



# Enhanced multifunctionality of sandy loam soil with co-application of biochar and organic manure is driven by microbial network complexity rather than community diversity

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## ABSTRACT

Despite the prevalent application of biochar and organic manure in agro-ecosystems, their combined effects on the restoration of sandy soils mediated by microbial mechanisms remain understudied. Here, we conducted a field experiment in the Mu Us sandy land to characterize the responses of soil microbiomes (bacteria, fungi, protists) and ecosystem functions (soil fertility, plant growth, material cycling, carbon mineralization potential) to co-application of biochar and organic manure with planting. The experiment incorporated three levels of biochar (0, 25, 50 t/hm<sup>2</sup>), two levels of organic manure (0, 10 t/hm<sup>2</sup>), and four levels of plants (1, 3, 5, 9 legume or grass species). The results indicated that both biochar and organic manure, but not plants, contributed to significant enhancement in soil multifunctionality. The greatest soil multifunctionality was observed at the high biochar rate with organic manure, which increased by 52 % compared to the lowest multifunctionality observed under no application of biochar and organic manure. This was manifested by increased contents of soil organic carbon (761 %), total nitrogen (207 %), and total phosphorus (67 %), alongside stimulated plant growth, despite greater carbon mineralization potential. Various microbial communities exhibited differential responses to biochar and organic manure treatments, with decrease in bacterial diversity and increase in protistan diversity. Fungal diversity positively responded to biochar application rate only. Soil organic carbon, total nitrogen, and total phosphorus emerged as the primary factors driving shifts in bacterial, fungal, and protistan communities. The complexity of microbial networks varied across treatments and positively correlated with the rate of organic manure (bacteria), biochar (fungi), or both (protist), but there was no response to planting. Linear regression analysis revealed a negative correlation between bacterial diversity and soil multifunctionality ( $R^2 = 0.14$ ,  $p < 0.01$ ). Protistan diversity ( $R^2 = 0.14$ ,  $p < 0.01$ ) and microbial network complexity (bacteria:  $R^2 = 0.25$ ,  $p < 0.001$ ; fungi:  $R^2 = 0.13$ ,  $p < 0.01$ ; protists:  $R^2 = 0.36$ ,  $p < 0.001$ ) were positively correlated with soil multifunctionality. Variance partitioning analysis indicated that microbial network complexity had a greater influence on soil multifunctionality than community diversity, underscoring the role of microbial interactions. Our results indicate that the co-application of biochar and organic manure, not plants, enhances the multifunctionality of sandy loam soil, with microbiome complexity serving as a critical predictor of changes in soil functions.

## 1. Introduction

Sandy desertification is a process of land degradation that widely occurs in arid, semi-arid, and sub-humid regions. This process is characterized by aeolian sand activities and primarily driven by human

behavior together with climate change (Wang, 2013). Sandy desertification can undermine ecosystem functions, as exemplified by the disruption of material circulation (Yao et al., 2023), reduction in carbon sink capacity (Lal, 2009), diminished nutrient supply for plant growth (Chen et al., 2023a), and loss of biodiversity (Zhang et al., 2023c). The

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resulting decrease in land carrying capacity and increased frequency of sandstorms threaten the livelihoods of nearly 1 billion people worldwide (UNCCD, 2024). Therefore, sandy desertification has emerged as a critical economic and environmental challenge that the global community must address (UNCCD, 2014). To combat this challenge, it is essential to formulate appropriate strategies that can effectively control sandy desertification.

Considerable research has been devoted to the control of sandy desertification using various restoration techniques. The popular techniques include: 1) reestablishment of vegetation by artificially reseeding and introducing plant species (Tian et al., 2023; Zhang et al., 2022); 2) improvement of sandy soil by mixing with different sandstone or clay materials (Chen et al., 2024); and 3) application of organic or inorganic fertilizers, humic acids, and soil amendments (Liu et al., 2022a; Zhang et al., 2023a). Studies using these techniques have yielded favorable outcomes. In particular, soil amendments offer a rapid and cost effective solution, since vegetation restoration takes a long time and soil amendments are mainly derived from agricultural and industrial waste.

Biochar is a widely recognized soil amendment with a porous structure and a high carbon content. It is produced through the thermochemical conversion of biomass under anaerobic conditions (Xie et al., 2022). Prior studies have established that biochar can efficiently adsorb and retain moisture because of its extensive porosity and substantial specific surface area. When incorporated into sandy soils, biochar markedly enhances soil water retention capacity (Glab et al., 2016; Uzoma et al., 2011). Furthermore, biochar exhibits outstanding adsorption capacity for soil nutrients and contains ash. These properties allow biochar to mitigate nutrient loss and improve nutrient stocks within sand soils, contributing to the restoration of nutrient supply function (Brockhoff et al., 2010; Uzoma et al., 2011). Given its high carbon stability, biochar persists in the soil for extended periods. Therefore, biochar is often considered effective as a soil amendment to sequester carbon and mitigate greenhouse gas (GHG) emissions (Karan et al., 2023; Lehmann et al., 2021). Biochar application has been shown to improve sandy soils regarding water retention, nutrient loss, and physical structure (Zhang et al., 2016). However, biochar has limitations, such as considerably lower nutrient content than that of fertilizers typically used in agro-ecosystems (Joseph et al., 2021). As such, applying biochar alone does not seem to provide a quick and effective solution to tackle nutrient deficiencies commonly found in sandy soils.

The issue arising from unbalanced nutrient stocks and inadequate nutrient levels in biochar can be solved by its co-application with organic manure—which contains sufficient nutrients (Jia et al., 2020). The high adsorption capacity of biochar allows it to mitigate nutrient loss from organic manure and facilitate a slow release of nutrients into soil. This in turn improves nutrient use efficiency and decreases GHG emissions during following application of organic manure (Kim et al., 2017; Tan et al., 2021). The combined effects of biochar and organic manure on soil quality are well documented for agricultural and horticultural systems (Bai et al., 2022; Hou et al., 2024). However, there has been a paucity of research demonstrating the feasibility of this approach for ecological restoration in sandy lands, and the role of biochar combined with organic manure in regulating the ecosystem functions of sandy soils remains ambiguous. Importantly, plants are integral to soil restoration, serving both as indicators and drivers of the restoration progresses (Jian et al., 2015; Liu et al., 2022b). Diverse plant species distinctly respond to identical restoration strategies, and their efficiency in soil restoration can vary when combined with other methods (Phillips et al., 2021; Slesak and Windmuller-Campione, 2024). Therefore, it is essential to integrate planting with co-application of biochar and organic manure in order to accurately evaluate the effects of soil amendments on sandy land productivity and subsequent vegetation restoration.

Soil microorganisms play a pivotal role in driving global biogeochemical cycles, including those of carbon, nitrogen, and phosphorus. Microbial metabolic activities facilitate the functioning of soil

ecosystems by enhancing the cycling of organic matter and nutrients, as well as contributing to soil structure formation (Sokol et al., 2022). These microbially mediated processes are intrinsically linked to vital ecosystem products (e.g., food, fiber, timber) and essential services (e.g., GHG emission regulation, carbon sequestration, erosion control, plant growth promotion) (Barrios, 2007; Coban et al., 2022; Jansson and Hofmockel, 2020). Thus, microbial activity is fundamental to the maintenance of healthy and fertile soils. Biochar exhibits variable effects on soil microorganisms, as a meta-analysis has revealed that the role of biochar in shaping soil microbial diversity is contingent upon both biochar characteristics and soil type (Deshoux et al., 2023). While the nutrient components of biochar are accessible for microbial proliferation, its extensive specific surface area and ample pore space create a conducive environment for microbial habitation (Palansooriya et al., 2019). Biochar induces alterations in soil microbiomes through various mechanisms, contributing to the fulfillment of specific soil functions (Ghiloufi et al., 2024; Phillips et al., 2021).

Substantial nutrient input from organic manure has also been shown to trigger a compositional transformation in soil microbiomes. Following the application of organic manure, soil microbiomes shift from a community structure that is well adapted to oligotrophic conditions to one that is better suited for the degradation of complex organic matter (Hartmann et al., 2015). Concurrent variations in nutrient availability and the introduction of microbial populations through organic manure regulate microbial interactions within the soil (Tang et al., 2023). This is accompanied by altered abundance of keystone microbial taxa that are essential for maintaining soil functionality (Han et al., 2022; Wang et al., 2021). Soil microbiome restructuring may have prominent influence on the overall health and functionality of the soil ecosystem. While the individual effects of biochar and organic manure are well documented, it remains elusive how their co-application shapes soil microbiomes, particularly in sandy soil environments. Deciphering the synergistic effects of biochar and organic manure on soil microbiomes can broaden our understanding of the mechanism underlying sandy soil restoration and offer a new possibility for desertification management.

This study was carried out in an experimental site of the Mu Us Sandy Land in northwestern China under restoration by co-application of biochar and organic manure with legume/grass planting. The aim of the present study was to address the following two questions: (1) Does co-applied biochar and organic manure with planting contribute to the ecosystem functions of sandy soil? And (2) What microbial mechanism drives the responses of sandy soil functions to the integrated restoration strategy? We analyzed the changes in soil functions and alterations in bacterial, fungal, and protistan communities under different treatments. The results could provide novel insight into the microbially mediated process of sandy soil restoration with co-application of biochar and organic manure.

## 2. Material and methods

### 2.1. Study area

The study was conducted at the Shenmu Experimental Station of Northwest A&F University ( $38^{\circ}48'36''$  N,  $110^{\circ}16'48''$  E) in Yulin, Shaanxi Province, China. The elevation of the study area ranges from 720.6 to 1437.8 m, with an average annual temperature of  $8.9^{\circ}\text{C}$ . The average annual precipitation is 440.8 mm, predominantly occurring between July and September. This area experiences a semi-arid continental monsoon climate characterized by ample sunlight, significant temperature variations, and favorable ventilation. Rainfall and heat occur during the same season, with distinct seasonal changes in a year. The primary natural hazards include drought and low-temperature frost, with secondary occurrence of strong winds and hails. There are three distinct topographic regions: the northern wind-sand grassland region, the central and southern loess hilly gully region, and the southeastern

canyon gully region. The predominant vegetation species in this area include *Salix cheilophila*, *Caragana korshinskii*, *Artemisia frigida*, *Amorpha fruticosa*, and *Melilotus officinalis*. The soil composition consists of 81 % sand, 16 % silt, and 3 % clay. Based on the International Union of Soil Science (IUSS), the soil texture is classified as sandy loam soil. For additional information regarding soil properties, please refer to Table S1.

## 2.2. Experimental design

A randomized block experimental design was employed to investigate the effects of three treatment factors: biochar, organic manure, and legume/grass plants. The biochar treatment consisted of three levels: B1: 0 t/hm<sup>2</sup>, B2: 25 t/hm<sup>2</sup>, and B3: 50 t/hm<sup>2</sup>. The organic manure treatment included two levels: O1: 0 t/hm<sup>2</sup> and O2: 10 t/hm<sup>2</sup>. The plant treatment involved single and mixed sowing of legumes and grasses under four regimes (Table S2), P1: single sowing of one species—sainfoin (*O nobrychis viciaefolia*); P3: mixed sowing of three species—sainfoin, wildryegrass (*Elymus dahuricus*), and smooth bromegrass (*Bromus inermis*); P5: mixed sowing of five species—sainfoin, wildryegrass, smooth bromegrass, yellow sweetclover (*Melilotus officinalis*), and perennial ryegrass (*Lolium perenne*); and P9: mixed sowing of nine species—sainfoin, wildryegrass, smooth bromegrass, yellow sweetclover, perennial ryegrass, alfalfa (*Medicago sativa*), chicory (*Cichorium intybus*), wormwood (*Artemisia ordosica*), and crested wheatgrass (*Agropyron cristatum*). Sainfoin, alfalfa, yellow sweetclover, wildryegrass, and wormwood are native plants or major locally cultivated forages. Smooth bromegrass, perennial ryegrass, crested wheatgrass, and chicory are selected species that adapt to the local climate. The economic and ecological values of plant species are taken into account, while ensuring a mixed sowing of legumes and grasses. This resulted in a total of 24 distinct treatment combinations, each replicated three times, culminating in 72 experimental plots (Table 1). Each plot measured 4 m × 4 m, with a 1 m aisle between plots.

The application procedures of biochar, organic manure, and planting in each plot are described in the Supplementary Method S1. Biochar (Biochar Future Eco-environmental Technology Co., Ltd., Guangzhou, Guangdong, China) was prepared at 500–550°C using corn straw, as corn is the predominant grain crop in northern Shaanxi, ensuring a reliable feedstock source. Composted sheep manure (Tunan Trading Co., Ltd., Jinan, Shandong Province, China) was used as an organic fertilizer, given the rapid development and large scale of sheep farming in northern Shaanxi, which facilitates the management of livestock waste. The properties of biochar and organic manure are listed in Table S1. All seeds were purchased from Rytway Ecotechnology Co., Ltd. (Beijing, China) and sown at a total rate of 60 kg/hm<sup>2</sup>. Establishment of the experimental plots started on 10 July 2022 and ended on 17 July 2022.

## 2.3. Sample collection

Soil and plant samples were collected in the peak growth period of plants (25–30 July, 2023). A 1-m<sup>2</sup> square sampling quadrat was established within each plot, and samples were obtained between plant rows

using a five-point method. Prior to soil sampling, surface debris was eliminated, and the subsequent sampling was conducted to a depth of 15 cm. The soil sample from the designated plot was prepared by mixing the collected soil from that quadrat. One portion of the soil sample was stored at -80°C until used for high-throughput sequencing. Another portion was stored at 4°C until used for the determination of soil enzyme activities and carbon mineralization. The remaining soil was sieved through 2-, 1-, and 0.15-mm mesh screens after air-drying and then used for the analysis of chemical properties. Whole plant samples were collected from the quadrat and weighed to determine their fresh weight. Then, the plant samples were oven-dried at 105°C for 0.5 h, followed by heating at 70°C until constant weight (Yang et al., 2024). The dry weight of plant samples was recorded as aboveground biomass (AGB).

## 2.4. Determination of soil chemical properties, enzyme activities, and carbon mineralization

Standard testing methods were employed to determine soil organic carbon (SOC), total nitrogen (TN), ammonia nitrogen (NH<sub>4</sub>N), and nitrate nitrogen (NO<sub>3</sub>N), total phosphorus (TP), available phosphorus (AP), and available potassium (AK) (Bao, 2000). The quantification of soil water-soluble potassium (K<sup>+</sup>), calcium (Ca<sup>2+</sup>), sodium (Na<sup>+</sup>), and magnesium (Mg<sup>2+</sup>) was conducted using an atomic spectrophotometer (PinAAcle500; Perkin Elmer Inc., Waltham, MA, USA), following sample extraction with ultrapure water (soil: water = 1:2.5, w/v). Microplate fluorescence assays were performed to measure soil enzyme activities involved in nutrient cycling: (1) carbon conversion related β-1, 4-glucosidase (BG), β-1,4-xylanidase (BX), and cellobiohydrolase (CBH); (2) nitrogen conversion related β-1,4-N-acetylglucosaminidase (NAG); and (3) phosphorus conversion related alkaline phosphatase (ALP) (DeForest, 2009; Saiya-Cork et al., 2002; Su et al., 2022).

Soil carbon cumulative mineralization (CM) was determined via microcosm incubation. Fresh soil samples (50 g each) were placed in 250-mL glass jars and adjusted to a 60 % field holding capacity with distilled water, followed by pre-incubation at 25°C for 3 days. Three empty jars without soil samples were used for reference readings. After pre-incubation, soil samples were continuously incubated and water content was monitored during the incubation period. Sampling was conducted at 1, 3, 5, 7, 14, 21, and 28 days of incubation. Prior to sampling, glass jars were sealed for 2 h. Then, headspace samples were collected using a 30-mL syringe, and the jars were opened for 1 h after sampling to maintain the oxygen level. The CO<sub>2</sub> concentration in headspace samples was measured using gas chromatography (GC-9790; Fuli Analytical Instruments, Wenzhou, Zhejiang, China). The carbon mineralization rate was calculated based on the increase in headspace CO<sub>2</sub> concentration over the 2-hour period and the volume factor of the glass jar (i.e., 250 mL). To obtain the carbon mineralization amount between two adjacent sampling time points, the average mineralization rate was multiplied by the time interval (2 or 7 days). The sum of carbon mineralization amounts over the 28-day incubation period was used to represent CM.

**Table 1**

Treatment combinations of biochar, organic manure, and plants. Biochar, B1: 0 t/hm<sup>2</sup>; B2: 25 t/hm<sup>2</sup>; B3: 50 t/hm<sup>2</sup>. Organic manure, O1: 0 t/hm<sup>2</sup>; O2: 10 t/hm<sup>2</sup>. Plants, P1: single sowing of sainfoin only; P3: mixed sowing of three species (sainfoin, wildryegrass, smooth bromegrass); P5: mixed sowing of five species (sainfoin, wildryegrass, smooth bromegrass, yellow sweetclover, perennial ryegrass); and P9: mixed sowing of nine species (sainfoin, wildryegrass, smooth bromegrass, yellow sweetclover, perennial ryegrass, alfalfa, chicory, wormwood, crested wheatgrass).

Treatment	B1		B2		B3	
	O1	O2	O1	O2	O1	O2
P1	B1O1P1	B1O2P1	B2O1P1	B2O2P1	B3O1P1	B3O2P1
P3	B1O1P3	B1O2P3	B2O1P3	B2O2P3	B3O1P3	B3O2P3
P5	B1O1P5	B1O2P5	B2O1P5	B2O2P5	B3O1P5	B3O2P5
P9	B1O1P9	B1O2P9	B2O1P9	B2O2P9	B3O1P9	B3O2P9

## 2.5. DNA extraction and 16S rRNA gene sequencing

Total genomic DNA was extracted from soil samples using the Soil Rapid DNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA) as per the manufacturer's protocol. After quality check, the extracted DNA served as a template for PCR. The 515 F/806 R primer pair was used to amplify the V4 region of the bacterial 16S rRNA genes (Miao et al., 2023). The internal transcribed spacer 1 (ITS1) region of the fungal rRNA genes was amplified with the ITS1F/ITS2R primer pair (Yu et al., 2021). The TAREuk454FWD1F/TAREukREV3R primer pair was used for PCR amplification of the protistan 18S rRNA genes (Wu et al., 2022). PCR products were purified and then sequenced at Majorbio (Shanghai, China). The sequencing was accomplished on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) using the 2 × 300 bp paired-end protocol.

The barcode and primer sequences were trimmed from sequencing reads using FLASH v1.2.7 (<https://ccb.jhu.edu/software/FLASH/>) (Magoc and Salzberg, 2011). The resulting reads were assembled to obtain raw sequences. All sequences were denoised using the DADA2 module in QIIME2 v2022.2 (Caporaso et al., 2010) to generate amplicon sequence variants (ASVs). The ASVs were taxonomically assigned based on the SILVA 138 database (<https://www.arb-silva.de/>) (Quast et al., 2013) using the classify-Sklearn module in QIIME2. Non-target taxa were eliminated from the bacterial, fungal, and protistan ASV count table before data analysis.

## 2.6. Data analysis

All data were statistically analyzed using R v4.3.3 (<https://www.r-project.org/>). Eighteen soil variables previously known to be regulated by the soil microbiome were considered, representing a wide range of ecosystem functions. Specifically, soil fertility was represented by SOC, TN, NH<sub>4</sub>N, NO<sub>3</sub>N, SAP, TP, AP, AK, K<sup>+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup>, and Mg<sup>2+</sup> contents; soil nutrient cycling was indicated by extracellular enzyme activities of BG, BX, CBH, NAG, and ALP; plant growth was exemplified by AGB; and the mitigation of carbon mineralization potential was characterized by reduction in CM. Single ecosystem functions were assessed by examining variations in individual soil variables. The capacity of the soil ecosystem to simultaneously deliver multiple functions and services, soil multifunctionality was calculated (Manning et al., 2018). The average multifunctionality index is a metric commonly used in numerous studies focused on soil multifunctionality (Chen et al., 2022; Wang et al., 2024; Ye et al., 2022). In this index, each ecosystem function is standardized to a range between 0 and 1 as follows:  $(X_{\text{raw}} - X_{\text{min}}) / (X_{\text{max}} - X_{\text{min}})$ , where  $X_{\text{raw}}$  denotes the raw value of the ecosystem function;  $X_{\text{min}}$  and  $X_{\text{max}}$  represent the minimum and maximum values of the ecosystem function observed across all experimental plots, respectively. As the mitigation of carbon mineralization potential was assessed by the reduction in CM, this function was transformed by  $1 - \text{standardized values}$ . The standardized values for all single ecosystem functions were averaged, giving the average multifunctionality index for each soil sample.

The "vegan" R package (Dixon, 2003) was used to estimate  $\alpha$ - and  $\beta$ -diversity (Bray-Curtis dissimilarities) of soil microbial communities. To ascertain variations in microbial community composition, principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities and permutation multivariate analysis of variance (PERMANOVA) were carried out (Chen and Zhang, 2022). Co-occurrence network analysis was used to reveal the treatment effects on microbial interaction patterns. First, ASVs that were present in no more than six samples were removed. Then, all pairwise Spearman correlations between ASVs were calculated, and weak correlations ( $|\rho| < 0.6, p > 0.05$ ) were removed. Network topological analysis and visualization were achieved using the "ggClusterNet" R package (Wen et al., 2022; Yuan et al., 2021). The following topological properties were analyzed: number of edges, number of nodes, connectance, average degree, average path length,

diameter, average clustering coefficient, centralization of degree, centralization of betweenness, and relative modularity.

Shapiro-Wilk and Bartlett tests were used to determine data normality and homogeneity of variance before statistical analysis. A three-factor analysis of variance (ANOVA) was implemented to determine the main effects of treatment factors and their interactions. In cases where data violated the assumptions of normality, an aligned rank transform ANOVA to elucidate the treatment effects. The significance of differences between group means was examined using t-test or Wilcoxon rank-sum test. Redundancy analysis (RDA) was used to determine the relationship between soil ecosystem functions and microbial community composition. ADONIS was used to test the difference in microbial community structure between paired samples. Linear regression was adopted to explore the relationship of soil ecosystem functions to microbial diversity and network complexity. Spearman correlations between soil ecosystem functions and network topological properties and key module composition were visualized using the "corplot" R package (Wei and Simko, 2021). Variance partitioning analysis (VPA) was used to assess the relative contribution of microbial diversity (Chao1 index) and network complexity (average degree) to soil multifunctionality.

## 3. Results

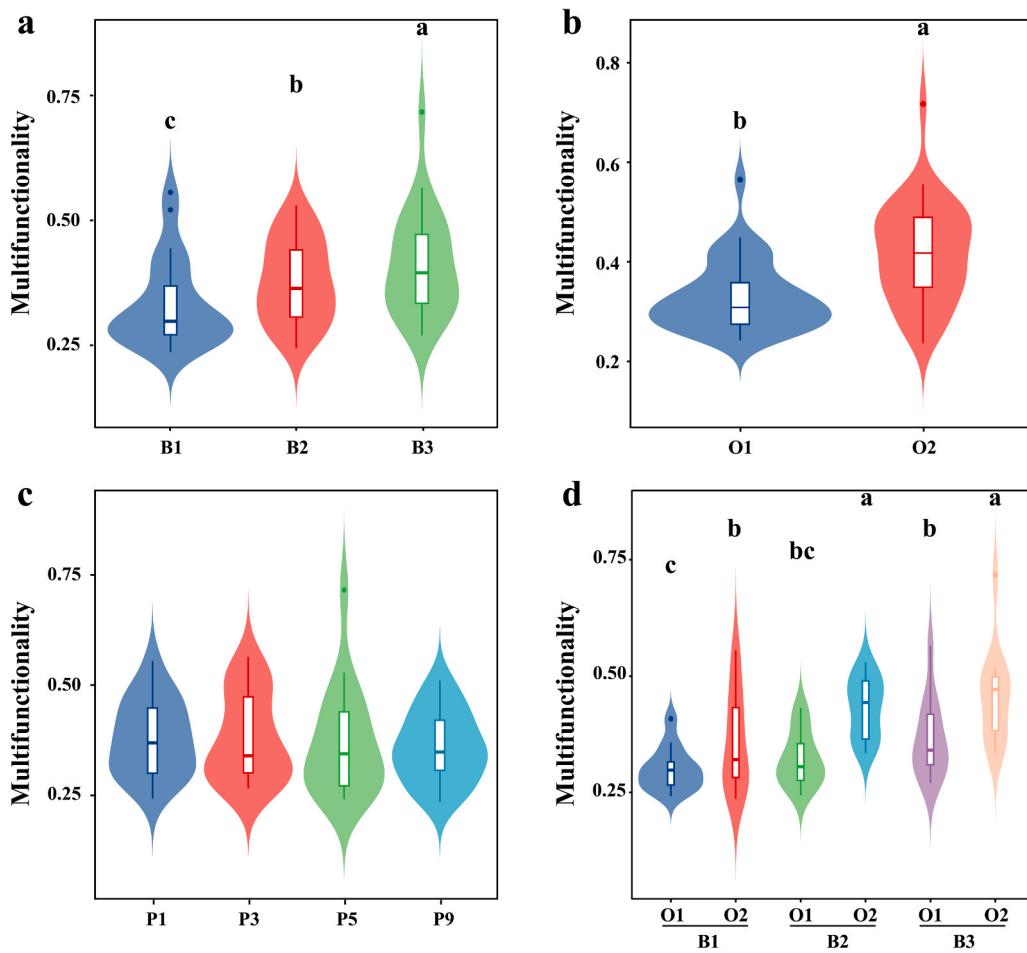
### 3.1. Single ecosystem functions and multifunctionality

Biochar, organic manure, and plant treatments exhibited distinct effects on soil ecosystem functions (Table S3). SOC, TN, and AK contents all significantly increased in response to biochar application depending on the rate applied. Compared to no biochar application (B1), the high biochar rate (B3) contributed to 395 %, 105 %, and 106 % increase in SOC, TN, and AK contents, respectively ( $p < 0.05$ ). Biochar addition also resulted in higher TP content than the no biochar treatment ( $p < 0.05$ ; Fig. S2). When organic manure was added, prominent increase was observed in the contents of SOC (33 %), TN (54 %), NO<sub>3</sub>N (229 %), TP (45 %), AP (264 %), Ca<sup>2+</sup> (20 %), and Mg<sup>2+</sup> (36 %) ( $p < 0.05$ ). This was accompanied by improved AGB (68 %) and CM (24 %), as well as enhanced NAG (34 %) and BX (22 %) activities, despite lower ALP activity (-33 %) ( $p < 0.05$ ; Fig. S3). In contrast, the effect of variations in plant communities was limited to AGB ( $p < 0.05$ ), excluding other ecosystem functions (Table S3).

As for the soil ecosystem multifunctionality, biochar and organic manure were two significant treatment factors (Table S4). The increase of biochar and organic manure application rates notably enhanced soil multifunctionality (biochar: B3 > B2 > B1; manure: O2 > O1; Fig. 1a and b), but no significant difference was observed among the mixed plant treatments (Fig. 1c). The collective results indicated that plants had little effect on either single ecosystem functions or multifunctionality (Tables S3 and S4, Fig. 1c). When plants were not taken into account, the co-application of biochar and organic manure showed a greater effect on soil multifunctionality than single application. The application of biochar and organic manure improved soil multifunctionality by 7 %–52 % compared to B1O1, and the highest multifunctionality was observed in B3O2 (Fig. 1d).

### 3.2. Variations in microbial diversity and community structure

The diversity of soil microbiomes responded differentially to biochar, organic manure, and plant treatments (Table S5). In particular, biochar was a major treatment factor for  $\alpha$ -diversity in all microbial communities. The co-application of biochar and organic manure had significant effects on  $\alpha$ -diversity in most microbial communities. The bacterial Chao1 index decreased with higher biochar and organic manure rates (Fig. 2a). Organic manure application negatively affected the bacterial Shannon index at the same biochar rate, and this effect was especially evident in B2O2 (Fig. 2d). Both the fungal Chao1 and Shannon indices increased with higher biochar rates, reaching their peak



**Fig. 1.** Differences in soil multifunctionality with application of biochar (a), organic manure (b), plants (c), and biochar combined with organic manure (d). B1 to B3 represent biochar application at rates of 0, 25, and 50 t/hm<sup>2</sup>, respectively. O1 and O2 represent organic manure application at rates of 0 and 10 t/hm<sup>2</sup>, respectively. Different lowercase letters indicate significant differences between treatments based on *t*-test ( $p < 0.05$ ).

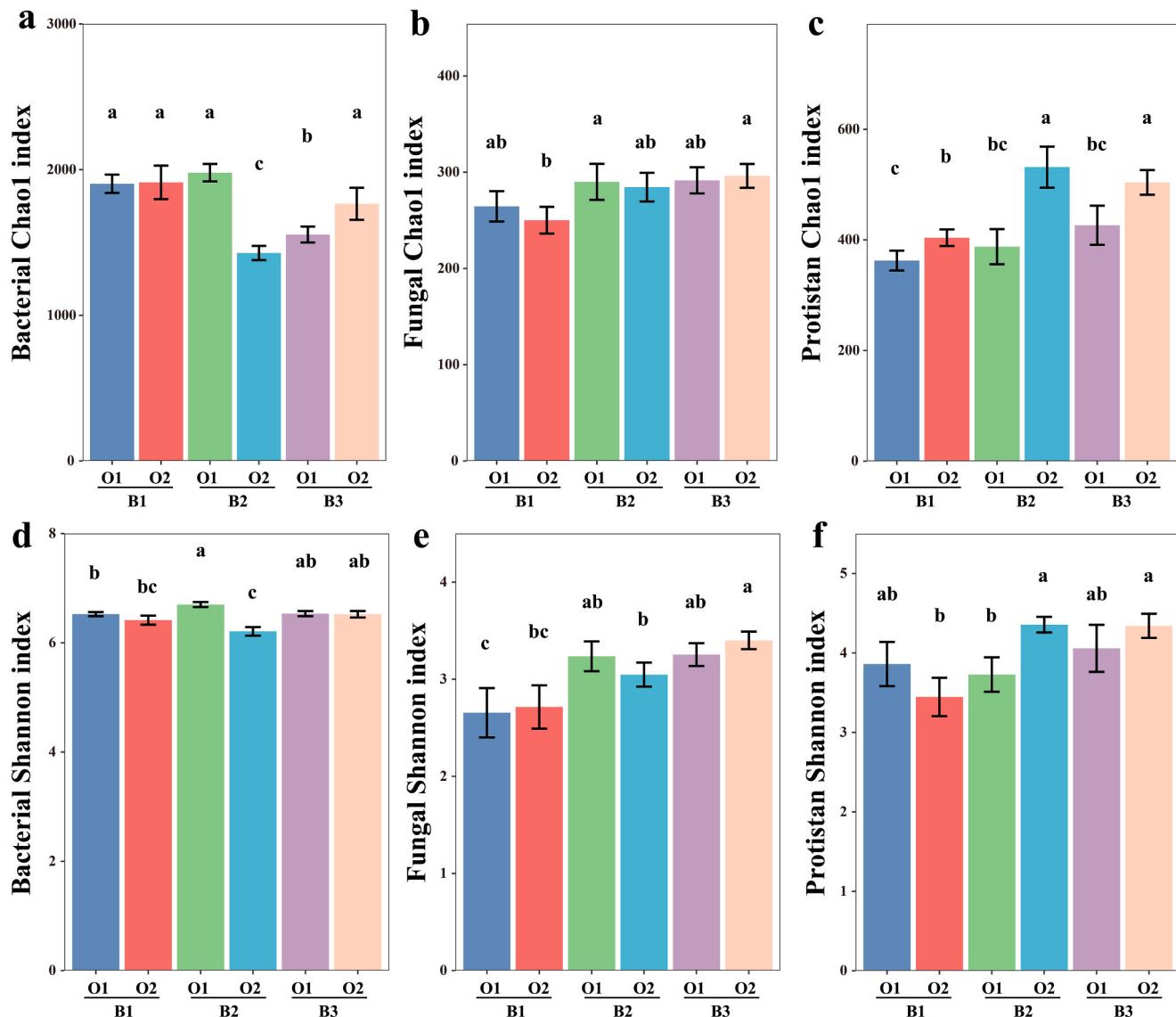
values in B3O2 (Fig. 2b and d). Compared to bacteria and fungi, protists responded more sensitively as indicated by notable increase in the Chao1 and Shannon indices following co-application of biochar and organic manure, and higher index values emerged in B3O2 (Fig. 2c and e).

PCoA revealed distinct differentiation of bacterial communities across biochar and organic manure treatments, with B3 and B1–2 samples (second axis), as well as O1 and O2 samples (first axis) clearly separated. In terms of fungal communities, B3 samples were also distinct from B1 and B2 samples, with less discrepancy between the latter two groups. Compared to bacteria and fungi, protists displayed a more intricate pattern in community structure (Fig. 3a). PERMANOVA results indicated significant treatment effects on the community structures of bacteria (biochar:  $R^2 = 0.05, p = 0.001$ ; manure:  $R^2 = 0.08, p = 0.001$ ), fungi (biochar:  $R^2 = 0.08, p = 0.001$ ; manure:  $R^2 = 0.02, p = 0.027$ ), and protists (biochar:  $R^2 = 0.04, p = 0.007$ ; manure:  $R^2 = 0.03, p = 0.001$ ). Planting primarily affected protistan community structure ( $R^2 = 0.12, p = 0.001$ ). Across various microbial communities, significant differences were observed in their compositions between B3 and B1 samples, as well as between O1 and O2 samples for bacteria, fungi, and protists (Table S6). Biochar and organic manure exhibited distinct effects on microbial community dissimilarities. Bacterial community dissimilarity trend higher with increasing rates of both soil amendments and was enhanced with organic manure addition regardless of biochar rate. Conversely, the dissimilarity of protistan communities diminished with biochar application (Fig. 3b).

### 3.3. Responses of microbial network patterns

To identify the potential interactions of soil microbiomes in response to various treatments, microbial co-occurrence networks were constructed (Fig. 4, Table S7) and the topological properties of each subnetwork were extracted. Biochar, organic manure, and their interaction had significant effects on multiple network properties of fungi, bacteria, and protists, whereas the effects of plants were not significant (Table S8). Various microbial networks responded differently to the treatments (Fig. 4). Several network properties of bacteria were significantly positively correlated with organic manure rate, but not with biochar rate or plant species number. The co-occurrence pattern of fungi was different to that of bacteria, with significant positive correlations between network properties and biochar rate. Protistan networks were more sensitive to the treatments and their topological properties were significantly positively correlated with both biochar and organic manure rates. Compared to single application, the co-application of biochar and organic manure improved microbial network properties more prominently, emphasizing a greater role of co-applied soil amendments in increasing network complexity (Figs. S4–6). The network robustness varied across treatments, which trended downward with higher biochar rate and increased with organic manure application (Tables S9, S10).

To pinpoint the keystone taxa possibly linked to soil ecosystem functioning, the taxonomic composition of the top 10 largest modules within the bacterial, fungal, and protistan networks was analyzed. Proteobacteria (27 %), Actinobacteriota (24 %), and Firmicutes (17 %) were found to be the dominant phyla within the bacterial modules.



**Fig. 2.** Changes in soil bacterial, fungal, and protistan  $\alpha$ -diversity in terms of Chao1 (a) and Shannon (b) indices following co-application of biochar and organic manure. B1 to B3 represent biochar application at rates of 0, 25, and 50 t/hm<sup>2</sup>, respectively. O1 and O2 represent organic manure application at rates of 0 and 10 t/hm<sup>2</sup>, respectively. Error bars represent standard error of the mean ( $n = 12$ ). Different lowercase letters above the error bars indicate significant differences between treatments based on Wilcoxon rank-sum test ( $p < 0.05$ ).

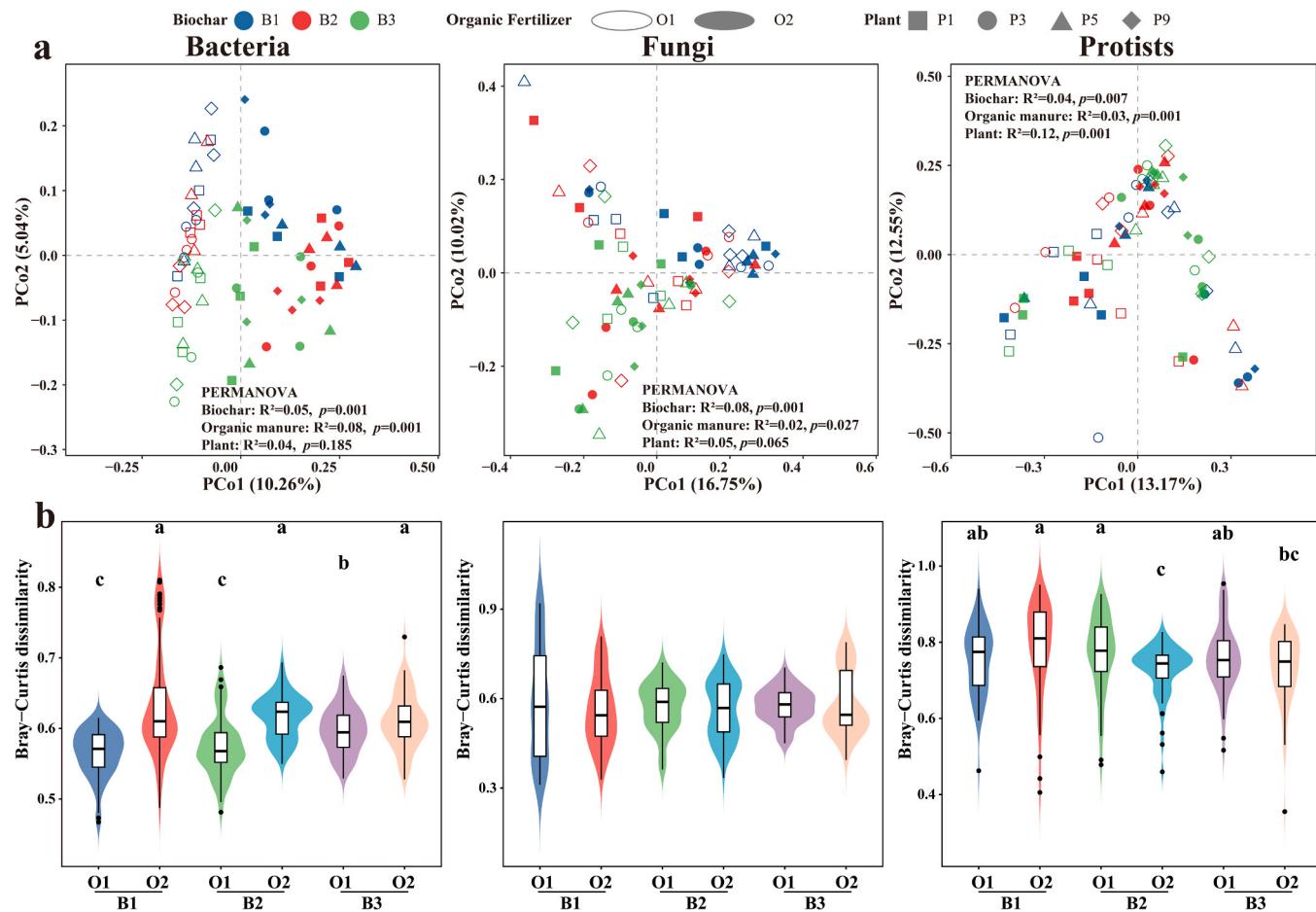
Ascomycota (61 %) and Cercozoa (21 %) were the major phyla in the fungal and protistan modules, respectively (Fig. S7). At a finer taxonomic resolution, genera such as *Rubellimicrobium* (bacteria), *Bacillus* (bacteria), *Alternaria* (fungi), and *Vermamoeba* (protists) showed high relative abundances within their respective networks (Fig. S8).

#### 3.4. Linkages between microbial diversity, network complexity, and soil functions

A linear regression model was employed to examine the correlation between the diversity of soil microbiomes and a range of ecosystem functions (Fig. S9). Regarding soil functions, only SOC, TN, and TP were significantly correlated with bacterial, fungal, and protistan diversity. Among the microbial communities, bacterial diversity was significantly negatively correlated with half (9) of the assessed soil functions, whereas a significant positive correlation emerged between fungal diversity and fewer than half (5) of the soil functions. Notably, protistan diversity displayed heightened sensitivity to soil functional changes, as indicated by its significant positive correlation with more than half (11)

of the soil functions. RDA identified SOC, Mg<sup>2+</sup>, NAG, and AGB as the principal soil functions shaping microbial community composition (Fig. S10). Correlation analysis between microbial network properties and soil functions revealed that the changes in network structure were principally driven by SOC, TN, TP, and AP (Fig. S11). Furthermore, the correlations between taxa relative abundances in key network modules and soil functions were assessed. A number of ASVs in module 1 of bacteria (Figs. S12–14), module 4 of fungi (Fig. S15), and module 1 of protists (Fig. S16) were identified as potential key taxa based on their significant positive correlations with multiple soil functions.

The relationship between soil microbial diversity and ecosystem multifunctionality exhibited distinct patterns (Fig. 5a). Bacterial and protistan diversity were significantly correlated with multifunctionality (bacteria:  $R^2 = 0.14$ ,  $p < 0.01$ , negatively; protist:  $R^2 = 0.14$ ,  $p < 0.01$ , positively). The correlation between fungal diversity and multifunctionality was weak ( $R^2 = 0.02$ ,  $p > 0.05$ ). Nevertheless, a significant positive correlation emerged between network complexity and multifunctionality across bacteria, fungi, and protists, with greater  $R^2$  values than those observed with diversity (Fig. 5b). Importantly, both the



**Fig. 3.** Responses of microbial community composition to various treatments revealed by principal coordinates analysis (PCoA, a) and community dissimilarity (b) based on Bray-Curtis distances. B1 to B3 represent biochar application at rates of 0, 25, and 50 t/hm<sup>2</sup>, respectively. O1 and O2 represent organic manure application at rates of 0 and 10 t/hm<sup>2</sup>, respectively. P1 to P9 represent single and mixed sowing of one to nine plant species, respectively. Significant differences between treatments are indicated by different lowercase letters ( $p < 0.05$ ).

diversity and network complexity of protists were positively correlated with multifunctionality (Fig. 5a and b). VPA revealed that the effect of microbial network complexity on multifunctionality was significant in all cases. Concerning microbial diversity and network complexity, protists exerted the most substantial influence on multifunctionality (Fig. 5c–e). The impact of protistan diversity on multifunctionality became negligible when the combined effects of protistan diversity and network complexity were not considered. These results indicate that soil microbial diversity did not always considerably affect ecosystem multifunctionality. A more pronounced relationship might exist between microbial network complexity and ecosystem multifunctionality, irrespective of bacteria, fungi, and protists.

#### 4. Discussion

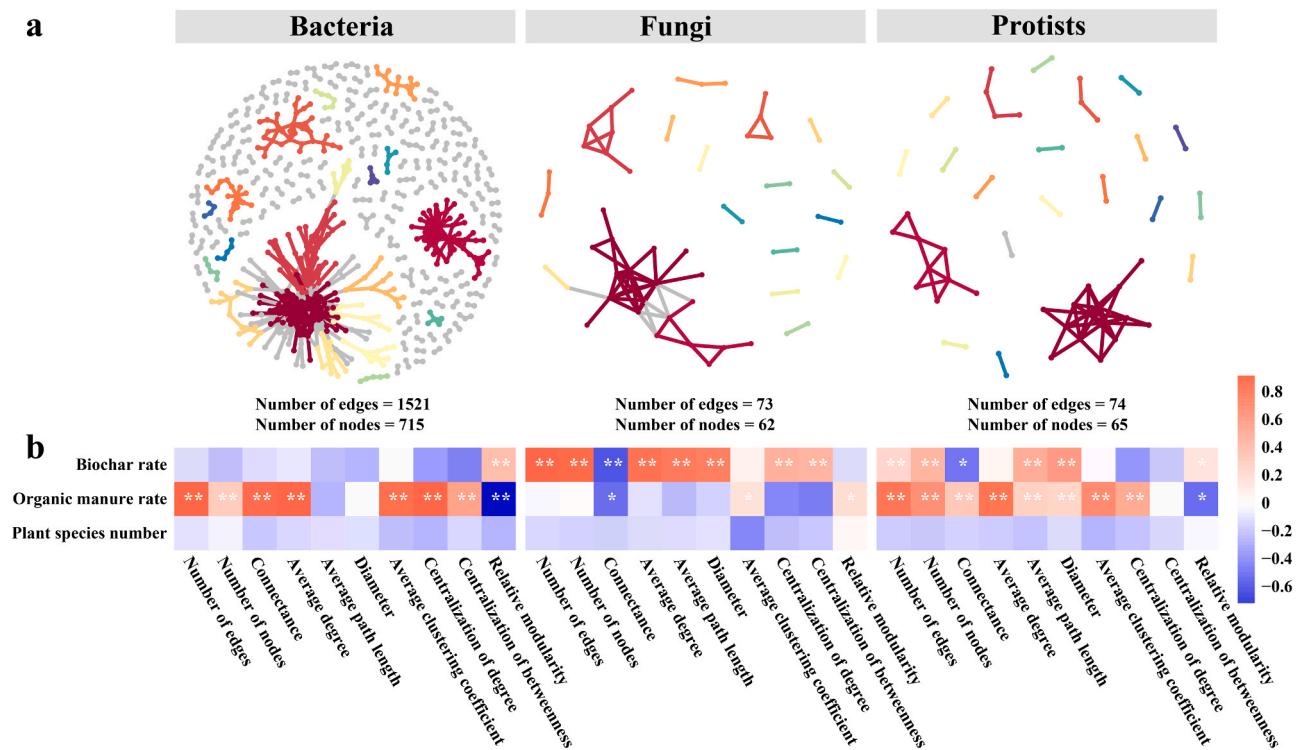
##### 4.1. Co-application of biochar and organic manure enhances soil ecosystem functions

Biochar and organic manure are widely used as soil amendments in agriculture (Clark et al., 2019; Garbowksi et al., 2023). Both materials contain notably higher nutrient contents (e.g., carbon, nitrogen, phosphorus) than natural soils, as indicated by empirical data from the present study and previous findings from other studies (Bekchanova et al., 2021; Cordovil et al., 2006; Meng et al., 2005). Thus, these soil amendments can substantially improve soil nutrient levels, boosting the ecosystem function of nutrient supply. Our findings align with previous studies, demonstrating that both biochar and organic manure treatments

markedly increased SOC, TN, and TP contents in sandy soil.

We observed that the addition of organic manure increased soil AP content while decreasing ALP activity. The manure-induced reduction in soil ALP activity is likely due to elevated phosphorus availability, which could facilitate microbial acquisition of inorganic phosphorus and diminish the necessity for organic phosphorus degradation (Chen et al., 2019). Nonetheless, the application of organic manure led to notable enhancement in soil NAG and BX activities, as well as in CM. This phenomenon can be attributed to the provision of a substantial carbon source by organic manure, which potentially stimulated microbial growth and metabolism, driving the dynamics of soil material cycling (Liu et al., 2025; Song et al., 2022). In summary, the co-application of biochar and organic manure substantially improves microbially mediated ecosystem multifunctionality of sandy soil.

However, we observed minimal effect of legume/grass plants on the ecosystem functions of sandy soil. This is contrary to many previous studies showing that plant diversity contributes to soil ecosystem multifunctionality over long time scales (Hu et al., 2022; Li et al., 2024). Plants influence soil functionality mainly through root exudates and litter inputs (Lei et al., 2023; Williams and de Vries, 2020). It takes time for this process to build up and produce a measurable impact on soil functions, leading to a potential lag effect, and the impact of plant diversity on soil functions increases over time (Eisenhauer et al., 2012). Furthermore, the inherent aridity of sandy soils can limit plant growth and, as such, affect the role of vegetation in soil functions (Hu et al., 2021). Disentangling the relationship between plant diversity and soil functions in sandy land under long-term vegetation restoration is of



**Fig. 4.** Co-occurrence networks of microbial communities (a) and relationships between treatment factors and network topological properties (b) based on Spearman correlation coefficients. \*  $p \leq 0.05$  and \*\*  $p \leq 0.01$ .

practical significance for desertification control. For the time being, microorganisms are essential contributors to various functions of soil ecosystems and their key role in regulating soil multifunctionality has been emphasized. Soil microorganisms mediate material cycling, facilitate the release of nutrients essential for plant growth, and contribute to carbon pool management (Chen et al., 2020; Ling et al., 2022). Future research should elucidate the long-term effects of plants on soil multifunctionality driven by microbial mechanisms.

#### 4.2. Soil microbiomes distinctly respond to co-application of biochar and organic manure

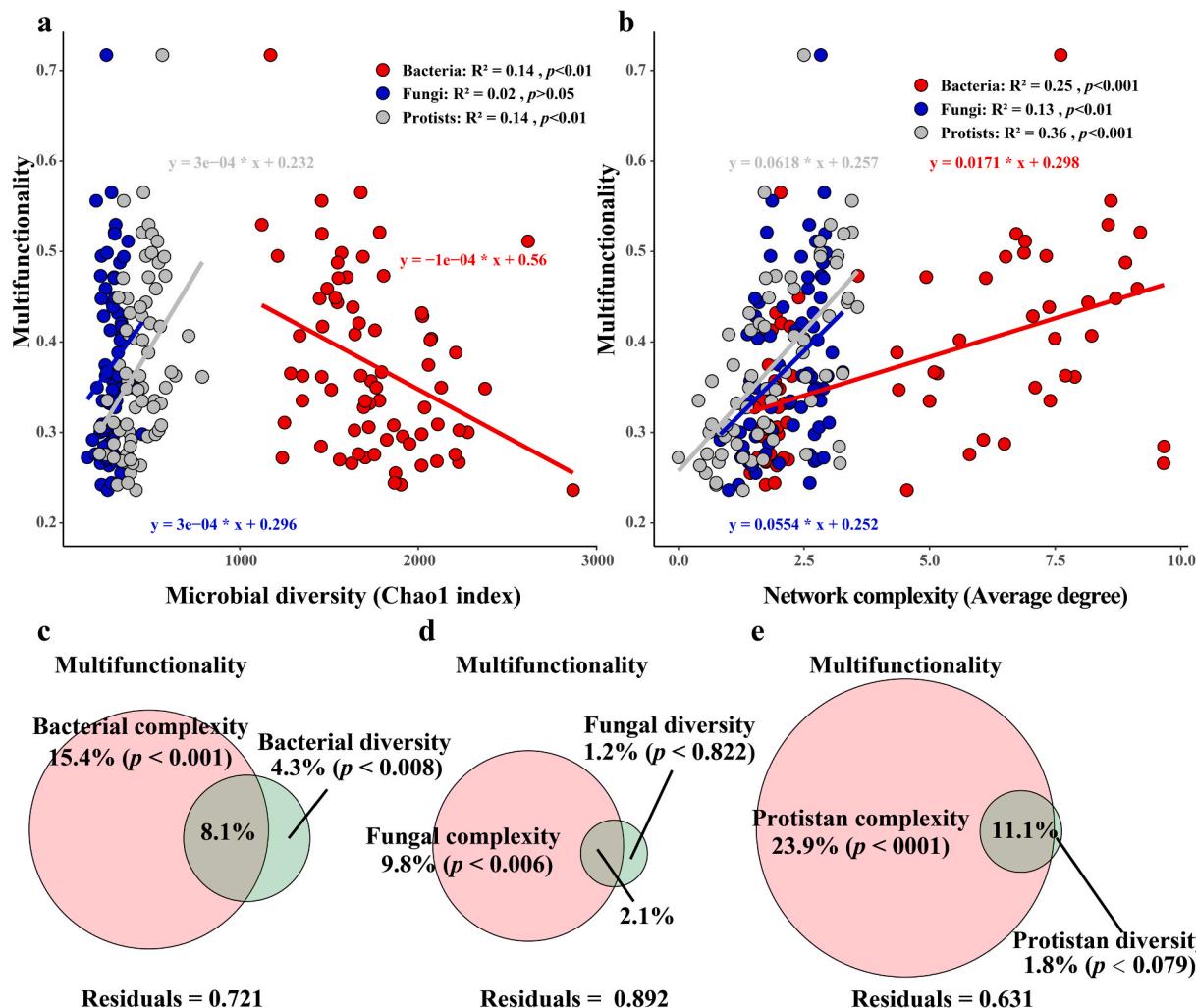
Biochar and organic manure have the potential to alter soil microbiomes by supplying nutrients and modifying ecological niches (Chen et al., 2016; Zhu et al., 2017). The co-application of biochar and organic manure led to diminished bacterial  $\alpha$ -diversity and enhanced fungal and protistan  $\alpha$ -diversity in sandy soil. Generally, the nutrients derived from soil amendments can stimulate microbial growth and reproduction (Sun et al., 2015; Wang et al., 2017). However, this effect is not absolute, because bacterial populations can be impaired by competitive interactions between soil bacteria and fungi (Bahrampour et al., 2018; Wang and Kuzyakov, 2024), as well as the predatory effect of protists on bacteria (Nguyen et al., 2023). Additionally, both biochar and organic manure treatments influenced the composition of soil microbial communities predominantly shaped by carbon, nitrogen, and phosphorus nutrients. We also observed a significant correlation between soil microbial diversity and various ecosystem functions (negative for bacteria and positive for protists). The collective results uncover that biochar and organic manure contribute to enhanced ecosystem multifunctionality by modifying soil microbial communities.

Microbial network analysis offered a novel perspective on ecosystem multifunctionality enhancement. Among the major genera we found in the microbial networks, many species of the genus *Bacillus* have the ability to promote plant growth and control soil-borne diseases (Prasad et al., 2023). *Alternaria* is a diverse group of saprotrophs and

*Vermamoeba* species feed on other microorganisms, both of which are large contributors to material cycling (Fernandes et al., 2023; Weger et al., 2024). Given their heterotrophic lifestyle, *Alternaria* and *Vermamoeba* are affected by nutrient inputs from the environment. This reveals a potential mechanism whereby the application of biochar with organic manure improves soil functions by shaping the microbial network. Notably, organic manure addition boosted the complexity of bacterial networks in sandy soil, despite decreased bacterial diversity. This observation suggests that soil bacteria might enhance their interactions to resist manure-induced environmental changes and species competition (Wang and Kuzyakov, 2024; Yuan et al., 2021). The complexity of fungal networks was more pronounced in response to biochar addition. When applied to the soil, biochar could create a favorable habitat for fungal growth (Zhang et al., 2023b), as fungi are generally less sensitive to changes in soil nutrient levels compared to bacteria (Huang et al., 2021). Protistan networks responded to both biochar and organic manure treatments. As protists are major microbial consumers in the soil, their abundance and interactions are influenced by the dynamics of bacterial and fungal communities (Zhao et al., 2019). The topological properties of microbial networks were found to be significantly positively correlated with a range of soil functions. In short, the co-application of biochar and organic manure promotes soil ecosystem multifunctionality by increasing microbial network complexity.

#### 4.3. Microbial network complexity surpasses biodiversity in driving soil multifunctionality

Microorganisms have been recognized as key contributors to the multifunctionality of soil ecosystems (Delgado-Baquerizo et al., 2016b; Hartmann and Six, 2023). However, prior research has mainly looked at the role of microbial diversity, overlooking the intricate interactions among microbial communities (Chen et al., 2022). The observed relationship between microbial diversity and soil multifunctionality is not consistently positive (Wagg et al., 2019; Zhou et al., 2020). Therefore, we employed regression analysis to disentangle the intertwined



**Fig. 5.** Microbial diversity and network complexity in relation to the average multifunctionality index (a) and their relative contributions to soil multifunctionality revealed by variance partitioning analysis (VPA, b).

relationship between microbial community diversity, network complexity, and ecosystem multifunctionality in sandy soil. The network complexity of bacteria, fungi, and protists was in more robust associations with soil multifunctionality than community diversity, consistently demonstrating a positive influence. Furthermore, VPA revealed that network complexity maintained a pronounced influence on multifunctionality, whereas the significance of community diversity diminished once the shared variance attributable to both variables was accounted for.

Our finding aligns with previous research, affirming that microbial network complexity, rather than community diversity, drives soil multifunctionality (Chen et al., 2022, 2023b). This pattern may be attributed to the presence of high functional redundancy in soil microbiomes (Delgado-Baquerizo et al., 2016a), coupled with the fact that not all microorganisms contribute to soil function maintenance (Bastida et al., 2016; Jiao et al., 2022). Correlation analysis revealed that only specific ASVs in individual network modules were significantly correlated with multiple soil functions. Consequently, the relationship between soil microbial diversity and ecosystem multifunctionality becomes increasingly unpredictable. Microbial interactions may indicate potential competitive or cooperative dynamics among microorganisms in soils (Guseva et al., 2022). The enhancement of soil multifunctionality is likely attributed to the activation of microbial species that are typically inactive or do not directly contribute to soil functions. Thus, integration of these species into the active co-occurrence network results in

increased community complexity.

This study underscores the prominent influence of microbiome complexity on the ecosystem multifunctionality of sandy soil under co-application of biochar and organic manure with legume/grass planting. To improve sandy soil conditions, the role of microbiome complexity should be considered when implementing restoration strategies aimed at enhancing soil multifunctionality. Microbiome complexity serves as a reliable predictor of variations in soil multifunctionality. However, bacteria, fungi, and protists do not operate in isolation within the sandy soil environment, and the interactions among various microbial communities remain to be thoroughly investigated.

## 5. Conclusions

This study unlocked the potential of biochar co-applied with organic manure for improving soil ecosystem functions, including nutrient supply and material cycling in sandy land. Unexpectedly, there was no effect of planting with single or mix legume and grass species over a shorter period. Given the escalating issue of soil desertification, this research provides empirical support for the use of biochar and organic manure in sandy soil management, broadening the scope of potential control strategies. Our findings indicate that microbiome complexity, rather than diversity, serves as a significant and stable predictor of alterations in the ecosystem multifunctionality of sandy soil. This insight elucidates a possible mechanism that co-applied soil amendments boost

ecosystem multifunctionality by reinforcing microbial interactions within sandy soil. Uncovering microbial interactions in sandy ecosystems, as well as other global ecosystems, will enhance our understanding of the microbial drivers of soil multifunctionality and foster the development of effective amelioration strategies.

### CRediT authorship contribution statement

**Jing Wang:** Investigation, Data curation. **Kang Zhang:** Investigation, Data curation. **Xing Ma:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Zhen Yang:** Methodology, Investigation. **Yuru Gao:** Investigation. **Aijiao Wu:** Project administration, Formal analysis. **Miaochun Fan:** Supervision, Resources, Project administration, Investigation, Funding acquisition. **Wenqing Chen:** Resources, Funding acquisition. **Zhouping Shangguan:** Resources, Funding acquisition.

### Declaration of Competing 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.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.agee.2025.109812](https://doi.org/10.1016/j.agee.2025.109812).

### Data Availability

Data will be made available on request.

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