl BSA (2 ng/μl), 1 μl forward primer (5 μM), 1 μl reverse primer (5 μl), 2 μl template DNA, and 5.5 μl ddH₂O. Cycling parameters were 95°C for 5 min, followed by 28 cycles of 95°C for 45 s, 55°C for 50 s and 72°C for 45 s, with a final extension at 72°C for 10 min. The PCR products were purified using an Agencourt AMPure XP Kit.

2.8 | High-throughput sequencing

Deep sequencing was performed on a Miseq platform at Allwegene Company (Beijing). After the run, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline Version 2.6.

2.9 | Sequence analysis

After allocation to each sample, the raw sequencing reads were trimmed using QIIME (version 1.8.0) with threshold of quality score higher than 20 over a 10 bp sliding window and the minimum length of 230bp. Paired-end reads were assembled using FLASH. The reads were compared with Gold Database using the UCHIME algorithm to detect chimera sequences. After chimera removal, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at a similarity level of 97% using the Uparse algorithm of Vsearch (v2.7.1) software. The representative sequences of each OTU for bacteria and fungi were selected and classified by the Ribosomal Database Project Classifier tool with a confidence threshold of 70%.

2.10 | Statistical analysis

The differences in soil physicochemical properties, enzyme activities, PLFAs, and soil microbial α-diversity were analyzed by the two-way analysis of variance (ANOVA) with Tukey multiple range test (p < 0.05). An OTU-based analysis was performed to calculate the richness and diversity of samples, including Chao1 and Shannon diversity indices.

| TABLE 1 | The types of phospholipid fatty acids (PLFAs) specific for various microorganisms |
|---|---|
| **PLFA types** | **Bacteria** |
| | i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, 16:1ω7c, 17:1ω8c, 17:1ω9c, 18:0, 18:1ω5c, 18:1ω7c and cy19:0 |
| **Gram-positive bacteria (G+)** | i15:0, a15:0, i16:0, i17:0 and a17:0 |
| **Gram-negative bacteria (G−)** | cy17:0, 16:1ω7c, 17:1ω9c, 18:1ω5c and 18:1ω7c |
| **Actinomycetes** | 10Me16:0, 10Me17:0 and 10Me18:0 |
| **Fungi** | 16:3ω6c, 18:1ω9c, 18:2ω6c and 9c |
Pearson correlation analysis was used to explore the relationships of (1) relative microbial abundance with soil properties and enzyme activities and (2) yield with soil properties, enzyme activities, and the relative abundance of microbial taxa. The pivotal and credible predictors of rice yield among different factors were evaluated using a Random Forest Analysis. All statistical analyses were performed using R version 3.5.1 and IBM SPSS version 25.0.

3  RESULTS

3.1  Soil physicochemical properties and rice yield

Biochar addition had a significant effect on SOC, TN, AP, and AK (Figure 1; Table S1). Compared with B0, biochar application significantly increased the SOC, TN, AP, and AK content (by 45%, 6.3%, 60%, and 53%, respectively) (Figure 1A–D). In addition, compared with N0, N fertilizer significantly (by 8.1%) increased TN content (Figure 1B). Total P was around 0.6 g kg⁻¹, nitrate-N 0.16 mg kg⁻¹, and ammonium-N 0.81 mg kg⁻¹, with no significant difference among the four treatments (Table S1). By contrast, MBC and MBN were significantly influenced by the interaction of biochar and N fertilizer (Figure 1E,F; Table S1). The MBC and MBN both increased significantly with biochar amendment at N0, but either remained unchanged or decreased with biochar addition in the N fertilization treatment (Figure 1E,F). Rice grain yield did not change with biochar amendment at N0, but increased significantly in the N fertilization treatment (Figure 1G).

3.2  Soil enzyme activity

Biochar addition had a significant effect on PPO (Figure 2B; Table S1). Compared with B0, biochar application significantly (by 31%) increased the PPO activity (Figure 2B). The N fertilization had a significant effect on activities of AG and BX. Compared with N0, N fertilizer significantly (by 82%) increased AG activity (Figure 2C), but significantly (by 18%) decreased BX activity (Figure 2F; Table S1).

The interaction biochar × N significantly influenced activities of PER, BG, CB, and LAP (Table S1). The PER, BG, CB, and LAP activities either remained

![Figure 1](image-url)
unchanged or increased with biochar amendment at N0 (Figure 2A,D,E,G), but the activity of both PER and LAP decreased significantly with biochar amendment in the N fertilization treatment (Figure 2A,G). The activity of BG did not change with biochar amendment in the N fertilization treatment (Figure 2D). The activity of NAG...
was around 0.66 nmol g$^{-1}$, with no significant difference among the four treatments.

### 3.3 The concentration of PLFA

Gram-positive bacteria, gram-negative bacteria, and total PLFAs were all influenced by the main effects of biochar and N fertilizer. Biochar application significantly decreased the abundance of gram-positive bacteria, gram-negative bacteria, and the concentration of total PLFAs (by 35%, 28%, and 29%, respectively), whereas N fertilizer significantly increased all three parameters (by 9%, 11%, and 7%, respectively) (Figure 3A,B,F). The concentration of fungal PLFAs was significantly influenced by the interaction of biochar and N fertilizer, with a significant decrease with biochar amendment in either the N0 or N fertilization treatment (Figure 3D). Compared with B0, biochar application significantly (by 28% and 27%, respectively) decreased the concentrations of PLFAs of bacteria and actinomycetes (Figure 3C,E).

**FIGURE 3** The gram-positive (A), gram-negative (B), total bacteria (Bacterial; C), total fungi (Fungal; D), actinomycetes (E) and total phospholipid fatty acids (Total PLFAs; F) amounts as influenced by the treatments with rates of biochar 0 (B0) and 13.5 t ha$^{-1}$ (B) with 300 kg N ha$^{-1}$ (N) or without nitrogen fertilizer (N0). Data are means $\pm$ SE ($n = 3$). When the interaction between biochar and N fertilizer was significant (fungal), different lowercase letters show statistically significant differences ($p < 0.05$). When the interaction was not significant (gram-positive, gram-negative, bacterial, actinomycetes, and total PLFAs), only the significant main effects were presented, and the t-test was run to assess the differences between the two rates. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. 
3.4 Soil bacterial and fungal community composition and diversity

The bacterial and fungal community composition was similar in different treatments, but the abundance was slightly different (Figure 4). For bacterial communities, BN increased the relative abundance of Chloroflexi by 52%–128% (*p* < 0.05) and decreased the relative abundance of Proteobacteria by 34%–61% (*p* < 0.05) relative to other treatments (Figure 4A). The relative abundance of Actinobacteriota increased by 2.3% (*p* < 0.05) in BN0 treatment and decreased by 31% (*p* < 0.05) in BN treatment relative to B0N0 (Figure 4A). Compared to B0N0, there was no significant effect of BN0, B0N, and BN on the relative abundance of Acidobacteriota, Gemmatimonadota, Myxococcota, Desulfobacterota, and Verrucomicrobiota (Figure 4A).

For fungal communities, BN0, B0N, and BN had no significant effect on the relative abundance of Ascomycota, Kickxellomycota, Mortierellomycota, Basidiomycota, and Chytridiomycota compared with B0N0 (Figure 4B). In comparison with B0N0, B0N and BN increased the relative abundance of unidentified phylum by 90% (*p* < 0.05) and 70% (*p* < 0.05), respectively (Figure 4B).

The Shannon diversity index of bacterial and fungal taxa was around 9.96 and 5.27, respectively, with no significant difference among the four treatments. The interaction biochar × N significantly influenced the bacterial and fungal Chao1 indices (Figure 5; Table S2). The bacterial Chao1 index was significantly lower in the BN treatment than B0N0 and B0N treatments (Figure 5A). For the fungal community, the Chao1 index was significantly higher in B0N treatment than other treatments (Figure 5B).

3.5 Relationships of the concentration of PLFAs and soil microbial community composition with soil physicochemical properties and enzyme activities

Gram-positive bacteria, total bacterial, and total PLFAs were correlated significantly and positively with NO$_3^-$-N, but negatively with AK (Figure 6A). There was a significant negative correlation between AK and gram-negative bacteria. Actinomycetes showed a positive correlation with NO$_3^-$-N (*p* < 0.05) (Figure 6A). The concentration of total bacterial PLFAs was correlated significantly and negatively with SOC (*p* < 0.05) and TP (*p* < 0.05) (Figure 6A).

The relative abundance of Gemmatimonadota had a significant negative correlation with SOC, TN, and NH$_4^+$-N, but a positive one with pH (Figure 6A). The relative abundance of Actinobacteriota showed a significant negative correlation with activities of AG and CB, and a positive correlation with pH (Figure 6A). The relative abundance of Campilobacterota had a significant negative correlation with activities of MBN, PER, and BG, whereas the relative abundance of Campilobacterota was correlated significantly and positively with BX activity (Figure 6A). There was a significant negative correlation of AG activity with the relative abundance of Elusimicrobiota and Proteobacteria, but a positive one with the relative abundance of Chloroflexi (Figure 6A). Moreover, the relative abundance of Chloroflexi showed a significant positive correlation with SOC, TN, and CB, but a significant negative correlation with pH (Figure 6A). The relative abundance of phyla Acidobacteriota and Verrucomicrobiota was correlated positively with AP activity (*p* < 0.05), and the relative abundance of phylum Nitrospirota was correlated negatively with CB activity.

**FIGURE 4** The relative abundance of the bacterial (A) and fungal phyla (B) as influenced by the treatments with no biochar and no nitrogen fertilizer (B0N0), biochar and no nitrogen fertilizer (B0N), no biochar and conventional nitrogen fertilizer (B0N), and biochar and conventional nitrogen fertilizer (BN).
The bacterial Chao1 index was correlated negatively with SOC ($p < 0.05$) and NO$_3^-$-N ($p < 0.05$) (Figure 6A). The bacterial Shannon index was correlated significantly and positively with MBC, MBN, PER, and LAP (Figure 6A).

The relative abundance of Mortierellomycota showed a significant negative correlation with PER activity (Figure 6A). The relative abundance of Glomeromycota was correlated positively with TP ($p < 0.05$), AP ($p < 0.01$), and AK ($p < 0.05$) (Figure 6A). The relative abundance
of Basidiobolomyctota showed a positive correlation with TN (p < 0.05), MBC (p < 0.01), MBN (p < 0.01), and LAP (p < 0.01), but a significant negative correlation with the activity of C-cycling enzyme BX (p < 0.05) (Figure 6A). NAG was correlated significantly and positively with the relative abundance of Ascomycota (Figure 6A). The fungal Chaol index was correlated negatively with BX activity (p < 0.05), but positively with MBC (p < 0.01), MBN (p < 0.01), and LAP activity (p < 0.01) (Figure 6A).

3.6  Relationships of rice yield with soil physicochemical properties, enzyme activities, and microbial community composition and diversity

Soil enzyme activities (AG and LAP) and soil properties (TN, MBN, and MBC) showed a significant positive correlation with yield (Figure 6B). The relative abundance of Proteobacteria was correlated significantly and positively with yield (Figure 6B). The relative abundance of bacteria Chloroflexi showed a positive correlation with yield (p < 0.01) (Figure 6B). Yield was correlated significantly and negatively with pH (Figure 6B).

The random forest model explained 63.4% of the variance in yield, with the activities of AG and LAP being the most important in influencing yield (Figure 6C). In addition, MBC, the relative abundance of Chloroflexi and Basidiobolomyctota, and bacterial Cha01 index were also important in influencing yield (Figure 6C).

4  DISCUSSION

4.1  Effects of biochar and N fertilizer application on soil physicochemical properties and enzyme activities

Biochar significantly increased SOC content (Figure 1A). Similar findings were reported by Liu et al. (2016) who observed an increased SOC content with the application of biochar. This can be explained by the high carbon content of biochar, which is mainly recalcitrant aromatic C, increasing the SOC stock (Zheng et al., 2016). Although our results are consistent with those of others showing that biochar addition significantly increased SOC, the higher increase in SOC in our study compared to that of Liu et al. (2016) may be due to the fact that important influencing factors, such as the biochar C:N ratio and soil pH, determine the effect size of biochar application on SOC (Zheng et al., 2016). Our field was alkaline soil, and the greatest effects of biochar on SOC were found in alkaline soils (Zheng et al., 2016).

Biochar can increase soil MBC, especially when applied together with fertilizer (Oladele et al., 2019). In this study, it was found that biochar application significantly increased MBC and MBN content (Figure 1E,F). This is consistent with the study of Liu, Zhang, et al. (2018). A potential explanation was that biochar, with its porous structure and huge specific surface area, can retain and balance soil moisture, air and nutrients and thus improve the microbial living environment. Moreover, biochar may become a new carbon source for microorganisms after degradation in soil, promoting microbial growth (Smith et al., 2010). Importantly, the combination of biochar and N fertilizer significantly increased MBC and MBN content compared to biochar alone (Figure 1E,F). This may be because biochar can adsorb nitrogen and other nutrients, thus creating a nutrient-dense microenvironment that provides ideal conditions for the growth of microorganisms (Oladele et al., 2019).

Organic material application can influence soil enzyme activities (Burns et al., 2013). The combined biochar and N fertilizer application significantly increased BG activity (Figure 2D). This is similar to Günel et al. (2018) who found that combination of dairy effluent biochar and mineral fertilizer significantly increased BG activity. In addition, the combined biochar and N fertilizer application significantly increased CB activity (Figure 2E), which might have been due to increased substrate availability and microbial activity increasing extracellular enzyme activity (Sekaran et al., 2020).

4.2  Effects of biochar and N fertilizer application on the concentration of PLFAs and bacterial and fungal community composition

The effect of biochar on soil microbial communities could be positive, neutral, or detrimental (Lehmann et al., 2011). We found that biochar application significantly decreased the concentrations of gram-positive, gram-negative, total bacterial, total fungal, and total PLFAs (Figure 3), likely due to a high aromatic C content in biochar that may adversely affect microbial growth and survival (Zhang, Guo, et al., 2018; Zhang, Jing, et al., 2018). The total PLFAs concentrations were the most sensitive indicators related to microbi ally mediated changes in soil nitrogen (Liu, Huang, et al., 2018). Our research found that the gram-positive, total bacterial, actinomycetes, and total PLFAs concentrations were positively correlated with NO3−-N (p < 0.05) (Figure 6A). Considering that the total PLFAs are usually strongly influenced by the total biomass of living cells, this identified relationship highlights the
link between microbial growth and available concentration of NO$_3^-$-N (Yang et al., 2022).

Changes in the soil properties after biochar and N fertilizer application were the major factors influencing the soil microbial community composition (Muhammad et al., 2014). In the present study, we observed some significant changes in bacterial and fungal community composition, with greater variation in bacterial than fungal community composition (Figure 4), perhaps because the labile C substrate supplied by biochar may favor fast-growing bacteria than fungi (Liu et al., 2019). For bacterial communities, Acidobacteria was the third most abundant phylum in the irrigated silt soil, associated with the decomposition of soil organic matter and the formation of humus (Yang et al., 2018). We found the increased relative abundance of Acidobacteria in BN treatment (Figure 4A). The possible explanation is that biochar includes more recalcitrant C, the types of C input have been found to be a major driver of changes in the soil bacterial community structure (Li et al., 2017).

Proteobacteria (including Alpha-, Beta-, Gamma-, and Deltaproteobacteria classes) are fundamental microorganisms in soil carbon cycle, and they thrive in soils with C and N contents (Trivedi et al., 2013). However, the relative abundance of Proteobacteria significantly decreased in the BN treatment (Figure 4A), which was further confirmed by the negative correlations between the SOC, TN, and the relative abundance of Proteobacteria (Figure 4A). Biochar may act as a substrate to change the growth and activities of some specific microbial taxa (Mitchell et al., 2015; Zheng et al., 2016). Prayogo et al. (2014) showed that biochar application may result in negative priming effect under field conditions over a long period of time.

The diversity of bacterial and fungal communities was affected by biochar and N fertilizer application (Figure 5). In this study, BN treatment had the lowest bacterial richness as indicated by Chao1 index (significantly lower than in the B0N treatment) (Figure 5A). Combined biochar and N fertilizer application generally reduced bacterial diversity relative to biochar alone, although biochar has the ability to retain N provided by the nitrogen fertilizer, biochar may not have enough sequestration capacity to retain much of the N provided by the fertilizer for microbial use (Li et al., 2020; Muhammad et al., 2014). In addition, B0N had the higher fungal Chao1 index than the other treatments (Figure 5B), which might be a compensatory effect of the lowest bacterial richness in that treatment. The increased competition between microbial communities may lead to an increase in community diversity (Ratzke et al., 2020).

### 4.3 Yield changes after long-term biochar and N fertilizer application

A number of factors, including changes in soil physicochemical properties, enzyme activities, microbial community composition and diversity, can influence crop yield (Lehmann et al., 2011). Compared to control (B0N0), rice yield was increased significantly in the treatments with biochar alone, N fertilizer alone and combined biochar and N fertilizer application (Figure 1). This finding was consistent with the results of Bai et al. (2022). Biochar alters soil physical and chemical properties as well as microbial properties, thus indirectly influencing crop growth (Diatta et al., 2020; Xu et al., 2021). In addition, our results showed that biochar with nitrogen fertilizer recorded the highest yield, increases compared with no nitrogen fertilized control (Figure 1). We support previous studies in which the biochar co-applied with both organic and inorganic fertilizer increased crop yield (Bai et al., 2022; Ye et al., 2020). This is because mixing biochar with organic and/or inorganic fertilizers can increase nutrient retention capacity, decrease nutrient leaching, facilitate improvements in soil pH, porosity and aggregate stability, and modulate the composition of the soil microbial community (Bai et al., 2022; Sun, Xiong, et al., 2021). Increased soil nutrient retention capacity and root colonization can also boost the ability of plants to acquire or assimilate more nutrients, leading to increased yields (Joseph et al., 2021). In our study, the changes in MBC and MBN were similar under biochar and N fertilizer application, and a strong positive relationship was found between yield and MBC or MBN (Figure 6B); this finding was consistent with the results of Iqbal et al. (2021), likely due to improved soil fertility supporting increased rice yield (Iqbal et al., 2021; Sun, Li, et al., 2021).

Rice yield was correlated significantly and positively to enzyme activities (AG and LAP) (Figure 6B). Combined biochar and N fertilizer application significantly increased the LAP activity; LAP hydrolyzes hydrophobic amino acids in the N segment of the polypeptide chain to produce free amino acids or inorganic nitrogen that can be used by plant growth (Awad et al., 2018), thereby increasing crop yields.

Combined biochar and N fertilizer significantly increased the relative abundance of *Chloroflexi* (Figure 4A). *Chloroflexi* is a deeply branched phylum of bacteria with diverse morphology and many ecosystem functions, including participation in cycling of carbon, nitrogen, and sulfur (Fan et al., 2021), which may contribute to plant growth (Figure 1G).
5 | CONCLUSIONS

This study characterized the soil physicochemical properties, enzyme activities, the concentration of PLFAs, microbial community composition and diversity, and rice yield in response to varying amounts of biochar and N fertilizer addition. Relative to B0N0, Biochar. The combined biochar and N fertilizer application significantly increased the activities of BG, CB, and LAP. Biochar significantly decreased the gram-positive bacteria, gram-negative bacteria, total fungi, total bacterial, and total PLFAs. Biochar alone, N fertilizer alone, and combined biochar and N fertilizer application increased rice yield. Yield had a significantly positive correlation with LAP activity, but a negative one with the relative abundance of Chloroflexi. In summary, biochar and N fertilizer application can influence yield by altering soil enzyme activities and soil microbial communities.

ACKNOWLEDGMENT
This study was supported by National Natural Science Foundation of China (31601834).

CONFLICT OF INTEREST
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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**How to cite this article:** Sun, J., Li, H., Wang, Y., Du, Z., Rengel, Z., & Zhang, A. (2022). Biochar and nitrogen fertilizer promote rice yield by altering soil enzyme activity and microbial community structure. *GCB Bioenergy*, 14, 1266–1280. https://doi.org/10.1111/gcbb.12995

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