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RESEARCH ARTICLE

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Biodiesel Co-Product (BCP) amendment drives beneficial soil microbiome assembly promoting acid soil health

Qunli Shen[1,2,3,4] | Paul Voroney[4] | Philip C. Brookes[5] | Ahmed S. Elrys[6,7] | Mengjie Yu[8] | Wei-Qin Su[2,9] | Lei Meng[7] | Meng Li[1,3]

[1]Archaeal Biology Center, Shenzhen Key Laboratory of Marine Microbiome Engineering, Institute for Advanced Study, Shenzhen University, Shenzhen, China

[2]Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Optoelectronic Engineering, Shenzhen University, Shenzhen, China

[3]Shenzhen Key Laboratory of Marine Microbiome Engineering, Institute for Advanced Study, Shenzhen University, Shenzhen, China

[4]School of Environmental Sciences, University of Guelph, Guelph, Ontario, Canada

[5]Soil Science Department, Rothamsted Research, Harpenden, UK

[6]Soil Science Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt

[7]College of tropical crops, Hainan University, Haikou, China

[8]College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, China

[9]McCormick School of Engineering, Northwestern University, Evanston, Illinois, USA

Correspondence
Meng Li, Archaeal Biology Center, Shenzhen Key Laboratory of Marine Microbiome Engineering, Institute for Advanced Study, Shenzhen University, Shenzhen, China.
Email: limeng848@szu.edu.cn

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Abstract

Biodiesel Co-Product (BCP) amendment has been shown to decrease both nitrate leaching and nitrous oxide (N_2O) emissions in acidic soil; however, the effects of BCP on the soil microbiome have not been investigated thoroughly. In this study, we investigated the response of prokaryotic and fungal communities in aspects of structure, diversity, and co-occurrence network to the BCP amendment following complete mixing application (0–18-cm depth) of 1.5 mg BCP-C g^{-1} and surface application (0–6-cm depth) of 4.5 mg BCP-C g^{-1} via high-throughput 16S rRNA and internal transcribed spacer (ITS) amplicon sequencing. The amendment altered microbial communities significantly by increasing the relative abundances of Proteobacteria (*Burkholderia*) and Ascomycota (*Trichoderma*) in prokaryotic and fungal communities, respectively. Only a higher rate application (4.5 mg BCP-C g^{-1}) decreased prokaryotic alpha diversity, whereas all rates of amendment decreased fungal diversity. The co-occurrence network of prokaryotes had more nodes and links and a higher average degree and clustering coefficient than the fungal network with BCP addition. The majority of keystone species in prokaryotic and fungal networks were from Proteobacteria and Ascomycota taxa. Of note, the BCP amendment significantly increased the OTU numbers of potential biocontrol agents, including *Trichoderma* (*T.*) *spirale*, *T. koningiopsis*, and *T. virens*, etc., while decreased OTU numbers related to plant pathogens species, particularly in the complete mixing application. Our work highlights the potential for BCP amendments to promote the assembly of a healthy soil microbiome by enhancing the abundance of

potential biocontrol microbes while reducing plant pathogens species, which may contribute to soil health.

KEYWORDS

biodiesel production, co-occurrence networks, keystone species, soil biocontrol agents, soil microbial community structure

1 | INTRODUCTION

Biodiesel, which is produced from the esterification of vegetable oils and animal fats (Hájek et al., 2021), is a large carbon (C) neutral product that can be used as an alternative to fossil fuels. Therefore, biodiesel could be renewable clean energy for mitigating climate change; however, significant amounts of biodiesel co-products (BCP) are generated during its synthesis. The BCP contains many processing residues, including a water-soluble mixture of glycerol, salts of fatty acids, methyl esters, methanol, potassium hydroxide, and water (Redmile-Gordon et al., 2014; Shen, Redmile-Gordon, et al., 2021; Shen, Song, et al., 2021).

Large amounts of nitrogen (N) fertilizers are applied to most Chinese croplands to increase crop production (Bei et al., 2018; Shen, Hu, et al., 2021); however, excessive applications of N fertilizers led to low fertilizer-use efficiency and soil acidification (Guo et al., 2010), and hence changes in microbial composition (e.g., a decline of bacteria/fungi ratio) (Shen et al., 2019; Zhao et al., 2020), which may negatively impact soil health (Chen et al., 2022) and the environment. Furthermore, soil acidification becomes the main threat to tea soil health and an imbalance of soil nutrients (Le et al., 2021), but BCP is an alkaline material, which could improve soil pH, mitigating soil acidification (Shen, Redmile-Gordon, et al., 2021; Shen, Song, et al., 2021). Our previous study showed that BCP had a high proportion of labile organic C substrates; so as a soil amendment, it significantly stimulated soil microbial biomass C (MBC) and adenosine 5′-triphosphate (ATP) contents and promoted the native soil microbes to immobilise inorganic N, thereby reducing inorganic-N losses from soil (Shen, Redmile-Gordon, et al., 2021), which may promote soil health (Fierer et al., 2021). Moreover, BCP additions improved the capacity of microbes to immobilise inorganic N in soils differing in moisture gradients. While maximum N immobilisation was at 60% water holding capacity (WHC), it significantly reduced nitrous oxide (N_2O) emissions from waterlogged soils by increasing copy numbers of the *nosZ* gene (Shen, Redmile-Gordon, et al., 2021). Likewise, BCP additions increased extracellular polymeric substances (EPS) and EPS-protein production efficiency (Redmile-Gordon et al., 2015), which promotes the formation of stable, erosion-resistant soil aggregates and the improvement of soil structure, which may contribute to soil health (Chang et al., 2022). Therefore, BCP amendments have considerable potential for solving problems caused by modern and intensive agriculture by improving soil health and minimising the deterioration of the environment.

Highlights

- BCP amendment caused larger changes in fungal than prokaryotic communities.
- BCP resulted in more complex prokaryotic networks and more centralised fungal ones.
- BCP amendment significantly increased the OTU numbers of *Trichoderma* spp.
- An increasing application rate of BCP may not get more beneficial microbes.

The soil microbiome, with its rich phylogenetic, taxonomic, and functional diversities (Chaparro et al., 2012), plays an important role in maintaining soil quality and ecosystem productivity through biochemical processes (Huang et al., 2013; Ling et al., 2016; Wang et al., 2006). Generally, additions of labile C substrates cause rapid increases in the soil microbial biomass and activity (De Graaff et al., 2010) and EPS production. Also, Cleveland et al. (2007) found that a labile C amendment resulted in a widely variable microbial community structure and composition, with increasing C availability favouring copiotrophic organisms. The response of the soil bacterial and fungal communities to labile C additions is further complicated by the fact that they possess different decomposition pathways and growth rates, with bacteria preferring to decompose easily available substrates while fungi tending to decompose more complex organic materials (Wardle et al., 2002). These changes in microbial community structure have also been related to changes in soil C and N cycling (Blagodatskaya et al., 2014; De Graaff et al., 2010; Fontaine et al., 2003); however, there is a limited understanding of the effects of

amendments of labile C derived from BCP on soil microbial diversity and community composition, and to our knowledge, the effects on the assembly of soil microbiome have not been investigated.

Our previous study revealed that MBC and microbial biomass N (MBN) was significantly stimulated by complete mixing and surface applications of BCP, as compared to non-BCP treatments (Shen, Song, et al., 2021); furthermore, surface application of BCP resulted in higher emissions of N_2O than complete mixing, which was attributed to denitrification from the subsoil without BCP applications, which had the highest ratio of $(nirK + nirS)/nosZ$. The effects of different applications of BCP on microbial communities and diversity have, however, not been investigated. Also, in this study, we used network analysis, an approach used to explore the ecological interaction patterns among microbial species in many different environments, including soils (Jiang et al., 2017), to decipher changes in soil community structure and assembly of complex microbial communities following BCP amendment (Barberán et al., 2012). Consequently, the main objectives of this study of soil amended with complete mixing and surface applications of BCP were: (i) to investigate how prokaryotic and fungal communities and diversities respond; (ii) to identify the microbial taxa mainly response to different incorporation methods of BCP; and (iii) to identify the differences in the co-occurrence network and keystone species in prokaryotes and fungi. According to our previous findings (Shen et al., 2023), BCP addition (1.5 mg BCP-C g^{-1} soil) led to a larger decline in fungal than bacterial diversities. In addition, it increased the OTU numbers of beneficial microbes and created more complex bacterial networks. Therefore, we hypothesised that (1) both BCP applications decreased the OTUs and diversities in fungal communities than prokaryotic ones, (2) surface applications (4.5 mg BCP-C g^{-1} soil) would assemble more beneficial microbes than the complete mixing one (1.5 mg BCP-C g^{-1} soil), and (3) surface applications more increase complexity and keystones of microbial networks than the complete mixing one.

2 | MATERIALS AND METHODS

2.1 | Soil sampling and experimental setup

The soil was collected from the topsoil (0–20 cm depth) of a tea field located in the Meijiawu tea region (30°20′ N, 120°09′ E), Hangzhou, Zhejiang Province, China. The setup of the experiment was as previously described in Shen, Song, et al. (2021) the soils were sieved moist to <5 mm, the water content was adjusted to 40% of WHC, and then the soils were incubated at 25°C for 7 days before determination of MBC and MBN as reported by Shen et al. (2018). The soil was transferred to soil lysimeters (24 cm in length, 6 cm in diameter). In total, 51 lysimeters were prepared with three lysimeters per treatment. Each lysimeter contained moist soil (equivalent to 350 g oven-dry soil) and received 80 μg urea-N g^{-1} soil at 5.18% ^{15}N atom excess when required. We have totally four treatments (each was separated as 0–6 cm and 7–18 cm lysimeter depth; totally, eight subtreatments are shown in Table 1. The total amounts of BCP applied were the same in treatments T3 and T4, although the concentrations in the 0–6 cm layer were three-fold higher.

Soil water content was adjusted to 50% WHC after the treatments were applied. The soils were leached at days 5, 10, 20, and 35 of the incubation with distilled water (100 mL). Lysimeters were destructively sampled at each measurement date. At each sampling time, three replicates of each treatment were sampled from 0 to 6 cm and 7 to 18 cm sampling depth (Shen, Song, et al., 2021).

Soil DNA was extracted after 5 and 35 days of incubation (see below). Biodiesel Co-Product was produced in

TABLE 1 The description of experimental treatments.

Treatments	Soil depth	Subtreatments	Urea	BCP
T1	0–6 cm	T1 (0–6 cm)	0 μg N g^{-1} soil	0 mg C g^{-1} soil
	7–18 cm	T1 (7–18 cm)	0 μg N g^{-1} soil	0 mg C g^{-1} soil
T2	0–6 cm	T2 (0–6 cm)	80 μg N g^{-1} soil (5.18% ^{15}N atom excess)	0 mg C g^{-1} soil
	7–18 cm	T2 (7–18 cm)	80 μg N g^{-1} soil (5.18% ^{15}N atom excess)	0 mg C g^{-1} soil
T3	0–6 cm	T3 (0–6 cm)	80 μg N g^{-1} soil (5.18% ^{15}N atom excess)	1.5 mg C g^{-1} soil
	7–18 cm	T3 (7–18 cm)	80 μg N g^{-1} soil (5.18% ^{15}N atom excess)	1.5 mg C g^{-1} soil
T4	0–6 cm	T4 (0–6 cm)	80 μg N g^{-1} soil (5.18% ^{15}N atom excess)	4.5 mg C g^{-1} soil
	7–18 cm	T4 (7–18 cm)	80 μg N g^{-1} soil (5.18% ^{15}N atom excess)	0 mg C g^{-1} soil

the laboratory from waste vegetable cooking oil following the procedure for biodiesel production reported by Shen, Redmile-Gordon, et al. (2021), and the main components of BCP were glycerol, which contained 73%, followed by potassium soap and volatile organics (both 11.7%), and potassium hydroxide (2.4%). The methodology used, measurements and results of soil physicochemical properties, N cycling genes, MBC, MBN, and greenhouse gas emissions were described in Shen, Song, et al. (2021).

2.2 | DNA extraction, amplicon sequencing, and sequencing data processing

The soil DNA was extracted from fresh soil samples using the FastDNASpin Kit for soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The quality and concentration of the DNA were checked by gel electrophoresis and a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), respectively. Soil DNA samples were stored at −80°C until further sequencing.

The DNA was diluted to 10 ng μL^{-1} using sterile water according to the concentration. The purified amplicons were sequenced on an Illumina Miseq sequencing platform (Illumina Inc., San Diego, CA, USA) at Novo-gene Co., Ltd, Beijing, China. Sequence analyses were carried out by Uparse software (Edgar, 2013). The V4 region of target prokaryotic 16S rRNA was amplified using the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′), and the reverse primer consisted of a seven bp barcode and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Also, the ITS1-ITS2 region of the fungal ITS gene was amplified using the primer pair ITS1-F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (Brabcová et al., 2016; Walters et al., 2016). All PCR reactions were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and ~10 ng of template DNA.

The raw sequencing data were quality assessed by QIIME 2 (Bolyen et al., 2019). A mean of 74,238 per sample and 26,029 per sample of high-quality prokaryotic and fungal sequences, respectively, were obtained after filtering low-quality reads, and quality scores were over 20. Lengths of read more than 200 bp were selected. After the selection and removal of the chimaera, the high-quality sequence data were clustered into operational taxonomic units (OTUs) with 97% similarity. Then the prokaryotic and fungal OTU taxonomic classification was, respectively, determined based on comparisons with the Silva 138 databases and the UNITE databases (Version 8), using the Ribosomal Database Project (RDP) classifier (Větrovský et al., 2020).

2.3 | Network analysis

Network analysis was used to explore the microbial co-occurrence patterns using Cytoscape 3.7.0 (Cline et al., 2007; Shannon et al., 2003) and was visualised using Gephi software, with six replicates per treatment. Briefly, pair-wise associations among OTUs were calculated using four methods (Spearman, Pearson, Bray–Curtis dissimilarity and Kullback–Leibler correlation methods) (Faust & Raes, 2012; Shen et al., 2023). A total of 1000 renormalised permutations and bootstrap scores were used to avoid compositional bias and false-positive correlations. The *p* values were then merged using Brown's method and corrected for multiple tests using Benjamini-Hochberg produce to mitigate the occurrence of false-positive results. Finally, the final correlation matrix was performed and visualised in the Gephi platform (Gephi 9.2) (Bastian et al., 2009). The statistics tool in Gephi was used to calculate the topological features of each network, and topological features for each node in the network were used in accessing the node role. For visualising the networks, the node represents a species or taxon, and the edge linking two nodes represents the positive or negative correlation between two nodes (Deng et al., 2012; Zhou et al., 2010; Zhou et al., 2011). Modularity, the key property of a network (Alon, 2003), is used to calculate an average clustering coefficient to show how well nodes are connected with their neighbours (Zhou et al., 2011). A *Zi-Pi* plot was used to classify keystone populations based on the node's role in the network (Deng et al., 2012). The threshold values of *Zi* and *Pi*, as proposed by Guimera and Amaral (Guimera & Nunes Amaral, 2005) and simplified by Olesen et al. (2007), were used for categorising OTUs were 2.5 and 0.62, respectively. Topological roles of different nodes were classified into four categories, (1) network hubs: nodes with $Zi > 2.5$ and $Pi > 0.62$; (2) module hubs: nodes with $Zi > 2.5$ and $Pi \leq 0.62$; (3) connectors: nodes with $Zi \leq 2.5$ and $Pi > 0.62$; and (4) peripheral nodes: nodes with $Zi \leq 2.5$ and $Pi \leq 0.62$.

2.4 | Statistics

All statistical analyses were conducted using Origin 9.0 and SPSS 22.0 software (SPSS Inc., Chicago, USA). Simple Spearman co-relationship analysis was performed using the R statistical programme (R Development Core Team, http://www.R-project.org) using the vegan

package (Oksanen et al., 2013). Nonmetric multidimensional scaling (NMDS) ordination plots were used to display differences in microbial community composition. One-way ANOVA was used to analyse the treatment effects, and differences with values of $p < 0.05$ were considered statistically significant, which were determined by the Turkey HSD test. Permutation multivariate analysis of variance (PERMANOVA) was used to evaluate the statistical significance of compositional differences between prokaryotic and fungal communities. Structural equation modelling (SEM) using the "lavaan" package in R was used to test causal relationships among BCP amendment, N fertilizer, prokaryotic alpha diversity, fungal alpha diversity, plant pathogen species, and biocontrol microbes. The model fit was tested by root-mean-square error of approximation (RMSEA), Chi-square (χ^2), and p-value of χ^2. The model has a good fit when RMSEA $0 \leq$ RMSEA ≤ 0.05 and $0.10 < p \leq 1.00$ (Schermelleh-Engel et al., 2003). The functional groups of fungi and bacteria were predicted by FUNGuild (Nguyen et al., 2016) and FAPROTAX (Louca et al., 2016), with confident levels of "highly probable" and "probable" selected for the analysis.

3 | RESULTS

3.1 | Response of the prokaryotic composition to BCP amendments

The dominant soil prokaryotic phyla in all treatments were Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, and Thaumarchaeota (Figure 1a). BCP significantly increased ($p < 0.05$) the relative abundance of Proteobacteria when treatment T4 (0–6 cm) was compared with treatment T4 (7–18 cm), which was 55% and 36% at day 5, and 57% and 38.9% at day 35, respectively. On the contrary, BCP significantly ($p < 0.05$) decreased the relative abundance of Acidobacteria when comparing BCP amended treatments with those nonamended with 18% and 32%, respectively, at day 5, 20% and 31.5%, respectively, at day 35. The Spearman analysis showed that BCP had a significant and positive relationship with Proteobacteria ($p < 0.001$) on days 5 and 35 (Figure S1), whereas Acidobacteria had a significant and negative relationship at those times (all $p < 0.01$). Similarly, Chloroflexi had a significant and negative relationship with BCP on days 5 and 35 (all $p < 0.001$; Figure S1). BCP significantly decreased ($p < 0.05$) the relative abundance of

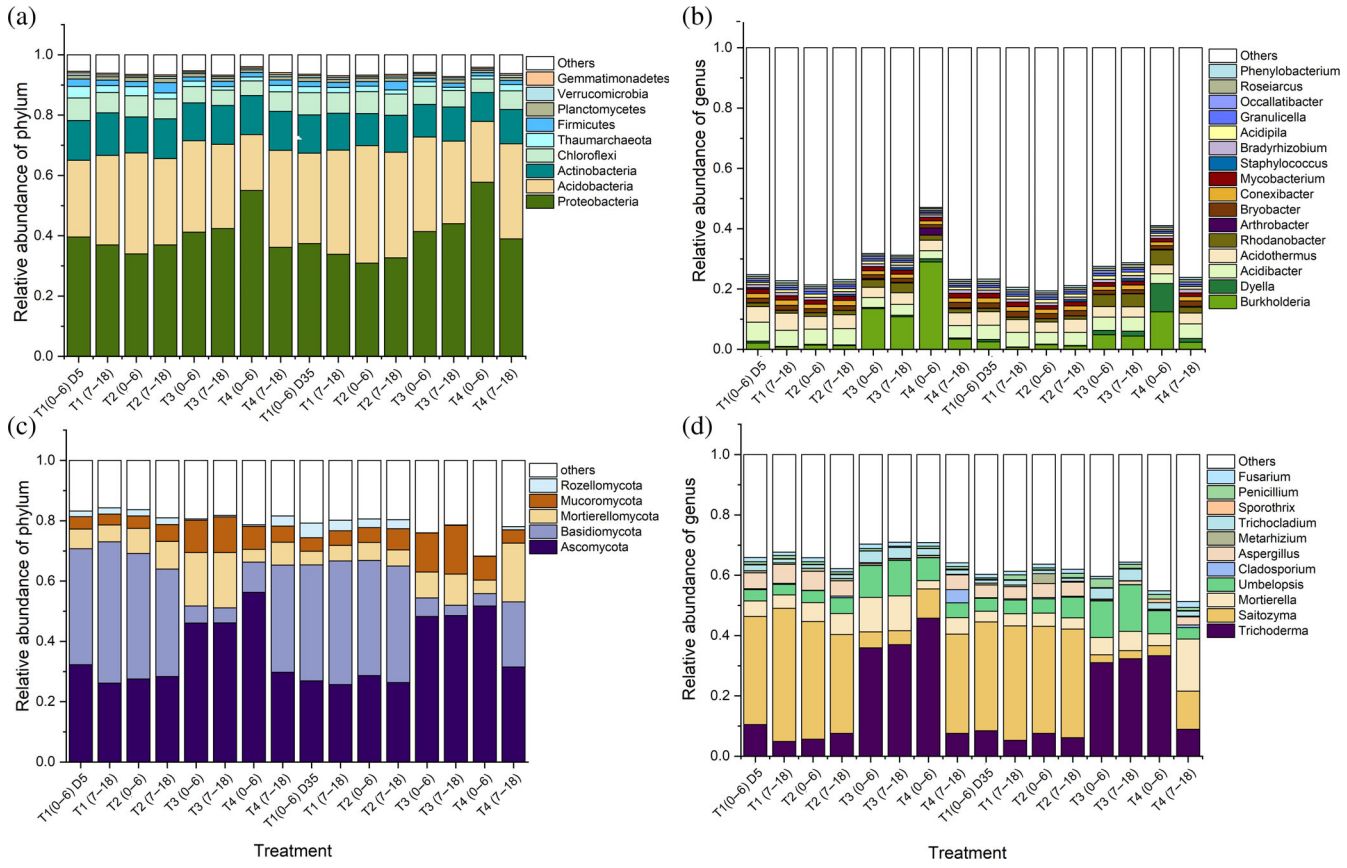

FIGURE 1 Relative abundance of prokaryotes (a and b) and fungi (c and d) taxa at phylum and genus levels in the different treatments on days 5 and 35.

Thaumarchaeota when comparing BCP-amended treatments with those nonamended with 1.4% and 2.5% at day 5, and with 1.0% and 1.8% at day 35, respectively.

At the genus level, the BCP addition significantly stimulated ($p < 0.001$) the relative abundance of *Burkholderia*. On day 5, it was 30% higher in the T4 (0–6 cm) treatment when compared with 3.4% in the T4 (7–18 cm), and 12% and 2.3% higher in the T4 (0–6 cm) and T4 (7–18 cm) treatments, respectively, on day 35 (Figure 1b). BCP addition significantly ($p < 0.01$) decreased the abundance of *Acidibacter* on day 5 (Figure S2), the relative abundance of *Acidibacter* was ~5% in the unamended treatment, but it was only 3% in amended treatments. There was no difference on day 35. Also, BCP significantly decreased the relative abundance of *Acidothermus* by days 5 and 35 ($p < 0.05$), with ~5% and 3% in unamended and amended treatments, respectively. BCP amendments only caused a significant increase ($p < 0.001$) in the relative abundance of *Rhodanobacter* in both depths of T3 treatments (2.7%) at day 5 (Figure 1b), when compared with unamended treatments (1.2%) (Figure 1b and Figure S2). On day 35, BCP addition significantly increased ($p < 0.001$) the relative abundance of *Rhodanobacter* when comparing BCP-amended treatments (4.5%) with those nonamended (1%). BCP addition significantly decreased ($p < 0.001$) the relative abundance of *Dyella*, which was about four times lower in BCP amended treatments than nonamended (0.22% vs 0.8%) at day 5. At day 35, the abundance was only 0.13% in the unamended treatments, 9.4% in the T4 (0–6 cm) about, and 1.5% in both T3 treatments (Figure 1b).

3.2 | Fungal composition altered by BCP amendments

The abundance of Ascomycota significantly increased ($p < 0.05$) with BCP additions by day 5, accounting for 56.2% in the T4 (0–6 cm); this was almost twice that of the T4 (7–18 cm) at 29.7%. Also, the abundance in both depths of T3 treatments 46% was significantly higher than nonamended (30%) (Figure 1c). By day 35, Ascomycota abundance in BCP treatments (50%) was still significantly higher ($p < 0.05$) than non-amended treatments (28%). In contrast, the abundance of Basidiomycota significantly decreased ($p < 0.05$) with BCP addition (Figure S3). On day 5, the abundance of Basidiomycota in BCP treatments was less than 10%, but it was more than 35% in non-BCP treatments. On day 35, it was only about 4% in BCP-amended treatments, but it was more than 20% in non-BCP treatments. The BCP addition significantly ($p < 0.05$) increased the abundance of Mucoromycota and Mortierellomycota only in both depths of T3 treatments (Figure 1c). The BCP significantly decreased ($p < 0.05$) the relative abundance of Rozellomycota when comparing BCP amended treatments with those nonamended with 0.4% and 2%, respectively, at day 5 and 0.01% and 3%, respectively, on day 35.

At the main genus level, BCP significantly increased the abundance of *Trichoderma* ($p < 0.001$; Figure S4). On day 5, in the BCP treatments, the abundances of *Trichoderma* were 36% in both depths of T3 treatments and 45.8% in T4 (0–6 cm), and only ~6% in non-BCP treatments. On day 35, it was about 30% in the BCP treatments and ~ 7% in non-BCP treatments (Figure 1d). BCP, however, significantly decreased ($p < 0.001$) the relative abundance of *Saitozyma* when comparing BCP amended treatments with those nonamended with 5% and 37%, respectively, at day 5, and 3% and 37%, respectively, at day 35. Likewise, average relative abundances of *Mortierella* (11%) in T3 significantly ($p < 0.05$) increased on day 5 compared with that in T2 (6.1%). Moreover, *Umbelopsis* abundances in both T3 treatments (13%) were higher than in others (3.5%) on days 5 and 35 (Figure 1d). Furthermore, BCP additions significantly increased ($p < 0.05$) the OTU number of soil biocontrol species such as *Trichoderma spirale*, *Trichocladium asperum*, *Trichoderma asperellum*, *Trichoderma virens*, *Trichoderma koningiopsis*, and *Trichoderma longibrachiatum* (Figure 2).

3.3 | Changes in microbial diversity and microbial communities in response to BCP amendments

BCP additions significantly decreased prokaryotic alpha diversity (represented by the Shannon index)

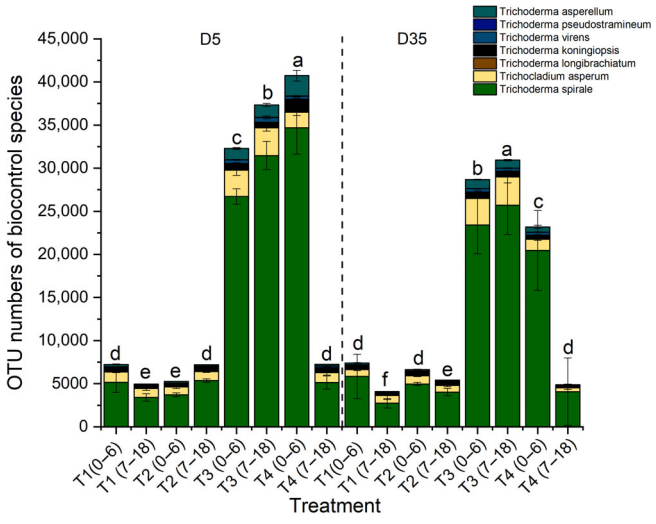

FIGURE 2 OTU numbers assigned to biocontrol agents on days 5 and 35. Lowercase letters indicate significant differences ($p < 0.05$) in different treatments.

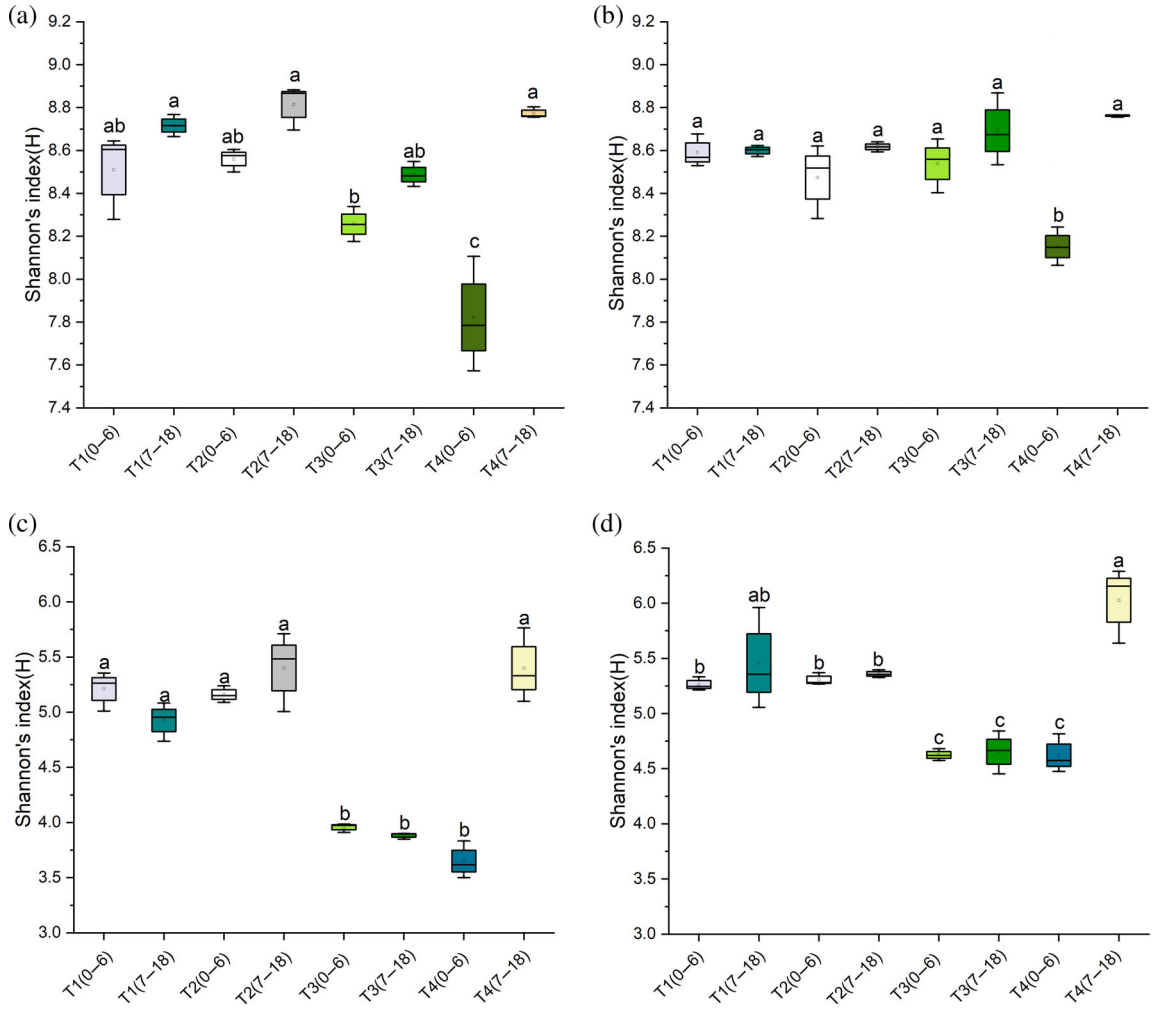

FIGURE 3 Diversity (Shannon's index) of prokaryotes (a and b) and fungi (c and d) in different treatments at days 5 and 35, respectively. Lowercase letters indicate significant differences ($p < 0.05$) among different treatments.

TABLE 2 The effects of BCP amendments and N fertilizer on prokaryotic and fungal communities based on PERMANOVA.

		BCP	N fertilizer	BCP*N fertilizer
Prokaryotic community	R^2	0.27	0.09	0.01
	p	**<0.001*** **	**<0.001*** **	0.3
Fungal community	R^2	0.28	0.18	0.01
	p	**<0.001*** **	**<0.001*** **	0.3

***Represents significant effects (marked in bold) at $p < 0.001$.

only in T4 (0–6 cm) ($p < 0.05$) at days 5 and 35 (Figure 3a,b). The fungal alpha diversity was significantly decreased in all BCP treatments ($p < 0.05$) at days 5 and 35 (Figure 3c,d). The PERMANOVA test showed that both prokaryotic communities ($R^2 = 0.27$, $p < 0.001$) and fungal communities ($R^2 = 0.28$, $p < 0.001$) were significantly affected by the BCP amendment (Table 2). Furthermore, NMDS analysis showed that BCP addition caused a greater separation of the fungal communities (stress = 0.0602) than the prokaryotic communities (stress = 0.1975) (Figure 4). Volcano plots showed that 64 upregulated OTUs and 4 downregulated OTUs after the comparison between T2 and T3, 47 upregulated OTUs and 1 downregulated OTUs between T2 and T4 (0–6 cm) in the prokaryotes; 211 upregulated OTUs and 427 downregulated OTUs after the comparison between T2 and T3, 165 upregulated OTUs and 165 downregulated OTUs between T2 and T4 (0–6 cm) in the fungi (Figure 5).

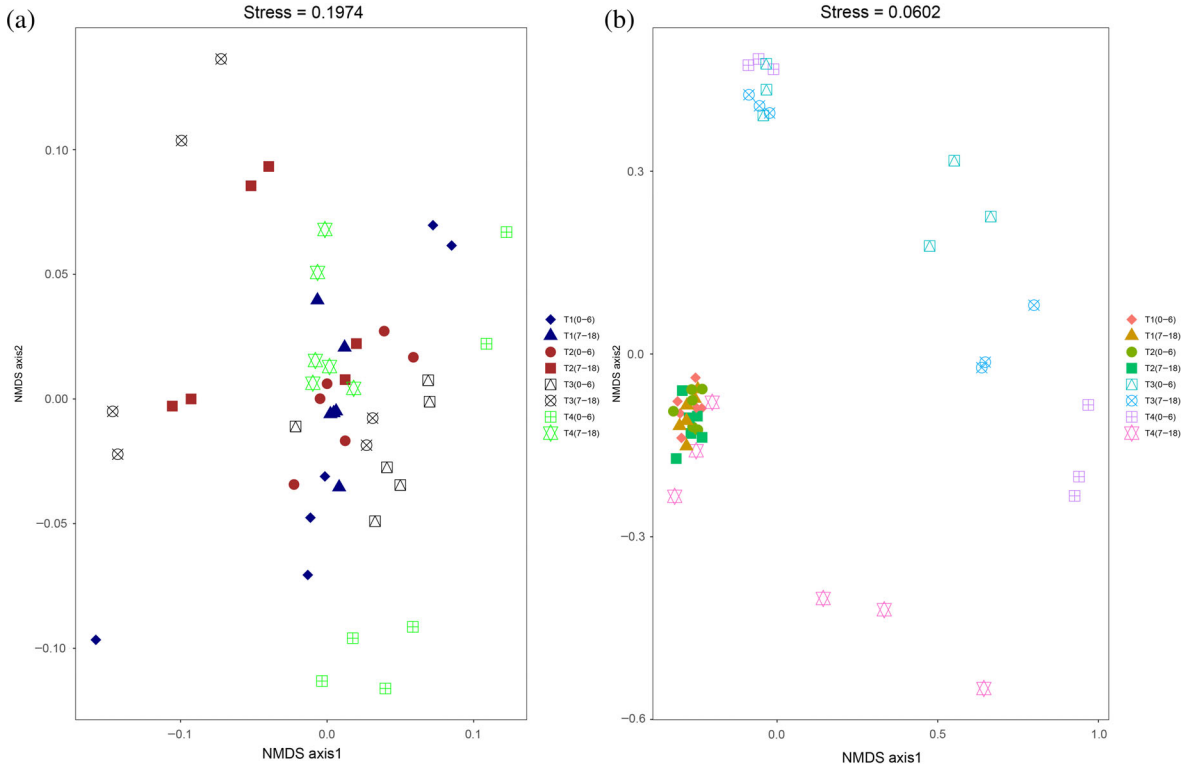

FIGURE 4 Nonmetric multidimensional scaling ordination plot (NMDS) based on the Bray-Curtis matrix of the soil prokaryotic community composition (a) and the soil fungal community composition (b) in different treatments.

3.4 | Network analysis of soil microbial communities

Network analysis revealed that BCP additions caused more edges and nodes in prokaryotic networks (Figure 6) (Table S1). The mean clustering coefficients of prokaryotic networks in BCP treatment (~0.34) were significantly higher than those in non-BCP (~0.24). The modularisation of T3 (0.937) was slightly higher than it was in T2 (0.936), while it was slightly lower in T4 (0.929) when compared with that in T2 (0.936) (Table S1). In contrast, fungal networks had fewer edges and nodes in BCP treatments compared to non-BCP treatments (Figure 6e–h; Table S1). The BCP amended fungal network (T3 and T4 had a significantly lower average clustering coefficient (0.602 and 0.296, respectively) than the T1 (0.68) and T2 (0.651)). The application of BCP significantly decreased the modularity of the network, with that of T3 at 0.732, T4 at 0.598, T1 at 0.941, and T2 at 0.939 (Table S1).

No network hubs were observed in both the prokaryotic or fungal networks, and with all nodes, four categories were identified (Figure 7a,b; Tables S2 and S3). Most of the nodes were specialists (fringing nodes and peripherals), as shown in both prokaryotic and fungal networks. We found eight module hubs in the prokaryotic network in the different treatments (Figure 7a), which were from both depths of T1 (two Chloroflexi and one unidentified OTU (NA)), both depths of T2 (one Firmicutes), both depths of T3 (one Proteobacteria and one Actinobacteria), and from both depths of T4 (one Proteobacteria and one Actinobacteria). Twenty-five connectors were found in the prokaryotic network in different treatments, which were from both depths of T1 (two Planctomycetes, one Firmicutes, three Actinobacteria, two Chloroflexi, and one NA), both depths of T2 (two Actinobacteria, five Proteobacteria, and one Planctomycetes), both depths of T3 (one Chloroflexi, two Proteobacteria, one Acidobacteria, and one Actinobacteria), and from both depths of T4 (three Actinobacteria and two Proteobacteria).

Only one module hub from both depths of T1 (one Rozellomycota) was found in the fungal network (Figure 7b). Sixty-four connectors were found in different treatments, which were from T1 (two Ascomycota and one NA), both depths of T2 (two Basidiomycota, four Ascomycota, two NA, two Mortierellomycota and three Rozellomycota), both depths of T3 (eleven Ascomycota, four NA, one Mortierellomycota, one Basidiomycota, two Rozellomycota and one Mucoromycota), both depths of T4 (three Basidiomycota, nine Ascomycota, eight NA, two Mortierellomycota, three Mucoromycota, and one Rozellomycota).

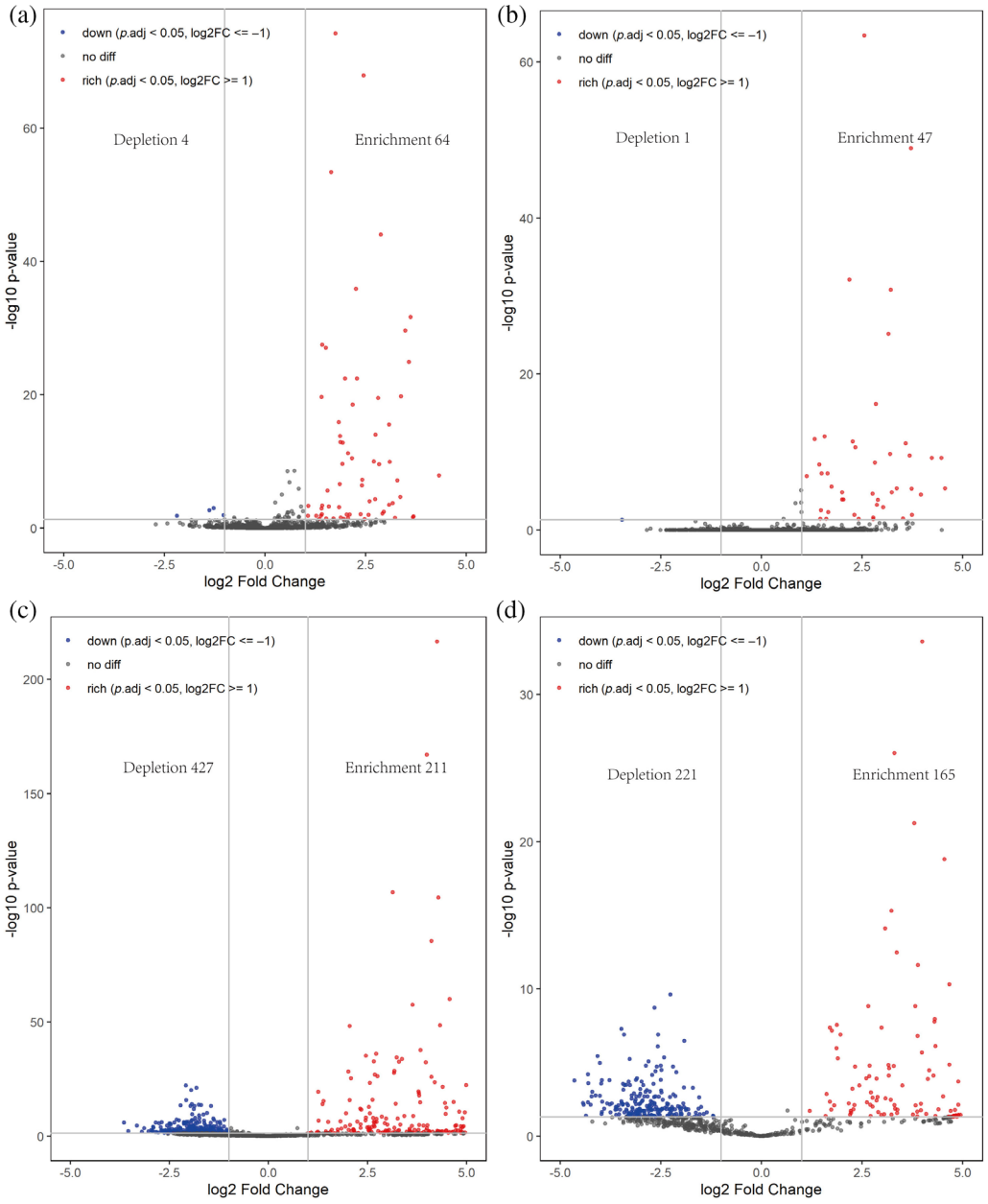

FIGURE 5 Volcano plots showing the log2-fold change (x-axis) in differential OTUs and the statistical significance of that change (y-axis). Differential OTUs in prokaryotes for T2 versus T3 (a) and T2 versus T4 (0–6 cm) (b). Differential OTUs in fungi for T2 versus T3 (c) and T2 versus T4 (0–6 cm) (d). Each point represents an individual OTU that increased/decreased more than (red) or less than (blue) 2-fold.

3.5 | Structural equation model (SEM) analysis and functional profiles

The SEM was conducted to further clarify the effects of BCP amendments and N fertilizer on the prokaryotic and fungal diversity, as well as on biocontrol microorganisms and plant pathogen species (Figure 7c,d). On days 5 and 35, BCP amendments significantly positively impacted biocontrol microbes but significantly negatively impacted plant pathogen, fungal and prokaryotic alpha diversity. On days 5 and 35, significant negative effects between biocontrol microbes and fungal alpha diversity were found. A significant and positive relationship between fungal alpha diversity and plant pathogen species was observed on day 5 only. Prokaryotic alpha diversity, however, significantly increased with increasing soil pH only on day 35. The N fertilizer had no influence on prokaryotic and fungal alpha diversity, biocontrol microbes, and soil-borne disease species.

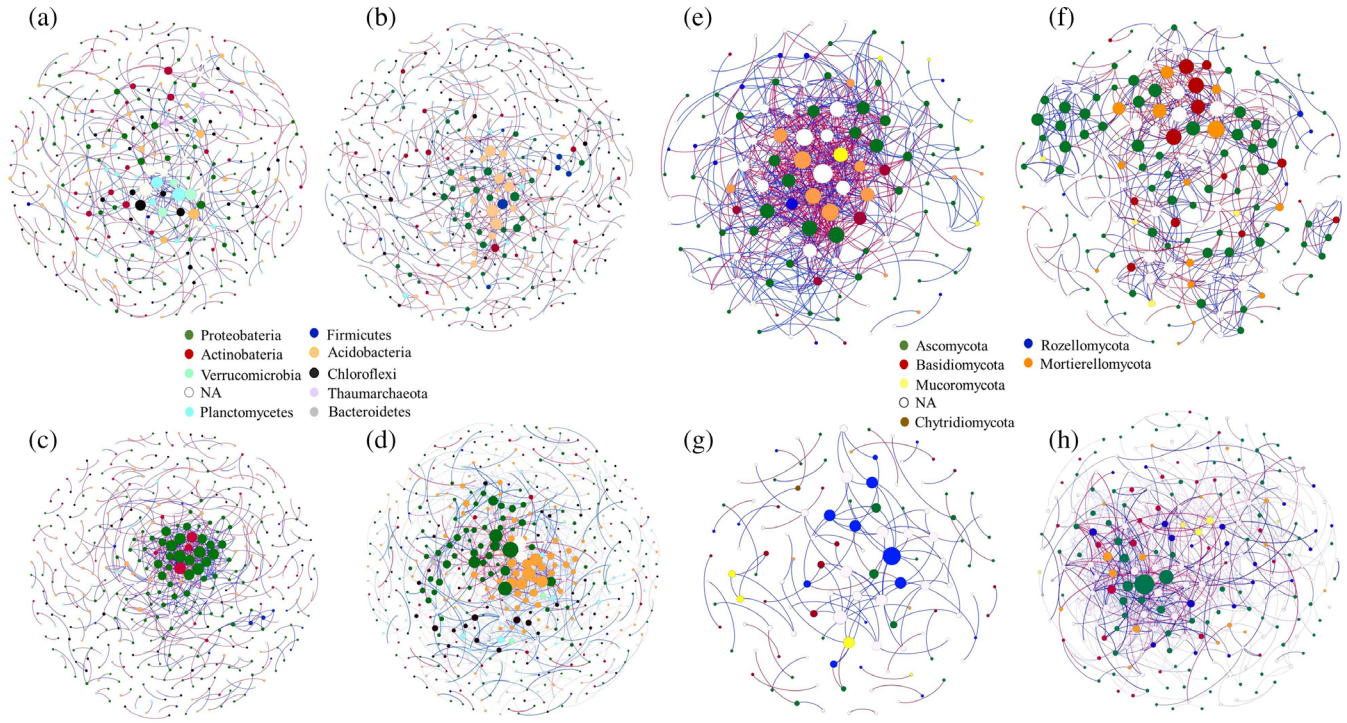

FIGURE 6 Co-occurrence networks of prokaryotic communities in treatments T1 (a), T2 (b), T3 (c), and T4 (d). Co-occurrence networks of fungal communities in treatments T1 (e), T2 (f), T3 (g), and T4 (h). Each node represents an OTU, and the size of each node is proportional to its relative abundance. Red and blue edges mark positive and negative correlations, and the thickness of each edge is proportional to the value of each correlation coefficient. Co-occurrence patterns of microbial communities treated in different treatments are shown in Table S1.

By using the FUNGulid to predict the functional group of fungal communities (Figure 8), the number of fungal OTU related to plant pathogen was significantly decreased over twice-fold after BCP additions in both depths of T3 treatments, but not in T4 (0–6 cm) at day 5, it was significantly decreased in all BCP-amended treatments at day 35. Also, fungal OTU numbers related to animal pathogens significantly decreased in amended treatments only on day 5. On days 5 and 35, the amended treatments had more fungal OTU numbers related to Saprotroph, Endophyte, and Epiphyte than in nonamended treatments. The FAPROTAX results (Figure 9) demonstrated that BCP significantly decreased prokaryotic OTU numbers related to ammonia oxidation, which, however, significantly boosted animal symbiotic OTU numbers.

4 | DISCUSSION

4.1 | Response of microbial diversity with BCP additions

Our results showed that fungal diversity was significantly decreased following BCP amendments, while there was only a significant decrease in prokaryotic diversity in T4 (0–6 cm) with a high-rate application (Figure 3). These results indicate that fungal communities are more sensitive than prokaryotic communities in response to BCP amendments; moreover, volcano plot results indicated that BCP additions enriched and depleted more than 600 and 300 OTUs from fungi in both depths of T3 and T4 (0–6 cm) treatments, respectively, but only 68 and 48 from prokaryotes. Likewise, the NMDS analysis (Figure 4), which showed greater separation of the fungal communities after BCP amendment. This is because fungi and prokaryotes in the soil microbial community have different growth rates and metabolisms (Chen et al., 2015; De Vries et al., 2006). For example, bacteria are more competitive than fungi in decomposing labile C (e.g., glucose), and bacteria populations are greater than fungi when labile C addition and BCP addition provide sufficient food sources for bacteria but not fungi, so it may have intensified competition between fungal communities. These data, therefore, support our first hypothesis that BCP amendments strongly depleted more OTUs in fungal than prokaryotic communities, and lowered fungal diversities than prokaryotic diversities. Prokaryotic and fungal communities changed less in the T4 (0–6 cm), i.e., high-rate application (4.5 mg BCP-C g^{-1} soil), than in T3 (1.5 mg BCP-C g^{-1} soil).

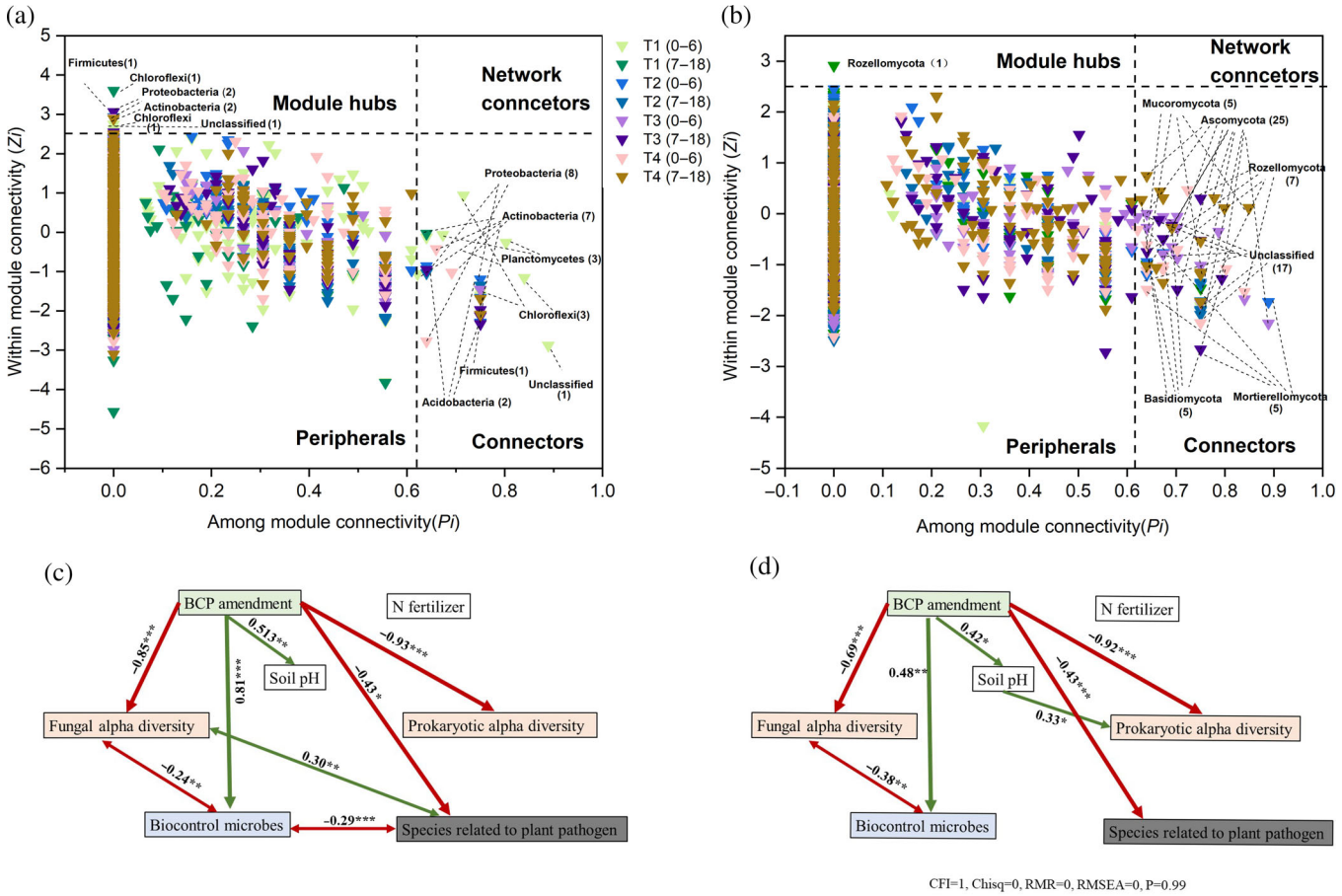

FIGURE 7 *Zi-Pi* plots showing the distribution of OTUs based on their topological roles in prokaryotic (a) and fungal (b) and networks. The module hubs and connectors are labelled with OTU taxonomic information, and their phylogenetic associations are in parentheses. The taxonomic information of these module hubs and connectors are shown in Tables S2 and S3 as T1 (0–6 cm) and T1 (7–18 cm), T2 (0–6 cm) and T2 (7–18 cm), T3 (0–6 cm) and T3 (7–18 cm), and T4 (0–6 cm) and T4 (7–18 cm). The threshold values of *Zi* and *Pi* for categorising OTUs were 2.5 and 0.62, respectively. Structural equation models showing the effects of BCP amendment, soil pH, N fertilizer, prokaryotic alpha diversity, fungal alpha diversity, and biocontrol microbes and plant pathogen species at days 5 (c) and 35 (d). The hypothetical model was satisfactorily fitted to the present data; significant paths are illustrated by the red arrow (negative) and green arrow (positive) with standardised path coefficients. Significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

4.2 | Changes to prokaryotic communities

Brant et al. (2006) have reported that substrate additions influence microbial community structure. Application of BCP significantly increased the relative abundance of Proteobacteria, the dominating prokaryotic phylum (Table 2; Figure 1a). A high abundance of Proteobacteria has previously been linked to high labile C (Fazi et al., 2005; Fierer et al., 2007), which was confirmed by our analysis. Proteobacteria contain multiple genera that suppress soil disease (Mendes et al., 2011). High concentrations of labile C can induce osmotic challenges and growing imbalances in cells of oligotrophic bacteria, such as Acidobateria and accumulation of toxic chemicals that impede DNA and protein synthesis (Koch, 2001). It may also improve ecological dynamics (Eichorst et al., 2007; Quaiser et al., 2003), genetic, as well as metabolic diversity. In our study, the lowest Acidobacteria abundance was in T4 (0–6 cm), suggesting that a high-rate BCP addition may harm their growth and contribute to the lowest microbial diversity (Figure 3). BCP significantly lowered the abundance of Chloroflexi (Figure S1), similar to Wang et al. (2021), who reported that labile C addition significantly decreased Chloroflexi abundance, and its oligotrophic nature explains its low ability to use BCP (Arcand et al., 2017).

Yuan et al. (2020) demonstrated that the diseased soil microbiome had greater higher relative abundances of Firmicutes, Choroflexi, and Gemmatimonadetes than healthy soils, which harboured higher relative abundances of Proteobacteria, Actinobacteria, and Acidobacteria. Consistent with our results, the relative abundances of Proteobacteria, Actinobacteria,

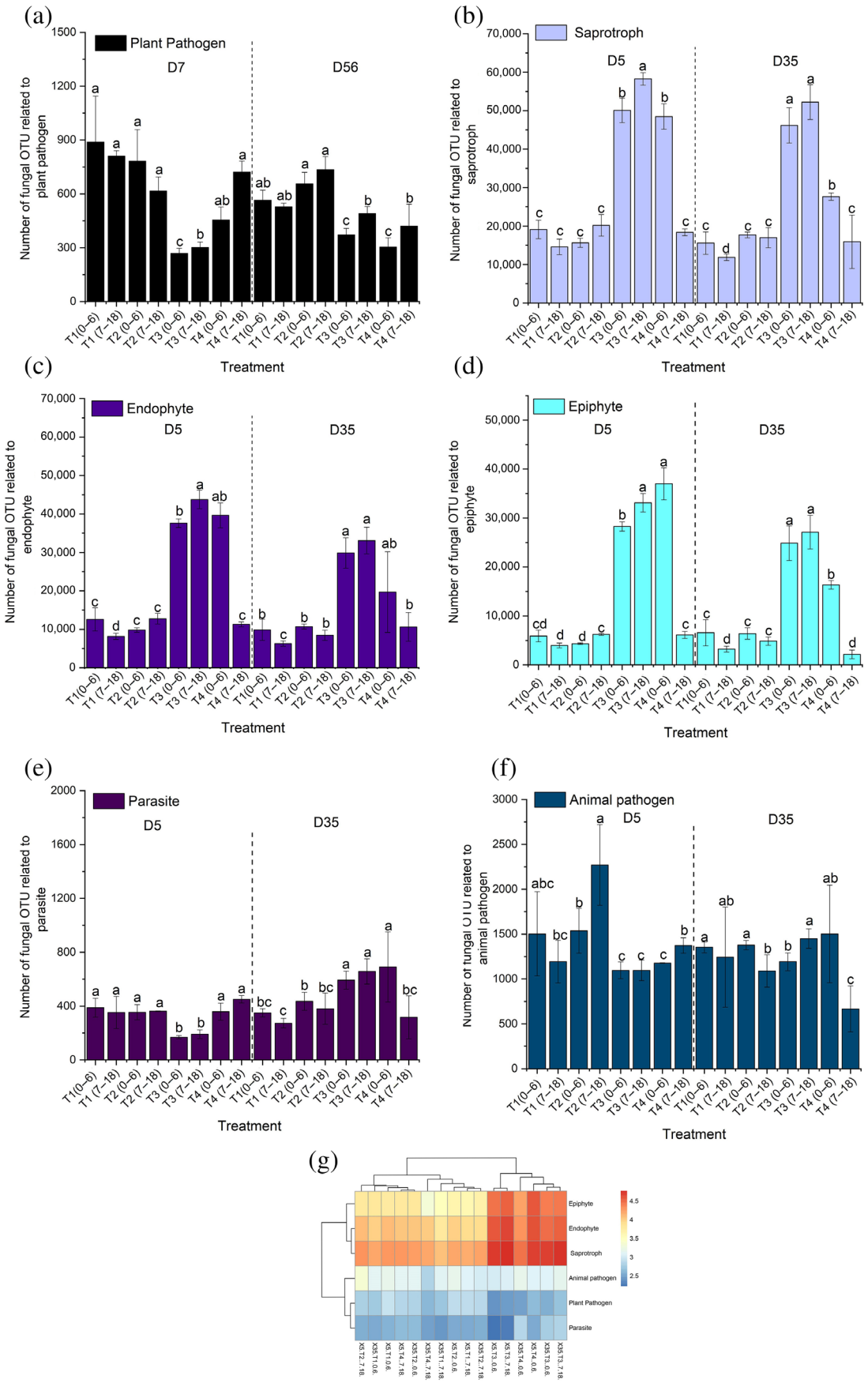

FIGURE 8 The OTU number and heatmap of dominant putative fungal (a–g) functional profiles based on FUNGuild were conducted to cluster with different treatments on days 5 and 35.

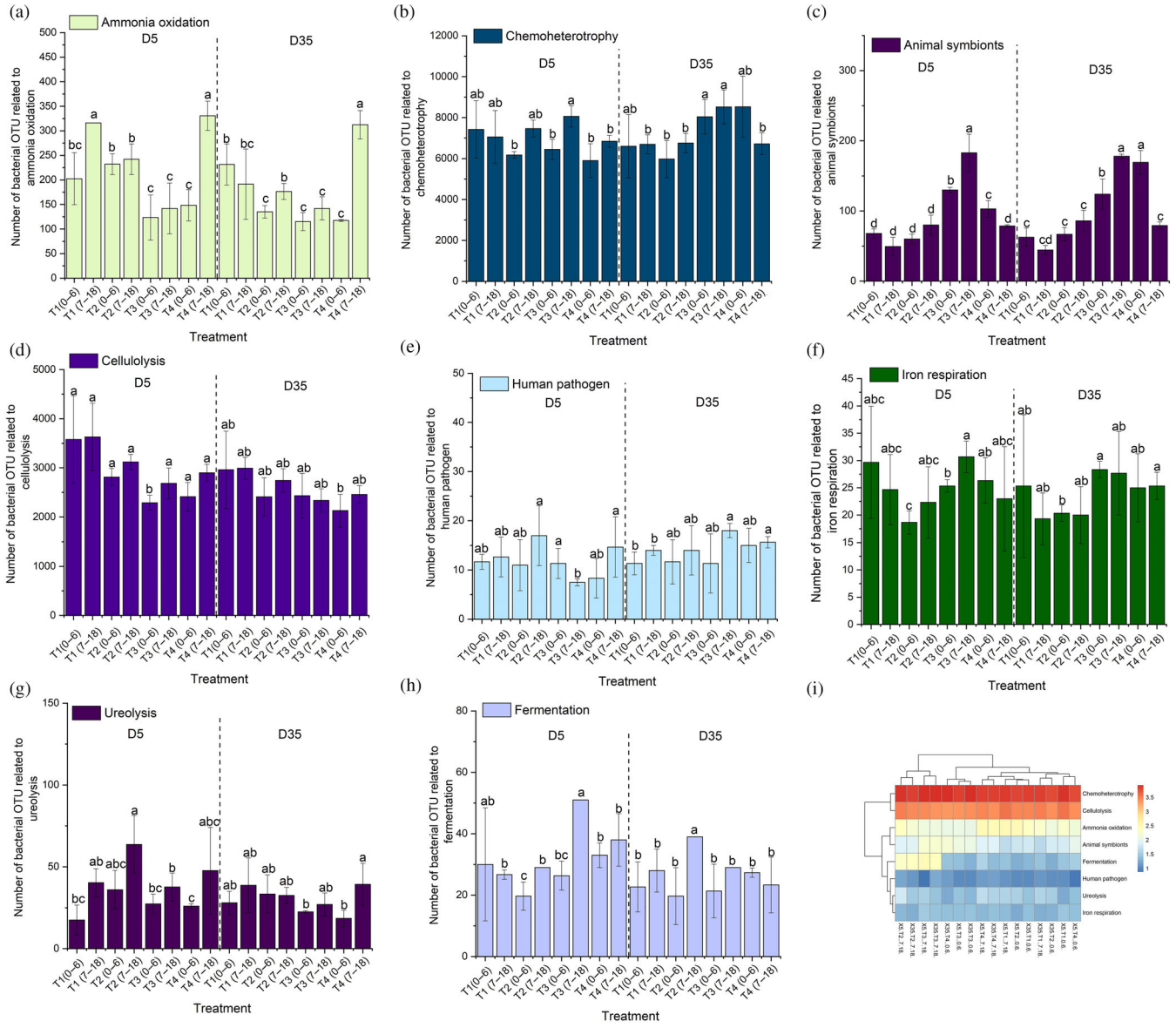

FIGURE 9 The OTU number and heatmap of dominant putative prokaryotic (a–i) functional profiles based on FAPROTAX were conducted to cluster with different treatments on days 5 and 35.

and Acidobacteria in BCP-amended treatments were abundant, while relative abundances of Firmicutes, Choroflexi, and Gemmatimonadetes were scarce; moreover, the abundances of Proteobacteria, Actinobacteria, and Acidobacteria were much more abundant in amended treatments (total of 85%) than in unamended treatments (total of 78%). These results imply BCP amendments have the potential to decrease N leaching (Shen, Song, et al., 2021), and promote soil health.

Burkholderia, *Dyella*, and *Rhodanobacter* were the primary Proteobacterial genera in our study (Figure 1b). Shin et al. (2017) found that the genera *Dyella* and *Rhodanobacter* were the major taxa in acidic soils, as found here. Van den Heuvel et al. (2010) suggested that *Rhodanobacter* may be responsible for most denitrification in low pH soils, despite its low initial abundance, which is in line with the present finding. Green et al. (2012) also found that *Rhodanobacter* was prominent in acidic soil environments, and, especially, its abundance was inversely correlated. *Rhodanobacter* appears to be the dominant producer of N_2O in this soil. BCP, however, significantly increased the abundance of N_2O-reducing *Dyella* (Figure S2) (Nishizawa et al., 2014; Pitombo et al., 2016) more than *Rhodanobacter*, which may explain why BCP additions decreased N_2O emissions (Shen, Song, et al., 2021); furthermore, BCP addition significantly enhanced *Burkholderiales* abundance (Figure S2), in which the main strain was *Paraburkholderia_unamae*, which boosts plant growth through N fixation (Levonian et al., 2019). BCP additions may,

thus, promote N fixation in tea garden soils, confirming the results of our previous study showing a significant increase in *nifH* gene copy numbers after BCP additions. *Acidothermus* and *Acidibacter* are known as acidophilic bacteria (Falagán & Johnson, 2014; Wang et al., 2018); hence, BCP amendment decreased their abundance as its addition increased soil pH (Shen, Redmile-Gordon, et al., 2021; Shen, Song, et al., 2021).

4.3 | Shifts in the composition of soil fungi

Amendment of BCP doubled the Ascomycota abundance but significantly decreased Basidiomycota abundance by nearly 50% (Figure S3), demonstrating that BCP was conducive to Ascomycota but not to Basidiomycota. Similar to Egidi et al. (2019), who detected that Ascomycota is diverse and dominant in global soils, we found Ascomycota abundance accounting for 50% and 25% in BCP and non-BCP treatments, respectively. Tian et al. (2017) and Tedersoo et al. (2014) found that soil inorganic N concentration was the main stimulator of Rozellomycota abundance in soil. BCP addition caused a sharp decrease in soil inorganic N concentrations by immobilising N, which is the main reason for its decreasing Rozellomycota abundance in this study. The abundance of the main genera of Mortierellomycotina and Mortierella significantly increased in both depths of T3 but decreased in T4 (0–6 cm) (Figure S4). Zhang et al. (2020) reported that healthy and high-quality tea soils always harbour a high relative abundance of *Mortierella*, suggesting that the application rate of BCP addition, 1.5 mg g^{-1} soil, was an optimal rate, which may improve tea soil healthy and quality but not with a high rate (4.5 mg g^{-1} soil).

Amendments of BCP also significantly stimulated the relative abundance of *Trichoderma* (Figure 1d and Figure S4), implying that it is more competitive for BCP than other genera. Likewise, *Trichoderma* species such as *Trichoderma spirale*, *Trichoderma koningiopsis*, *Trichoderma longibrachiatum*, and *Trichoderma virens* are beneficial fungal species that operate as biological control agents or prime plant defence systems by excluding incoming plant pathogens and by attacking or inhibiting the growth of plant pathogens directly (Harman et al., 2004; Sallam et al., 2009). Additionally, the OTU number of *Trichocladium asperum* significantly increased with BCP addition (Figure 2), and Antoniou et al. (2017) proved it exhibited biological control agent capabilities; moreover, BCP boosted *Umbelopsis* abundance (Figure S4), which was also resistant to plant pathogens (Zhao et al., 2021). T4 (0–6 cm) and both depths of T3 had more biocontrol agents than those in unamended treatments. The research, taking these evidences together, reveals that BCP addition was conducive to the growth of biological control agents, which may improve soil health. Adding labile C decreases populations of soil-borne plant pathogens and suppresses the occurrence of plant diseases, as previously reported (Yao et al., 2017).

Likewise, BCP addition significantly decreased fungal OTU numbers related to plant pathogen species, especially in both depths of T3 treatments (Figure 9a). Furthermore, SEM results revealed that BCP amendments boosted biocontrol microbes and lowered plant pathogen species. These results indicate that BCP amendments play an important role in the suppression of plant pathogens, thereby improving soil health.

Notably, T4 (0–6 cm), i.e., a high-rate application, assembled fewer beneficial microbes than both depths of T3 (i.e., 1.5 mg BCP-C g^{-1} soil) at the end of the experiment (Figure 2), suggesting that the increasing application of BCP may not improve beneficial microorganism assembly. This is contrary to the second hypothesis.

Fierer et al. (2021) found that more soil microbial biomass and less soil N leaching indicated healthier soil conditions, which is consistent with our previous results, which demonstrated significant increases in a range of biological variables, including MBC, MBN, and ATP contents owing to BCP application (Shen, Song, et al., 2021); furthermore, as we mentioned in section 4.2, fewer nitrifier abundances indicate fewer losses of soil N via nitrification, and greater *nifH* gene abundances indicate greater N-fixation following BCP inputs, both of which may benefit soil health (Fierer et al., 2021). Overall, BCP additions have the potential to improve soil health conditions.

4.4 | Response of microbial topological properties and keystone species by BCP additions

Barberán et al. (2012) reported that soil microbial community composition shifts microbial networks. In this study, BCP additions increased nodes and edges of the prokaryotic network (Figure 6a–d and S8), suggesting a more complex network and a more stable prokaryotic community. Likewise, BCP increased the mean clustering coefficient (*avgCC*) in prokaryotic networks, as demonstrated in Tables S1 and S4, which suggests a prokaryotic network that highly matched the rule of "scale-free, small-world" (Barabasi & Oltvai, 2004), and produced a better connection degree between nodes and adjacent nodes (Wang et al., 2017). Albert et al. (2000) showed that a small-world model consisting of several highly connected nodes would make the bacterial network more resistant to random interruptions. BCP

additions, however, decreased the nodes, edges and *avgCC* of the fungal network (Figure 6e–h and S9; Tables S1 and S4), showing that prokaryotes were more tightly linked than fungi under the BCP amendment; BCP may reduce competition between prokaryotes. Also, prokaryotic communities may be able to maintain a relatively stable state through corporation or competition under BCP addition, which may explain why fungal communities vary more. Yuan et al. (2020) indicated that the diseased fungal feature network had more nodes and edges, clustering coefficient values and a higher average degree than a healthy feature network. Notably, consistent with our previous study (Shen et al., 2023), lower average degree and clustering coefficient values were found in the fungal networks with BCP addition. In summary, this confirms the amendments with BCP were conducive to the formation of healthier soil microbial ecosystems. Negative linkages in prokaryotic and fungal networks for each treatment accounted for >50% of the total links, implying that competitive exclusion rather than mutual cooperation may play a key role in shaping the microbial community structure, even under the BCP amendment. Negative correlations of both networks in T3 were lower than those in T2 (Figure S5), implying that BCP-amended microbial communities cooperated more. In addition, the *avgCC* of prokaryotic-fungal co-occurrence networks and positive edges increased by BCP addition (Figure S7 and Table S5), indicating that it stimulated the mutualism of the soil microbial community.

Montoya et al. (2006) and Deng et al. (2012) found that the module hubs and connectors in *Zi-Pi* plots were analogous to microbial keystone species in the microbial communities. Keystone species, in our study, belong mostly to Proteobacteria in soil prokaryotic networks (Table S2), and Ascomycota played a dominant role in the fungal network, and most of the OTU nodes that were keystone species belonged to Ascomycota (Table S3). The keystone species belong mostly to Proteobacteria in soil prokaryotic networks, according to others (Banerjee et al., 2018; Ma et al., 2020). Also, most keystone species in fungal networks belong to Ascomycota, which agrees with Ji et al. (2021) and is presumably influenced by their numerical dominance across a diversity of ecosystems. *Mortierella*, the keystone species in all BCP-amended treatments (Table S3), is a saprophytic oleaginous fungus possessing various agricultural benefits (Li et al., 2020), implicating that BCP additions may have direct agricultural benefits. The prokaryotic network in the T4 (0–6 cm) was more complex (e.g., higher *avgCC*, links, and edges), but fewer keystones (e.g., module hub and connector) were found in the fungal networks than those in T3 (Tables S3 and S4). By taking them together, our last hypothesis is rejected.

The keystone species had rare abundances (Figure S6). Brown et al. (2009) found that low-abundance microorganisms can store genetic and functional diversity, as well as buffer the ecosystem against environmental change, suggesting that rare microorganisms may be involved in biogeochemical processes in this soil. Examples of this include *Penicillium*, which is identified as a fungal antagonist used as a biocontrol agent (Meng et al., 2019) and *Rhodanobacter*, which plays an important role in N cycling.

5 | CONCLUSION

Our study found that BCP amendments increased the relative abundances of Proteobacteria (e.g., *Burkholderia*) and Ascomycota (e.g., *Trichoderma*) in the prokaryotic and the fungal communities, respectively, indicating that BCP has the potential to significantly change the assembly of the soil microbiome. Notably, BCP additions resulted in a significant decrease in fungal diversity and led to a higher number of enriched and depleted OTU in fungal communities. This suggests that BCP amendments exert a stronger influence on fungal communities. We found that BCP additions increased the *avgCC*, as well as the number of nodes and edges in the soil prokaryotic co-occurrence network. Conversely, these metrics decreased in the fungal co-occurrence network. These findings imply that, in response to BCP amendments, prokaryotes exhibit a higher degree of interconnectedness and tighter associations compared to fungi. We highlighted that BCP additions significantly increased the OTU numbers of potential biocontrol agents such as *Trichoderma* (*T.*) *spirale*, *T. koningiopsis*, *T. longibrachiatum*, and *T. virens* and decreased OTU numbers related to plant pathogens. The BCP amendment, thus, has the potential to promote soil health by increasing biocontrol agents and reducing disease species; ultimately contributing to the C-neutral goal if it could be applied extensively. Nevertheless, it is worth noting that the effectiveness of BCP amendments may not necessarily improve with higher application rates; thus, whether BCP could assist in the formation of biocontrol agents for improving soil health and which rate is optimal should be explored in future research.

AUTHOR CONTRIBUTIONS

Qunli Shen: Writing – original draft; methodology; visualization; software; data curation; formal analysis; investigation. **Paul Voroney:** Conceptualization; methodology; writing – review and editing. **Philip C. Brookes:** Conceptualization; funding acquisition; supervision; writing – review and editing; methodology; resources. **Ahmed S. Elrys:** Writing – review and editing. **Mengjie Yu:** Methodology. **Wei-Qin Su:** Methodology. **Lei Meng:** Methodology; writing – review and editing. **Meng Li:**

Conceptualization; funding acquisition; supervision; validation; writing – review and editing; methodology.

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CONFLICT OF INTEREST STATEMENT

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.

ORCID

Qunli Shen https://orcid.org/0000-0002-4906-4430

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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