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# Unearthing and harnessing the power of the soil microbiome and mycorrhizas to enhance plant nutrient utilization under climate stress

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# Unearthing and harnessing the power of the soil microbiome and mycorrhizas to enhance plant nutrient utilization under climate stress

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# Editorial: Unearthing and harnessing the power of the soil microbiome and mycorrhizas to enhance plant nutrient utilization under climate stress

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KEYWORDS

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Editorial on the Research Topic
Unearthing and harnessing the power of the soil microbiome and mycorrhizas to enhance plant nutrient utilization under climate stress

The population of the Earth, estimated at 7.86 billion currently, is projected to reach 9.8 billion by 2050 and 11.2 billion by 2100, according to the United Nations Department of Economic and Social Affairs (1). This growth is expected to increase the demand for agricultural land to achieve food security, however, the sustainability of agriculture continues to be hindered by its over-reliance on chemical fertilizers, pesticides, and herbicides, which can lead to severe negative environmental consequences, such as a decrease in soil organic matter and a reduction in soil microbial diversity, ultimately impacting the production of food. This problem is further compounded by climate change, soil health degradation, and a variety of biotic and abiotic stresses (2). Adapting the soil microbiome can lead to improvements in biotic and abiotic stress management, increased crop yields, optimized nutrient cycling, and enhanced natural resource utilization (3). Identifying and harnessing beneficial soil microorganisms is essential for sustainable agricultural production, especially given the challenges posed by biotic and abiotic stressors. Among the various potentially beneficial microorganisms, plant growth-promoting microbes (PGPM) and arbuscular mycorrhizal fungi (AMF) are often considered safe and environmentally friendly solutions for addressing a range of stressors (4). A better understanding of interaction and application of PGPM and AMF in agricultural systems can help to develop novel methods that enhance crop yield, soil health, and fertility (5).

In this Research Topic, the potential and prevalence findings was observed in the area of plant-soil-microbial interactions and these findings emphasize the primary factors involved in the biological, physical and chemical changes that occur in the rhizosphere, and the positive impact these changes may have on plant nutrient acquisition. Additionally, the study sheds light on how the soil microbiome and specific species can promote nutrient uptake, plant-growth and development, and alleviate climate stress. The study of Wan et al. found that soil microbial communities are vital for terrestrial ecosystem functions and indicate grassland health. It observed that drought stress significantly impacts bacterial and fungal diversity, with bacteria being more sensitive than fungi. This implies that bacterial community diversity or structure could indicate alpine grassland health status. The study identified soil moisture, plant diversity, and soil organic matter as the key factors influencing soil fungal & bacterial community and composition of functional potential. Importantly, microbial functional potential can be predicted through taxonomic profiles. This research elucidates the mechanisms behind microbial community composition and functional responses to climate change, specifically prolonged drought, in semiarid alpine grasslands. Similarly, the implications of drought brought on by the increase of groundwater depth on soil microbiota and multi-functionality remain unclear, hindering our comprehension of the sustainability of water-deficient ecosystems that heavily depend on ground-water resources. This research examined the effects of modified ground-water depths, induced by hydrological processes or anthropogenic activities, on rhizospheric microbiota and multi-functionality in a semi-arid area. The composition of soil microbiota was altered due to the rising ground-water depth, resulting in a decrease in the relative abundance of dominant phyla, such as Proteobacteria and Ascomycota (Zhao et al.). Mukhongo et al. conducted a study using morphotyping and enumeration to assess the composition and spore abundance of Arbuscular Mycorrhizal Fungi (AMF) in sweet potato cultivation areas in eastern Uganda. They identified six AMF genera: *Gigaspora, Entrophospora*, *Glomus*, *Archaeospora, Scutellospora*, and *Acaulospora*. The study found that soil parameters impacted AMF spore abundance differently across genera, with pH, N, SOC (soil organic carbon), Na, silt, and Mg, having the most significant effects. The predominant AMF species, *Glomus* and *Acaulospora*, have potential for local bio-inoculant production. Huang et al. investigated microbial diversity and its relationship with soil properties and enzyme activities along an elevational gradient in Southwest China. While specific soil properties varied significantly among sites on the elevational gradient, overall soil chemistry was not clearly differentiated by elevation. Conversely, bacterial and fungal communities differed significantly on the elevation gradient. Fungal diversity exhibited an N-shaped pattern, while bacterial diversity decreased with elevation. Microbial abundance peaked at 1,800 m, but only bacterial variations correlated with soil pH. Soil enzyme activities were elevation-dependent and linked to specific microbes: Acidobacteria and Planctomycetaceae bacteria were associated with catalase and ACP (extracellular acid phosphatase) changes, respectively. Fungi were mainly associated with β-glucosidase, sucrase, and urease changes. Herpotrichiellaceae positively correlated with β-glucosidase and sucrase, and Sebacinaceae with urease. Li et al. identified Actinobacteriota, Chloroflexi, Firmicutes, Acidobacteriota and Proteobacteria as the dominant bacteria in non-organic, organic, and intercropping soil groups, with variations in their relative abundance. Acidobacteria bacterium particularly increased in the organic and intercropping soils. Venice et al. examined a mature silver fir stand affected by windstorm-induced uprooting and compared it to a nearby undamaged area. The site, impacted by root rot agents *A. ostoyae* and *H. abietinum*, showed that these agents were not the sole cause of damage. The study found that tree uprooting increased alpha diversity, primarily due to wood-decay fungi, likely resulting from the rise in dead plant material. Additionally, certain mycorrhizal taxa responded to plant succession, marked by increased grasses and shifts in plant symbionts. According to Li et al, intricate decomposition pathways within soil micro-food webs play a crucial role in facilitating the cycling of soil organic carbon and nutrients, ultimately impacting the productivity, quality, and sustainability of soil systems. Ducousso-Détrez et al. highlighted P insufficiency in croplands, even in regions using P fertilizers, prompting interest in microorganisms that convert unavailable P into plant-accessible forms. *Bacillus* and *Pseudomonas*, the most common genera identified, are promising candidates for biofertilizers, potentially forming bioinoculant consortia to improve P nutrition and growth in soils rich in reactive P. Another finding by Liu et al. showed that the soil samples from Fujian Province and Xinjiang Autonomous Region exhibited a rich fungal diversity, comprising thirty-one classes, two hundred families, eleven fungal phyla, eighty-six orders, three hundred eighty-eight genera, and five hundred fifteen species. These samples revealed dominant fungal phyla that play crucial roles in energy cycling and organic matter degradation, such as *Agaricomycetes*, *Leotiomycetes*, *Sordariomycetes*, and *Archaeosporomycetes*. Ji et al. reported that the influence of halotolerant nitrogen-fixing bacteria (HNFB) on AMF community in the rhizosphere of apple plants is affected by soil nitrogen levels. It was found that the inoculation of HNFB resulted in an increase in microbial biomass and the relative abundance of beta-glucosidase-related genes. According to research by An et al., Astragalus-cultivated soils exhibit an enhanced presence of AMF. The study identified a total of seventy-four OTUs and classified them into a single phylum, Glomeromycota; 1 class, Glomeromycetes; 4 orders; 4 families; and 6 genera. Liu et al. examined the impact of exogenous organic acid composite biological substrates on bacterial community structures in beach soils. Their findings showed that these substrates did not significantly alter bacterial alpha diversity ($p \geq 0.05$). Nonetheless, the fulvic acid composite pine needle treatment slightly increased alpha index values relative to the control. Ng et al. demonstrated that PGPR and elevated $CO_2$ ($eCO_2$) enhance plant yield and quality *via* interactions with rhizosphere microorganisms. For *Pseudostellaria heterophylla*, PGPR (*Bacillus subtilis* and *Pseudomonas fluorescens*) under 1000 ppm $eCO_2$ positively influenced the plant and its rhizosphere microbes. This resulted in a 38% increase in yield and a 253% higher in the accumulation of active ingredient polysaccharides in the tuber.

Finally, we believe that this Research Topic on "Unearthing and Harnessing the Power of the Soil Microbiome and Mycorrhizas to Enhance Plant Nutrient Utilization under Climate Stress" will offer valuable insights into the latest developments and advantages of soil microorganism application to attain sustainable agricultural production, as well as the utilization of microbial inoculants to boost crop yields while conserving soil health.

## Author contributions

DM: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. MP: Supervision, Writing – original draft, Writing – review & editing. AR-S: Project administration, Supervision, Writing – original draft, Writing – review & editing. PA: Conceptualization, Writing – original draft, Writing – review & editing. SS: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. PP: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. MG: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Different and unified responses of soil bacterial and fungal community composition and predicted functional potential to 3 years' drought stress in a semiarid alpine grassland

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**Introduction:** Soil microbial communities are key to functional processes in terrestrial ecosystems, and they serve as an important indicator of grasslands status. However, the responses of soil microbial communities and functional potential to drought stress in semiarid alpine grasslands remain unclear.

**Methods:** Here, a field experiment was conducted under ambient precipitation as a control, −20% and−40% of precipitation to explore the responses of soil microbial diversity, community composition, and predicted functional potential to drought stress in a semiarid alpine grassland located in the northwest of China. Moreover, 16S rRNA gene and ITS sequencing were used to detect bacterial and fungal communities, and the PICRUST and FUNGuild databases were used to predict bacterial and fungal functional groups.

**Results:** Results showed drought stress substantially changes the community diversity of bacteria and fungi, among which the bacteria community is more sensitive to drought stress than fungi, indicating that the diversity or structure of soil bacteria community could serve as an indicator of alpine grasslands status. However, the fungal community still has difficulty maintaining resistance under excessive drought stress. Our paper also highlighted that soil moisture content, plant diversity (Shannon Wiener, Pieiou, and Simpson), and soil organic matter are the main drivers affecting soil bacterial and fungal community composition and predicted functional potential. Notably, the soil microbial functional potential could be predictable through taxonomic community profiles.

**Conclusions:** Our research provides insight for exploring the mechanisms of microbial community composition and functional response to climate change (longer drought) in a semiarid alpine grassland.

KEYWORDS

**bacteria and fungi, drought stress, microbial community, diversity, functional potential, alpine grasslands**

## 1. Introduction

Precipitation is the main water resource of arid and semiarid land, and it plays an important key driving factor for various biological processes at different spatial and temporal scales (Harpole et al., 2007). The intensification of human activities has substantially affected the global atmospheric circulation pattern (IPCC, 2013) and enhanced the variation of global precipitation (Easterling et al., 2019). Climate models have also predicted the variability in global precipitation increases with a considerable increase in the intensity, frequency, and duration of drought events in the future (Huang et al., 2016). Arid and semiarid land grassland ecosystem plays an important role in maintaining the stability of the ecosystem structure and service function (Prevey, 2019). Numerous previous studies have shown drought has a profound effect on plant diversity (Grman et al., 2010), nutrient cycling (Haverd et al., 2017), and microbial communities (Yuste et al., 2011) in arid and semiarid land ecosystems, which restricts its sustainable development.

Soil microorganisms play an important role in nutrient cycling and organic matter decomposition in soil–plant systems. Water is indispensable for the growth, metabolism, and reproduction of soil microorganisms, and precipitation changes directly affect soil microbial communities (Wardle et al., 2004). In general, evidence suggests bacterial diversity decreases under drought stress with resource constraints (Raúl et al., 2018). Soil fungal community shows higher stability compared with bacteria under drought conditions (Yuste et al., 2011). For example, a 3-year experiment showed precipitation variation has a strong effect on bacteria but not on fungi in a meadow grassland in northeastern China (Yang et al., 2021). Furthermore, fungal community diversity increases because of high drought tolerance, particularly in extreme arid environments (Preece et al., 2019). However, some studies have shown fungal and bacterial communities are resistant to drought stress (Abbasi, 2020). Drought does not affect the community diversity of bacteria and fungi in semiarid temperate grassland ecosystems (Li et al., 2022a). Based on existing research, the response of soil microbial community diversity to drought stress remains unclear. Therefore, understanding the mechanism how drought affects soil microbial community diversity is essential for predicting the effects of climate change in alpine grassland ecosystems.

Soil microorganisms adapt or resist external drought stress by changing their community composition. The observed changes in soil microbial community composition involve variations in the relative abundance of the dominant phyla, which could be a consequence of drought stress. For example, drought stress increases the relative abundance of the Ascomycota phylum fungal community in a semiarid grassland (Chen et al., 2019). The relative abundance of Actinobacteria, as a dominant phylum in arid soils, decreased substantially with more mean annual precipitation in the grassland of the Loess Plateau (Li et al., 2020). Soil microbial communities are highly variable in natural and experimental environments because of the different duration of drought stress. More tolerant soil microbial bacterial and fungal phyla (i.e., Actinobacteria, Glomeromycota, and Ascomycota) are selected under long-term periodic drought to achieve a resistance memory to drought (Canarini et al., 2021). Therefore, understanding the responses of soil microbial communities to various degrees of drought stress in sensitive alpine grasslands is lacking.

Drought-induced changes in microbial communities may be regulated through two pathways. Firstly, soil properties such as soil moisture (Naidoo et al., 2022), pH (Wang et al., 2015) and temperature (Zhang et al., 2013) are major drivers of microbial communities. In addition, drought may affect soil microbial communities by regulating plant characteristics, such as plant coverage (Maestre et al., 2016), plant diversity (Spehn et al., 2000), and plant biomass (Na et al., 2019). The diversity and abundance of soil bacteria and fungi are decreased under drought stress because of reduced plant cover and soil organic carbon input (Maestre et al., 2016). The variation in plant diversity affects plant products and organic components, thereby influencing soil microbial composition (Spehn et al., 2000). However, studies have indicated that soil microbial composition is not substantially related to plant species diversity (Meier and Bowman, 2008) but significantly correlated with multi-species litter mixtures (Mathieu et al., 2017). Therefore, considering the soil–plant–microbial relationship, the regulating characteristics of abiotic factors (i.e., soil properties) and biotic factors (i.e., plant characteristics) to soil microbial community in alpine grassland under drought stress must be comprehensively explored.

Microbial functional potential is mainly affected by soil microbial community structure and composition. A research stated that taxonomy and function are coupled (Fierer et al., 2012), indicating that microbial functional potential changes can be directly predicted by monitoring changes in microbial community classification. A study showed that changes in precipitation patterns can affect the microbial community composition and functional potential (β-diversity) in desert soils; for example, Acidobacteriota and "resistance to antibiotics and toxic compounds" related genes are relatively more abundant under an increased precipitation zone (Naidoo et al., 2022). Another research determined that microbial functional (at the β-diversity level) is strongly correlated with taxonomic and phylogenetic β-diversity in many soils, including cold deserts, hot deserts, forests, grasslands, and tundra (Fierer et al., 2012). However, the relationship between soil microbial community composition and functional potential under drought stress in arid and semiarid regions is still unclear. Thus, the responses of soil microbial community composition and functional potential under drought stress must be explored, and the coupled mechanism must be clarified.

A 3-year field *in situ* control experiment was conducted to select three precipitation gradients (100% referred to ambient precipitation, −40% and −20%) of drought treatment in the northern slope of Kunlun Mountains. Moreover, 16S rRNA gene and ITS sequencing were used to detect bacterial and fungal communities, and the PICRUST and FUNGuild databases were used to predict bacterial and fungal functional groups. Here, we predict that (1) drought stress will change soil bacterial and fungal community diversity and composition, and bacterial community is more sensitive to drought compared with fungal community structure; (2) biotic and abiotic factors together influence soil microbial community; and (3) there is a coupling between soil microbial community structure and microbial functional potential.

# 2. Materials and methods

## 2.1. Study area description and experimental design

This paper is based on the rainfall experiment platform of the national grassland fixed monitoring station of Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences. The experimental site is located on the northern slope of the Kunlun Mountains (80°35′08″ E, 36°08′02″ N) at an altitude of 3,236 m, which is influenced by a typical continental arid climate. The mean annual precipitation is approximately 335 mm, which occurs during the growing season (April to September, Supplementary Table S1). The soil is a moderately mature gray desert soil, and 0–20 cm of the soil is sandy loam (Wan et al., 2022). The predominant vegetation types are *Seriphidium rhodanthum*, *Stipa capillata*, *Astragalus polycladus,* and *Allium chrysanthum* Regel.

The three treatments in the rainfall platform as described by Zhang et al. (2019) and modified for this study included a control (CK, natural precipitation), −20% (D20, 20% reduction of precipitation), and −40% (D40, 40% reduction of precipitation) with each experimental treatment replicated four times. Supplementary Figure S1 shows a rainfall platform with 2 m × 3 m size was randomly assigned in the study area with a 6 m buffer established between each neighboring plots, ensuring excess precipitation from the rainfall platform dropping into the buffer. The rain shelters were installed at 1.2 m above ground, and 20% and 40% of the plot area were covered by a transparent tempered glass (Supplementary Figure S1), minimizing light blockage and avoiding temperature increase. The whole experimental area was flat, and the natural slope was less than 1%.

Temperature and humidity sensors were installed in each sample plot at a 15 cm soil depth to enable the real-time collection of soil moisture and temperature conditions (Supplementary Figure S2). The experimental treatments were conducted over 3 years starting in April 2019 and ending in September 2021 for the artificially controlled rainfall alteration experiment. To avoid differences in vegetation composition and soil properties caused by spatial heterogeneity, uniform grassland was fenced and divided into three blocks before the experiment. Moreover, four plots similar in vegetation composition were established in each block.

## 2.2. Plant and soil sample collection

In September 2021, as the end of the drought treatment, 1 m × 1 m subplots were randomly selected within the treatment plots, and the indicators of the vegetation survey included the composition and density of each species (Supplementary Table S2). In the vegetation survey, all the surviving aboveground plant individuals in each subplot were collected and dried at 65°C until constant weight to obtain aboveground biomass (AGB). Five soil samples were collected randomly at 0–20 cm by using a soil corer (2.1 cm inner diameter) in each plot and mixed into one composite sample. Four duplicate soil samples were collected of each treatment, producing 12 soil samples. Living root samples obtained from randomly selected soil plots (20 cm × 20 cm × 20 cm) were cleaned with deionized water and dried to a constant weight at 65°C to obtain belowground biomass (BGB). Soil samples passed through a 2 mm sieve were equally divided into two groups. The first group, which was dried in air at room temperature, was used to determine the soil total nitrogen (TN) and total phosphorus (TP), and the second fresh soil group was stored at −80°C for soil microbial community.

## 2.3. Soil physicochemical characteristics

Soil moisture was measured by the drying method (105°C for 48 h). Soli organic matter (SOM) and TP were determined by potassium dichromate heating and acid digestion (Kalembasa and Jenkinson, 1973), respectively. TN was determined by using an automatic elemental analyzer (Vario EL Cube, Elementar, Langenselbold, Germany). Total potassium (TK) and pH (PHS–3C; Shanghai) were determined by flame photometry and electrode potentiometry (Walker and Adams, 1958), respectively. Soil available phosphorus (SAP) was leached with 0.5 mol $L^{-1}$ of $NaHCO_3$ (pH = 8.5) and determined by molybdenum blue colorimetry (Kjeldahl, 1883). Twelve samples including four replicates per treatment were analyzed. All changes in soil and plant properties under different stages of precipitation were collected as shown in Supplementary Table S3.

## 2.4. Soil DNA extraction and bacterial community composition analysis

Total soil DNA was extracted using the TIANamp Soil DNA Kit (TIANGEN) according to the manufacturer's protocol, and each treatment included four samples with each sample extracted once. Concentration quality and DNA purity were evaluated using a NanoDrop One spectrophotometer (Thermo Scientific, Wilmington, DE, United States) and through agarose 1% gel electrophoresis (180 V, 25 min). Bacterial 16S rRNA gene and fungal ITS sequences were used for PCR amplification using different primers. For bacterial diversity analysis, the primer sets 338F and 806R (Supplementary Table S4) were used to amplify 16S rRNA gene (Canarini et al., 2021). The fungal sequences of the ITS-V1 gene (Yuste et al., 2011) were amplified using the universal primers ITS5 and ITS2 (Supplementary Table S4). The PCR products for each sample were mixed after completing PCR amplifications using the same template with three replicates and then purified using the Thermo Scientific GeneJET PCR Purification Kit (Li et al., 2014). High-throughput sequencing analysis of the target genes was applied using the Illumina NovaSeq PE250 platform (Shanghai Personalbio Technology Co., Ltd.) with the paired-end 300 bp strategy (Reyon et al., 2012). Bioinformatic analysis was performed using QIIME2 (2019.4). The raw data were obtained after sequencing. Firstly, the primer fragments were cut, and the mismatched primer sequences were discarded through the function of qiime cut-adaptive trim-pair. Then, the Divisive amplicon Denoise Algorithm 2 (DADA2) was used to perform sequence quality control, denoising, splicing, and chimera removal through the qiime dada2 denoise-pair function (Callahan et al., 2016). DADA2 no longer clustered in similarity, and only dereplication or clustering in 100% similarity was performed (Callahan et al., 2016). Based on QIIME2 (2019.4), Vsearch (v2.13.4 linux_x86_64) and cutadapt (v2.3) were used for subsequent analysis, which clustered high-quality sequences at 97% similarity level and output representative sequences and

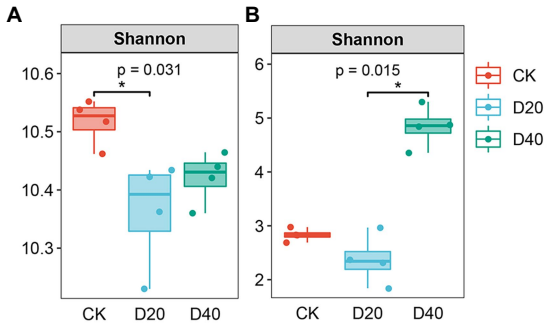


FIGURE 1
Alpha diversity index of soil microorganisms based on AVS. Changes in alpha diversity of bacteria and fungi in different stages of drought gradient. Shannon index of bacteria **(A)** and fungi **(B)**, respectively. CK: natural precipitation, D20: 20% precipitation reduction, and D40: 40% precipitation reduction.

amplicon sequence variant (AVS) tables (Edgar, 2017). Singletons were removed from AVS tables and their representative sequences for downstream analysis. Bacteria and fungi were performed using QIIME 2's classify-sklearn algorithm (Bokulich et al., 2018) based on Greengenes and UNITE databases, respectively, and unleveled ASV sequence was selected for species annotation in QIIME2 software through the pretrained Naive Bayes classifier. The raw data were submitted to the National Center for Biotechnology Information (NCBI).[1]

## 2.5. Statistical analysis

Single-factor analysis of variance was performed using Duncan's multiple range test ($p < 0.05$) in SPSS 26.0 (SPSS Inc., Chicago, IL, United States). The alpha diversity of the microbial community was estimated with Shannon index based on the Bray–Curtis distance.[2] Nonmetric multidimensional scaling (NMDS) analysis was performed in accordance with the Bray–Curtis distance matrix to visualize the microbial communities, and the differences in microbial community composition were presented by performing an ordination plot using "ggplot2." The significance of the separation between stages of microbial community structure was tested by the "ADONIS" function of the vegan R software package (999 permutations). Mantel test was used to investigate the relationship between the Shannon index of bacteria and fungi with environmental factors based on 9,999 permutations using the vegan R software package. Redundancy analysis (RDA) was used to assess the relationship between environmental factors and bacterial and fungal community structure using the vegan package (Oksanen et al., 2014). The significance of RDA correlations was tested Monte Carlo permutation test. Spearman's correlation coefficient was used to test the relationship between environmental factors (plant and soil properties) with relative abundance of the top 10 bacteria/fungi at the phylum level. All data were processed using QIIME2 (2019.4) and Excel (2019), and plots were performed using Origin (Origin Laboratories, Ltd., Northampton City, MA, United States) and R (version 3.6.1). Bacterial function prediction was analyzed using PICRSt software and the closed AVS tables obtained by QIIME were compared with the KEGG database to obtain different database function prediction information (Langille et al., 2013). FUNGuild (Fungi Functional Guild) V1.0 online platform was used to classify fungi ecologically and functionally. OTUs obtained from high-throughput sequencing were uploaded to the FUNGuild platform for analysis, and the results were downloaded for screening fungal communities and linking fungal species classification to functional guild classification by bioinformatics methods (Sun et al., 2016). Heat maps and histograms were plotted by using the heatmap package in R. Microbial network analysis was performed by the genes cloud tools.[3] Only the top 100 abundance at the genus level were selected, and the co-occurrence patterns were explored based on strong (Spearman's $\rho > |0.6|$) and significant correlations ($p < 0.05$). Cytoscape 3.4.0[4] was used to visualize network.

# 3. Results

## 3.1. Soil microbial diversity under drought stress

The Illumina NovaSeq PE250 platform was used to filter the obtained raw data, obtaining 1,132,993 bacterial and 1,395,635 fungal high-quality sequences with averages of 94,416 and 116,302 sequences, respectively. The average coverage of all samples was more than 97%, and the rarefaction curve of each sample was flat (Supplementary Figure S3), indicating that the sequencing depth was saturated and could reflect the vast majority of microbial diversity information in the samples (Edgar, 2017). Drought stress (D20 and D40) significantly reduced bacterial species richness ($p < 0.05$), and the D20 treatment showed the lowest fungal species richness ($p < 0.05$, Supplementary Figure S4).

The Shannon diversity index of bacteria and fungi in different treatments showed similar trends with species richness (Supplementary Figure S4). The Shannon diversity index for soil bacteria was highest in CK and lowest in D20 ($p < 0.05$, Figure 1A), indicating drought stress significantly reduced bacterial community diversity. Moreover, the Shannon diversity index for fungi was highest in D40 and lowest in D20 ($p < 0.05$, Figure 1B). Fungi were more resistant to drought compared with bacteria, especially in D20, but excessive drought (D40) may lead to the increasing of specific taxa and reorganization of fungal communities.

## 3.2. Soil microbial community structure under drought stress

The NMDS results for bacterial community and fungi community showed a stress value of 0.175 (Figure 2A) and 0.089 (Figure 2B),

1 http://www.ncbi.nlm. nih.gov/PRJNA881479
2 http://scikit-bio.org
3 https://www.genescloud.cn
4 https://cytoscape.org/

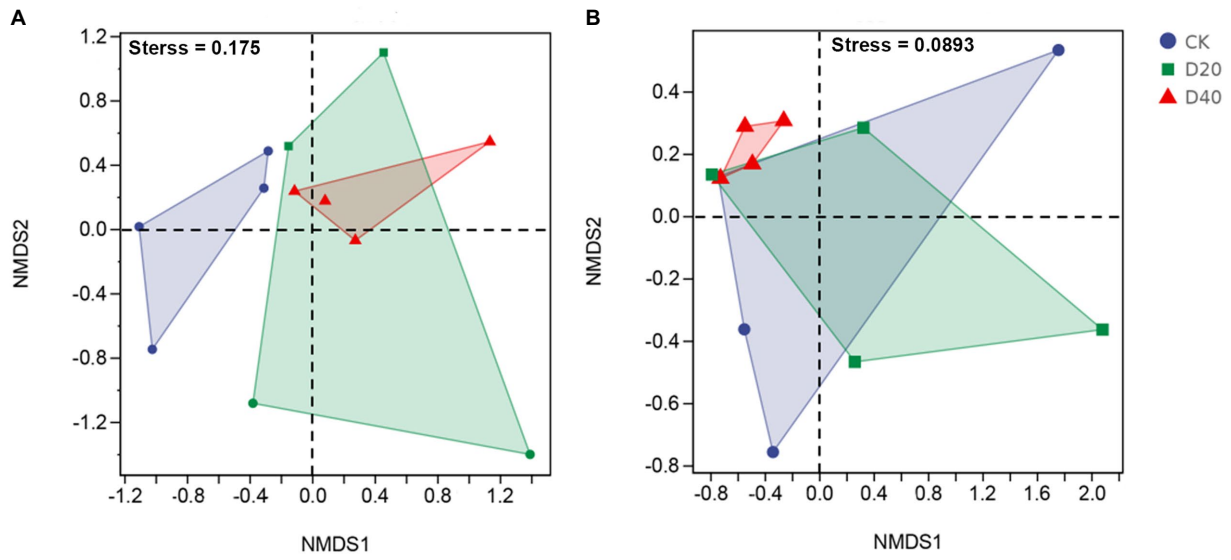


FIGURE 2
The non-metric multidimensional scaling (NMDS) analysis of bacterial and fungal communities at different stages of degradation. **(A)** bacterial community structure, **(B)** fungal community structure.

which are suitable for NMDS analysis. In addition, nonparametric multivariate statistical tests (Adonis) indicated that bacterial ($p = 0.086$) and fungal ($p = 0.106$) community structures have no significant differences.

Further refinement of community structure showed the dominant bacterial phyla in soil for all treatments were Actinobacteria (36.30%–42.45%), Proteobacteria (24.17%–28.31%), Chloroflexi (10.86%–14.88%), and Acidobacteria (11.02%–13.90%), followed by the variable occurrence of Gemmatimonadetes, Bacteroidetes, Firmicutes, Patescibacteria, Planctomycetes, and Verrucomicrobia. Drought stress increased the relative abundance of Actinobacteria and Chloroflexi ($p < 0.05$) but reduced the relative abundance of Acidobacteria and Bacteroidetes ($p < 0.05$, Figure 3). At the genus level, only three of the top 10 bacterial species were influenced by drought stress. Subgroup-6 and A4 both declined, while Sphingomonas increased with drought gradient ($p < 0.05$, Supplementary Figure S5). The predominant phyla of fungi included Basidiomycota (30.56%–75.38%) and Ascomycota (13.97%–51.03%), followed by the variable occurrence of Mortierellomycota, Glomeromycota, and Chytridiomycota. In the fungal community, drought stress increased the relative abundance of Ascomycota and Glomeromycota ($p < 0.05$) but reduced the relative abundance of Basidiomycota ($p < 0.05$, Figure 3). At the genus level, only two of the top 10 fungi species were influenced by drought stress, showing as Hygrocybe declined with drought gradient, and Gibberella was lowest in D20 ($p < 0.05$, Supplementary Figure S5).

## 3.3. Environmental factors associated with soil microbial diversity and community structure

In soil properties, soil bacterial Shannon index was significantly correlated with TN ($p < 0.05$), and fungal Shannon index was significantly correlated with SWC ($p < 0.05$, Figure 4). In addition, for plant factors, the Shannon indexes of the soil bacterial and fungal communities were significantly correlated with Shannon Wiener and BGB, respectively ($p < 0.05$, Figure 4). Furthermore, under drought treatments, no same environmental factor was correlated with the soil bacterial and fungal communities, indicating that the internal mechanisms of changes in soil microbial diversity and community structure under drought stress are different.

RDA was applied to analyze the relationship between environmental factors and microbial community composition under drought stress. The RDA results explained 50.62% (37.79% for axis 1 and 12.83% for axis 2) and 47.08% (26.03% for axis 1 and 21.05% for axis 2) of the relationship between soil bacterial community composition with soil factors and plant factors (Figures 5A,B). In the soil bacterial community, soil C:P ($R^2 = 0.41$, $p = 0.047$), SAP ($R^2 = 0.46$, $p = 0.034$), and SWC ($R^2 = 0.43$, $p = 0.044$) are the main soil factors affecting the bacterial community structure. Moreover, plant diversity [Simpson ($R^2 = 0.53$, $p = 0.027$), Shannon Wiener ($R^2 = 0.34$, $p = 0.042$), and Pieiou ($R^2 = 0.49$, $p = 0.033$)] and BGB ($R^2 = 0.51$, $p = 0.031$) are important plant factors controlling the soil bacterial community structure. However, under different drought stress, the positive correlation between SWC and D20 was significantly greater than that with CK and D40 (Figure 5A). In addition, D40 showed a higher positive correlation with plant factors (Pieiou, Shannon Wiener, and Coverage) than CK and D20 (Figure 5B). For the fungal community, the RDA results explained 41.73% (32.72% for axis 1 and 9.01% for axis 2) and 53.33% (41.65% for axis 1 and 11.68% for axis 2) of the relationship between soil fungal community diversity with soil factors and plant factors (Figures 5C,D). In the soil fungal community, SWC ($R^2 = 0.45$, $p = 0.047$), soil C:P ($R^2 = 0.47$, $p = 0.041$), and SAP ($R^2 = 0.46$, $p = 0.043$) are the main soil factors affecting the fungal community structure. Shannon Wiener ($R^2 = 0.43$, $p = 0.042$), BGB ($R^2 = 0.51$, $p = 0.029$), and AGB ($R^2 = 0.41$, $p = 0.045$) are important plant factors controlling the soil fungal community structure. Moreover, under different drought stress, the positive correlation between SWC and CK was the highest (Figure 5C), and the positive correlation between CK and plant factors (AGB and Shannon Wiener) was higher than that

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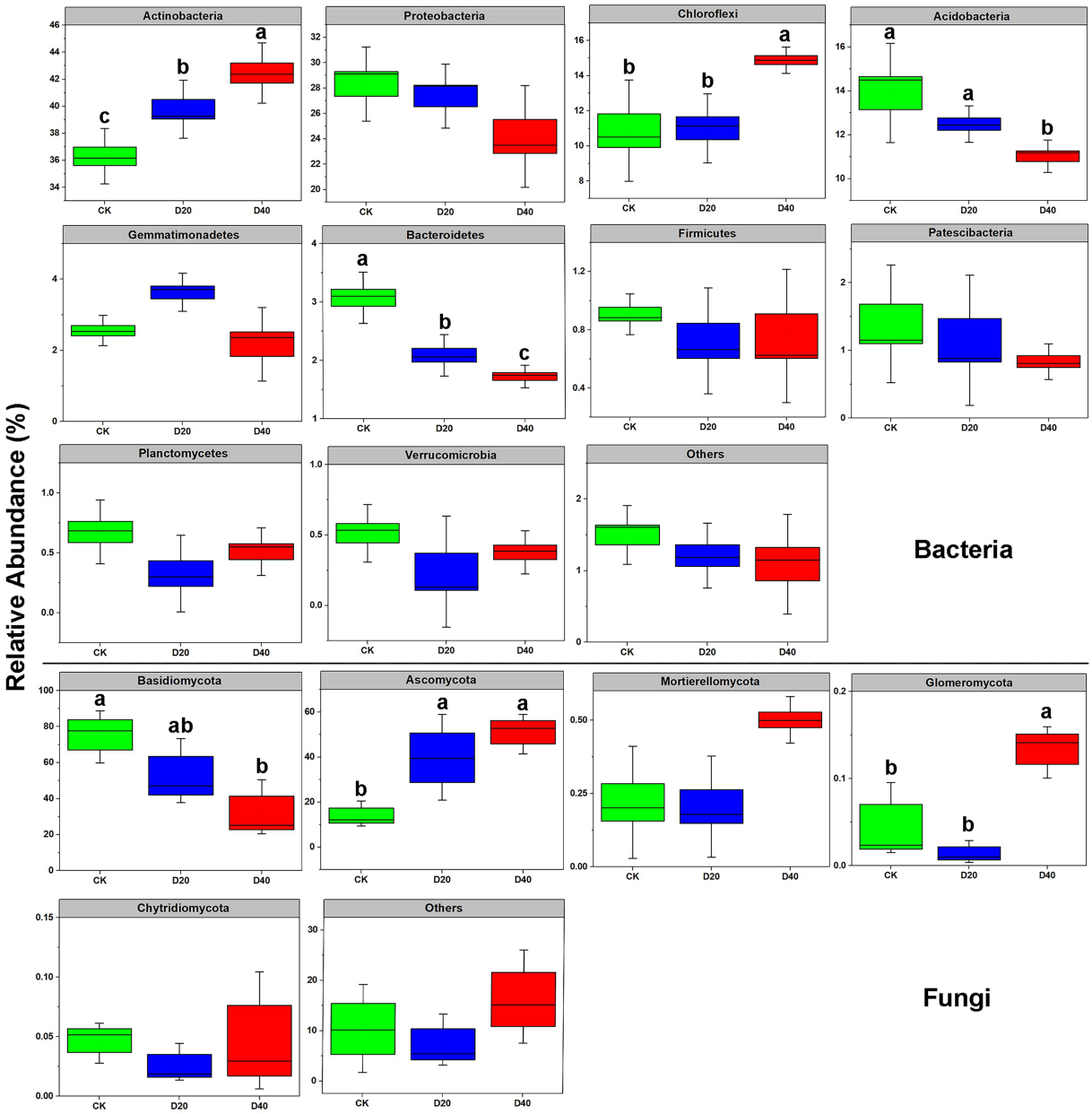


FIGURE 3
Relative abundance of bacterial and fungal phyla at different drought levels. Different lowercase letters (a, b, ab, c) indicate significant differences among the three drought levels ($p<0.05$).

between D20 and D40 (Figure 5D). Thus, among all environmental attributes, especially under drought treatment, SWC and plant diversity are important determinant of the bacterial and fungal community structure.

Further refinement of the Spearman's correlation between bacterial and fungal phylum with environmental factors showed the relative abundances of drought-tolerant bacterial phyla; for example, Acidobacteria ($r=-0.7973$, $p=0.0018$) and Chloroflexi ($r=-0.6116$, $p=0.0345$) showed a significant negative correlation with SWC. The relative abundances of drought-sensitive bacterial phyla, for example, Proteobacteria ($r=0.6208$, $p=0.0312$) and Bacteroidetes ($r=0.8582$, $p=0.0003$), were positively correlated with SWC, but Bacteroidetes ($r=-0.6343$, $p=0.0003$) also showed a negative correlation with plant Pieiou. Moreover, the relative abundance of Firmicutes showed a significant negative correlation with plant Simpson richness ($r=-0.8054$, $p=0.0015$; Figure 6). About fungal community composition, the abundance of Basidiomycota was positively correlated with SWC ($r=0.7797$, $p=0.0027$) in soil factors but negatively correlated with Simpson richness ($r=-0.5385$, $p=0.0071$). However, the abundance of Ascomycota was positive correlation with Simpson richness ($r=0.6448$, $p=0.0023$) and negatively correlated with SWC ($r=-0.7313$, $p=0.0068$). Furthermore, the abundance of Mortierellomycota was significantly and negatively correlated with BGB ($r=-0.7161$, $p=0.0088$) of plants and SWC ($r=-0.7509$, $p=0.0048$) but positively correlated with Shannon Wiener ($r=0.5059$, $p=0.0439$) of plants. Thus, the main environmental limiting factors

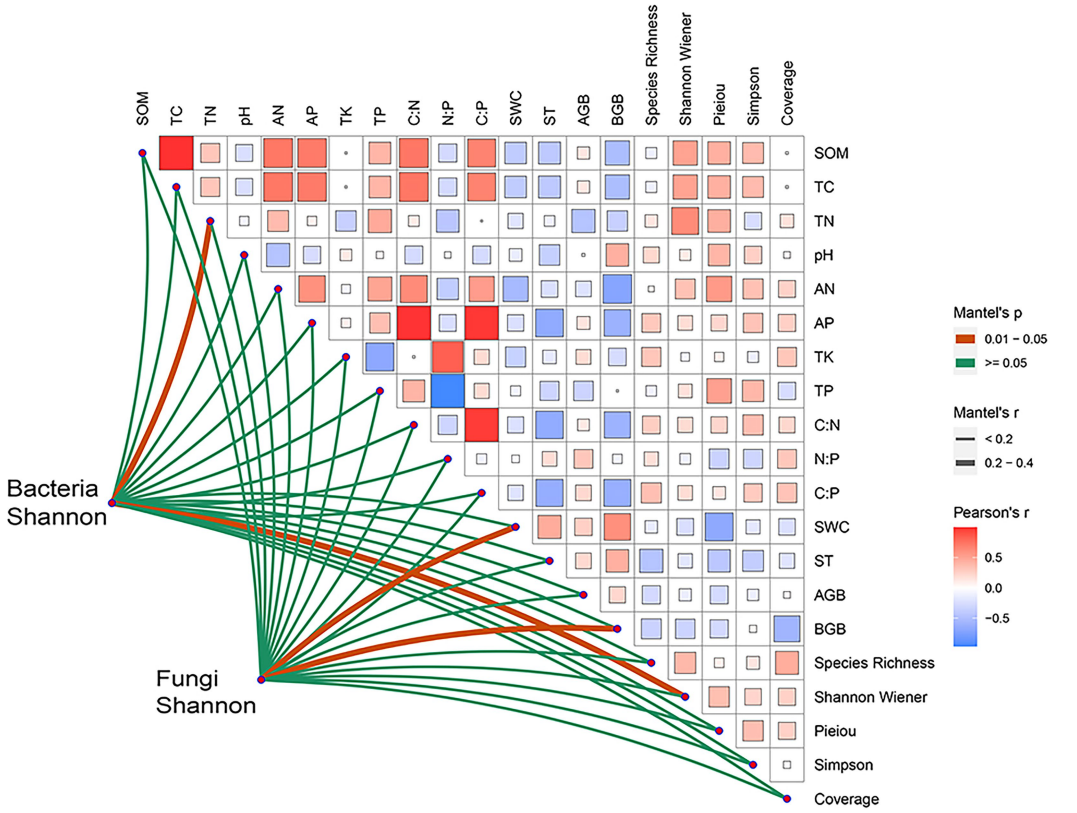


FIGURE 4
Relationship between the Shannon index of bacteria and fungi with environmental factors. SOM, soil organic matter; AN, soil alkali–hydrolysable nitrogen; AP, available phosphorus; TK, total soil potassium; TC, total soil carbon; TN, total soil nitrogen; TP, total soil phosphorus; soil total C: total N, soil total C: total P and soil total N: total P; SWC, soil moisture content; ST, soil temperature; AGB, aboveground biomass; BGB, belowground biomass.

(SWC and plant diversity) are consistent with the RDA results (Figure 5).

## 3.4. Soil microbial predicted functional potential under drought stress

PICRUSt was used to predict the bacterial community functions based on KEGG pathways genes, and six types of biological metabolic pathways were obtained: Metabolism, Genetic information processing, Environmental information processing, Cellular processes, Organismal systems, and Human diseases. Among them, the top two most abundant functions were Metabolism and Genetic information processing, accounting for 82.13%–83.08% and 11.23%–11.45%, respectively (Figure 7). The relative abundance in the secondary predicted functional layer was analyzed, and the heat map of all 25 predicted functions showed nine subfunctions with substantial differences under drought stress were mapped to three level-1 functional categories (Metabolism, Genetic information processing, and Cellular processes; Supplementary Table S5). Among them, the frequency of three predicted level-1 functional categories (Cell growth and death, Amino acid metabolism, and Carbohydrate metabolism) increased with drought gradient ($p < 0.05$, Supplementary Table S5). The frequency of three predicted level-1 functional categories (Cell motility, Replication and repair, and Lipid metabolism) declined with drought gradient ($p < 0.05$, Supplementary Table S5).

The functional classification of the fungi and the abundance of each functional classification in different treatments samples were obtained from the FUNGuild functional prediction (Table 1; Supplementary Figure S6). In the above functional classification, saprotroph and pathogen–saprotroph fungi were overwhelmingly represented in all treatments (Table 1; Supplementary Figure S6), consistent with the increased relative abundance of Ascomycota under drought stress (Figure 3). The fungal function under D40 treatment showed that the proportion of pathogen–saprotroph, endophyte–saprotroph, and parasite–saprotroph fungi increased, indicating that the fungal community still had difficulty maintaining resistance under excessive drought stress.

## 3.5. Environmental factors associated with soil microbial functional potential

To determine the major environmental factors associated with predicted soil microbial functional potential, RDA also was applied to analyze relationship between environmental factors, which significantly correlated with microbial community diversity and structure (Figure 5), with soil microbial functional potential. The RDA results explained 59.80% (46.32% for axis 1 and 13.48% for axis 2) and

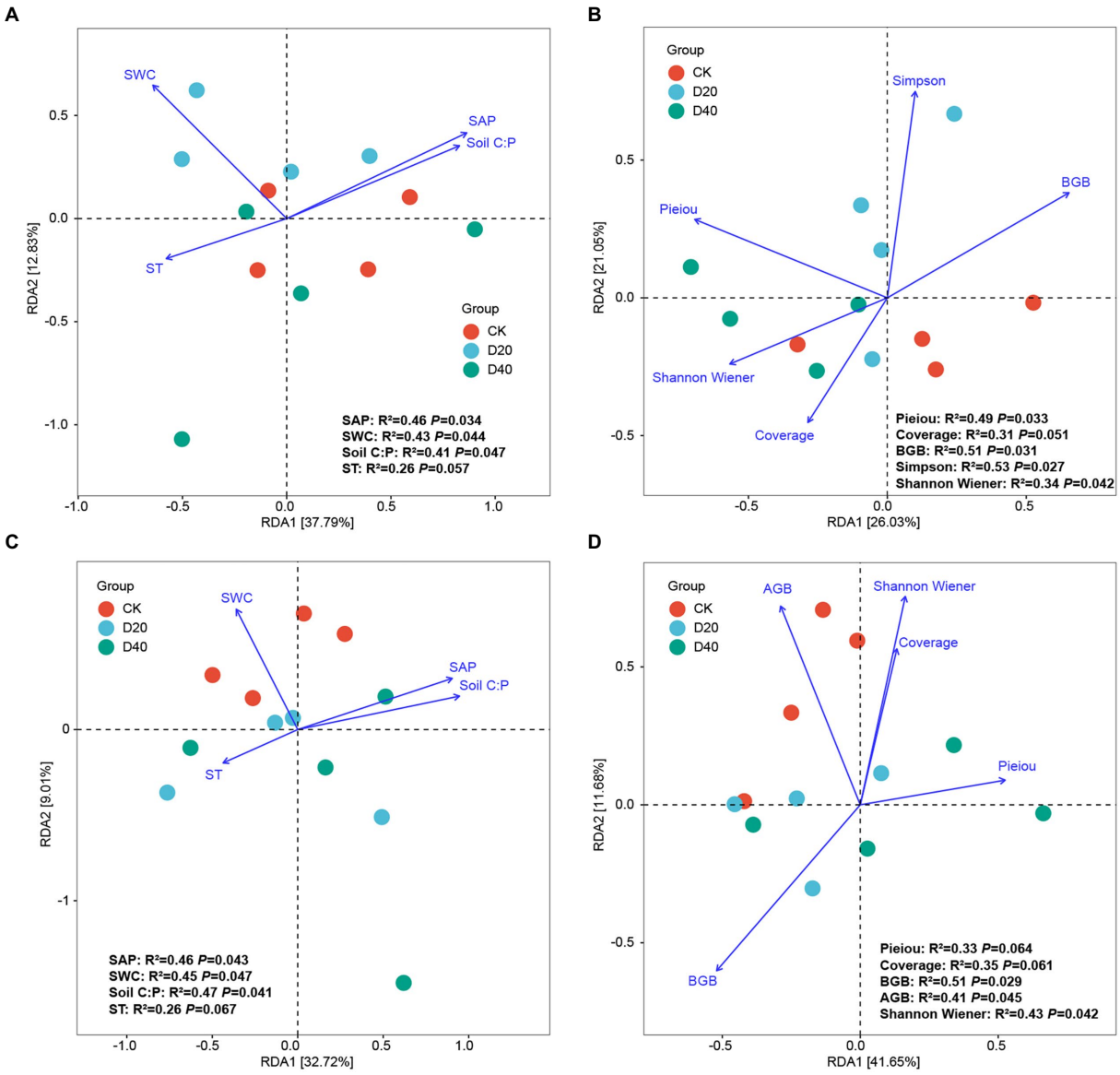


FIGURE 5
Ordination plots of RDA between microbiology communities, soil and plant properties at the AVS. **(A)** Bacterial community and soil factors, **(B)** Bacterial community and plant characteristics, **(C)** Fungal community and soil factors and **(D)** Fungi community and plant characteristics. SAP, available phosphorus.

68.34% (54.60% for axis 1 and 13.74% for axis 2) of the relationship between bacterial community functions (Figure 8A) and fungal community functions (Figure 8B) with environmental factors. The main important environmental factors controlling predicted bacterial and fungal functions were SWC, SOM, BGB, Species Richness, and Shannon Wiener. For the bacterial community functions, under different drought stress, compared with CK and D20, D40 showed higher correlation with SOM ($R^2 = 0.44$, $p = 0.028$), Species Richness ($R^2 = 0.36$, $p = 0.041$), and Shannon Wiener ($R^2 = 0.41$, $p = 0.037$). For the fungal community functions, under different drought stress, D40 also showed higher correlation with SOM ($R^2 = 0.31$, $p = 0.031$) and Shannon Wiener ($R^2 = 0.37$, $p = 0.027$) than CK and D20. The functions of the bacterial and fungal communities under excessive drought stress (D40) both showed a correlation dependence on SOM and Shannon Wiener, which may be closely related to the death and reuse of plants and soil microorganisms.

# 4. Discussion

## 4.1. Microbial diversity of the dominant microbial phylum

The soil microbial diversity index is an important indicator for evaluating soil microbial community (Zhang et al., 2016). Previous studies have shown bacterial community diversity is more susceptible to drought compared with fungi (Kaisermann et al., 2017). Our results showed drought stress reduces the Shannon diversity index of soil

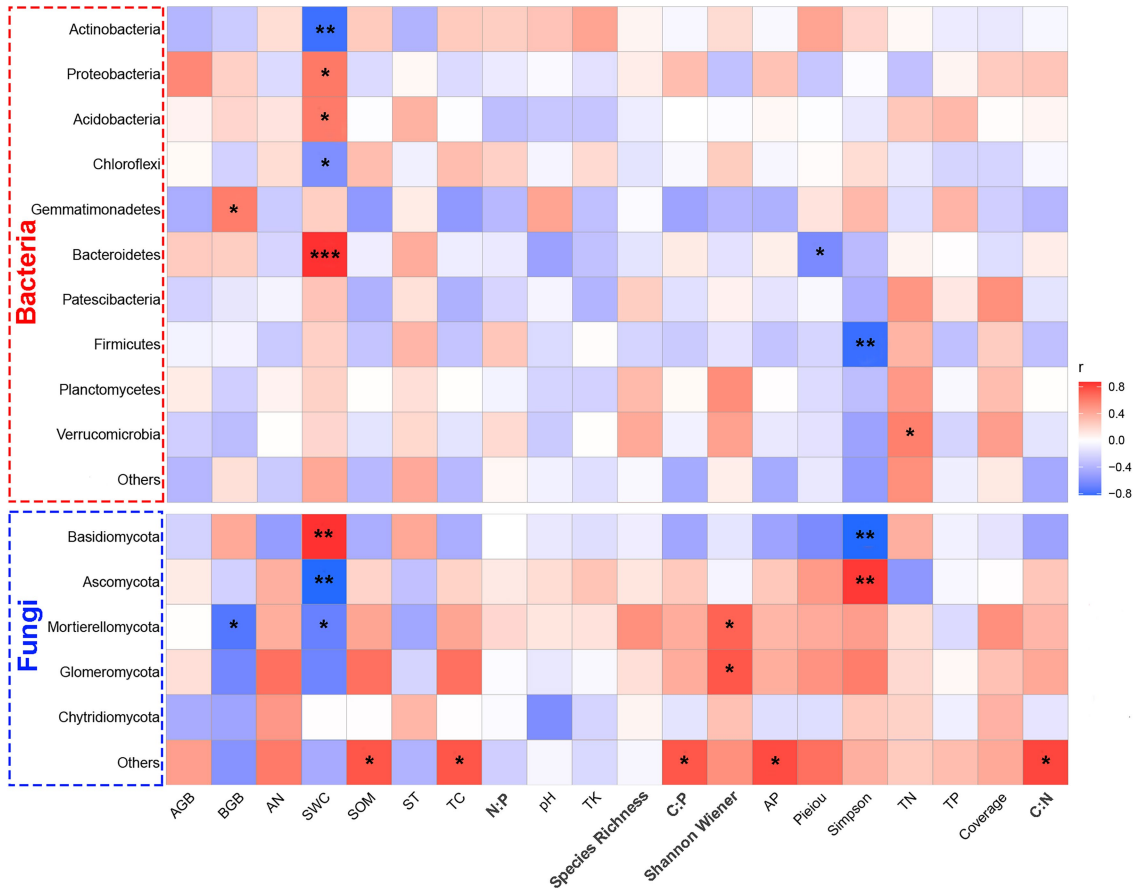


FIGURE 6
Correlations of plant and soil properties with relative abundance of the top 10 bacteria/fungi at the phylum level. The right side of the legend is the color range of R-values. SOM, soil organic matter; AN, soil alkali-hydrolysable nitrogen; AP, available phosphorous; TK, total soil potassium; TC, total soil carbon; soil total C: total N, soil total C: total P and soil total N: total P; ST, soil temperature; SWC: soil water content; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; AGB, aboveground biomass; BGB, belowground biomass. Significant correlations are reported as: *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$.

bacteria (Figure 1A). Bacterial species have an osmotic adjustment function, but they are more vulnerable to drought because they require water membranes in soil aggregates and on soil surfaces for substrate dispersion and diffusion (Esther and Joseph, 2016). Under drought conditions, the decrease of soil moisture will affect soil porosity, thereby inhibiting the growth and multiplication of bacteria (Farooq et al., 2009). Our paper showed TN substantially affects bacterial diversity (Figure 4). Drought reduces plant productivity and nitrogen fixation capacity (Ma et al., 2020), thereby decreasing the supply of soil TN and limiting the growth and multiplication of bacteria. Under drought stress, soil moisture mobility is poor, affecting soil nutrient mobility (Moyano et al., 2013), and bacteria die because of a lack of sufficient energy sources. In addition, plant diversity considerably affects bacterial diversity (Figure 4), which is consistent with the results of previous studies (Schlatter et al., 2015). Plant diversity induces species-specific effects that may affect bacterial diversity through changes in root exudates, plant litter, and plant secondary metabolites (He et al., 2008).

Microbial responses to drought depend on their metabolic flexibility and physiological conditions. Fungi can remain active at a lower water potential compared with bacteria. The above results also verified our first prediction. Fungi are more resistant to drought than bacteria because they can establish large water absorption networks, which promote long-distance water transfer and enable them to explore water-filled soil pores or obtain water from small soil pores (Sun et al., 2020). In this paper, fungal diversity increased significantly because of drought treatments (Figure 1B), which is consistent with the results of previous studies (de Oliveira et al., 2020). Drought may promote the growth of potentially slow-growing, drought-adapted soil microbes. Changes in fungal community diversity result from fungal redistribution, water use (Barnard et al., 2013), or mycelial contraction (Hossain et al., 2007), leading the community to adopt ecological strategies appropriate to different drought conditions. Our study found that BGB significantly affected fungal diversity (Figure 4), which is consistent with the results of previous studies (Li et al., 2022b). This result may be attributed to two aspects. Firstly, BGB affects belowground nutrient and energy exchange, and the inputted organic and inorganic material by inter–root secretions promotes or inhibits the growth and diversity of soil fungi (Graham and Mendelssohn, 2016). Secondly, mycorrhizal (plant root–fungal symbiosis) mycelium expands the root uptake area to utilize deep soil water (McHugh and Gehring, 2006).

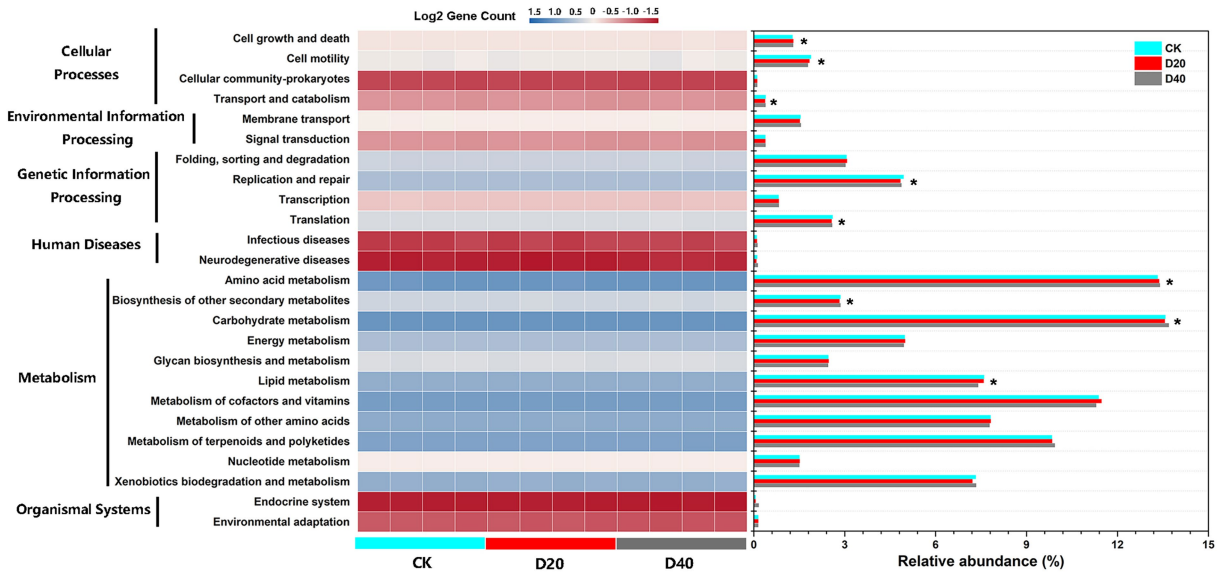


FIGURE 7
Relative abundance of predicted functional categories for different treatments in three origins bacterial microbiome using PICRUSt grouped into level-2 functional categories. * indicates the level-2 functional categories show a significant difference among the three drought levels.

## 4.2. Soil microbial community structure

In this study, dominant bacterial phyla in drought stress treatment were Actinobacteria, Proteobacteria, Chloroflexi, and Acidobacteria, which are common bacterial phyla in soil subjected to drought stress, similar to a meadow steppe (Yang et al., 2021). Drought stress changed the abundance of the microbial dominant phylum (Figure 3), significantly increasing Gram-positive bacteria (i.e., Actinobacteria and Chloroflexi). The relative abundance of the microbial community of Gram-positive bacteria increased in response to drought stress (Fuchslueger et al., 2014). This result may be related to the cell structure of Gram-positive and unique physiological characteristics. Gram-positive bacteria have a thick, tough cell wall outside the cell membrane (Schimel et al., 2007), which is less susceptible to water loss and death under drought stress (Manzoni et al., 2012). On the contrary, the relative abundance of Gram-negative bacteria (i.e., Proteobacteria) were decreased under drought due to loss of sporulation capacities during the course of evolution and poor adaption to soil moisture disturbance (Denef et al., 2009).

Related studies have found that changes in the external environment will influence the function of soil microorganisms and soil microbial community structure until reaching a new nutrient balance condition (Petra et al., 2003). Actinomycetes were significantly and negatively correlated with SWC (Figure 6) and drought tolerance, and they were well enriched in arid environments (Xu et al., 2018). Actinobacteria can adapt to soil environments under prolonged water and nutrient stress because of their ability to decompose soil litter and a variety of organic compounds, including aromatics, cellulose, wood, and other complex compounds (Van Bergeijk et al., 2020) to maintain its growth and reproduction. By contrast, drought reduces the relative abundance of oligotrophic bacteria (i.e., Acidobacteria) as this community is unable to synthesize all important nutrients, promotes decomposition of difficult-to-degrade C sources and acid uptake and grows slowly (Zengler and Zaramela, 2018). Previous studies have shown that Acidobacteria readily multiply in acidic soils (Kim et al., 2021). In the present paper, no significant correlation was observed between Acidobacteria and soil pH probably because the soil pH in the study area was neutral, and drought did not significantly change the pH (Supplementary Table S3). Bacteroidetes are well known degraders of polymeric organic matter, and they are important components of some organic carbon recycling and decomposition (Thomas et al., 2011). Drought reduced the relative abundance of this bacterium group, which is consistent with the results of the Inner Mongolia arid grassland study (Shao et al., 2018). This result may be due the patchiness of grasslands because of drought (Hoffman et al., 2017) and reduced net primary productivity of plants (Shaw et al., 2022). Bacteroidetes lack energy sources to readjust growth strategies of species distribution because of reduced organic carbon input.

Compared with bacteria, fungi have more unique survival skills or physiological structures to increase tolerance (Kaisermann et al., 2015). The effects of drought stress on fungi were mostly concentrated in Basidiomycota and Ascomycota. In this study, drought reduced the relative abundance of Basidiomycota ($p < 0.05$, Figure 3). Some of the Basidiomycota colonies form symbiotic associations with root systems of specific plants (Lareen et al., 2016), and reduced water input may weaken the cooperative relationship. The results of this study showed that SWC was significantly positively correlated with Basidiomycota (Figure 6), confirming the existence of ectomycorrhizal mycorrhizal cooperation between Basidiomycota and plant roots. Drought reduced water transport and nutrients by fungal mycelium for plants outside the root system, and the regulatory and storage role of plant roots is reduced (Naylor and Coleman, 2018), thereby affecting Basidiomycota growth. However, drought increased the relative abundance Ascomycota ($p < 0.05$, Figure 3). Thus, ascomycete fungi might produce ascospores adapted to the drought environment (Lozano et al., 2021).

TABLE 1 Changes of predicted soil fungal functional groups in different treatments.

| Fungal functional groups | CK (%) | D20 (%) | D40 (%) |
|---|---|---|---|
| Wood saprotroph | 0.15 ± 0.01b | 0.06 ± 0.01c | 0.22 ± 0.01a |
| Saprotroph-undefined biotroph | 71.76 ± 7.89b | 81.70 ± 8.32a | 43.09 ± 4.67c |
| Undefined saprotroph | 10.11 ± 1.63b | 6.14 ± 0.78c | 27.51 ± 2.71a |
| Plant pathogen-wood saprotroph | 7.12 ± 0.78b | 9.11 ± 0.81a | 2.01 ± 0.23c |
| Plant pathogen | 3.22 ± 0.22a | 0.33 ± 0.01b | 3.37 ± 0.31a |
| Leaf saprotroph-plant pathogen | 2.05 ± 0.23b | 1.16 ± 0.14c | 6.00 ± 0.89a |
| Fungal parasite-plant pathogen-plant saprotroph | 1.40 ± 0.12a | 0.08 ± 0.01c | 0.76 ± 0.05b |
| Endophyte-litter saprotroph-soil saprotroph | 0.28 ± 0.01b | 0.22 ± 0.01c | 0.81 ± 0.03a |
| Endophyte | 0.02 ± 0.01b | 0.01 ± 0.01b | 0.24 ± 0.01a |
| Dung saprotroph-plant saprotroph | 1.62 ± 0.15b | 0.08 ± 0.01c | 10.11 ± 1.26a |
| Animal pathogen-undefined saprotroph | 0.25 ± 0.01a | 0.03 ± 0.01c | 0.07 ± 0.01b |
| Animal pathogen-plant saprotroph | 0.90 ± 0.01b | 0.62 ± 0.02c | 2.72 ± 0.51a |
| Animal pathogen-plant pathogen-soil saprotroph | 0.09 ± 0.01b | 0.09 ± 0.01b | 0.31 ± 0.01a |
| Animal pathogen-fungal parasite-undefined saprotroph | 0.38 ± 0.01c | 0.08 ± 0.01b | 0.98 ± 0.08a |

Different letters (a, b, c) indicate significant differences among different treatments ($p < 0.05$). Values are means ± SD ($n = 4$).

Drought stress affects the community structure of soil microorganisms (bacteria and fungi) by affecting various environmental factors such as soil organic carbon, organic nitrogen, soil aeration, and pH value (Yang et al., 2021). In this study, the RDA correlation analysis in Figure 5 shows that SWC is the main soil factor affecting the soil microbial community structure (Figures 5A,C). Water primarily affects the growth and vitality of plant roots and changes the content of root exudates (Fan et al., 2019). It also affects the bacterial and fungal diversity in soil. Moreover, the plant diversity (Shannon Wiener and Pieiou) and AGB were significantly associated with the bacterial and fungal community structure (Figures 5B,D). Plant diversity enriches the soil microbial community structure, and microbial community affects plant growth by changing nutrient supply (Bijalwan et al., 2022). When plant community diversity is poor, the composition of litter and root exudates decreases, and the structure of soil microbial community changes (Mendes et al., 2013). The above results also verified our second prediction. Consequently, the change in precipitation gradient (drought stress) plays an important role in the construction of soil microbial diversity in the alpine grassland region.

## 4.3. Soil microbial predicted functional potential

Drought stress changed the nutrient balance of the soil microbial community structure, inevitably causing the soil microbial community function shift until maintaining a certain nutrient balance (Petra et al., 2003). For the bacterial predicted functional potential, drought stress showed substantially influence on three level-1 functional categories (i.e., Metabolism, Genetic information processing, and Cellular processes), and nine level-2 functional categories were significantly different under CK, D20, and D40 (Supplementary Table S5). The reason maybe that due to the weak resistance of bacteria to drought (Zhang et al., 2016; Kaisermann et al., 2017), the diversity decreased, and the community structure composition began to change under D20 treatment (Figures 1A, 3), which also can be confirmed by results of the co-occurrence networks of bacterial taxa at the genus level (Supplementary Figures S7–S9; Supplementary Table S6). The numbers of nodes, total links, positive links, network centralization, and network density in the bacterial networks are decreased with drought degree (Supplementary Table S6), indicating that the stability and interaction of bacterial communities were severely impaired by drought stress. Research showed the microbial functional potential is largely determined by microbial community composition (Naidoo et al., 2022). Furthermore, under excessive drought treatment (D40), more dead bacterial residues were transformed into SOM and nutrients, which can be utilized by saprophytic fungi (Sokol et al., 2022). Thus, compared with CK and D20, D40 showed a higher correlation with SOM, Species Richness, and Shannon Wiener (Figure 8A). Drought stress changed SWC and plant diversity in the plots, altered the bacterial community composition and further indirectly affected the cell movement, metabolism, and genetic information processing in the bacterial community.

For the fungal predicted functional potential, with the aggravation of drought, the proportion of pathogen-saprotroph, parasite–saprotroph, and endophyte–saprotroph fungi functions increased (Supplementary Figure S6; Table 1), indicating that the resistance of fungi to drought was disintegrated. The function shift in fungal community was also mainly due to the changes of community composition (Naidoo et al., 2022). Excessive drought led to a substantial increase in the proportion of Ascomycete and Glomeromycota fungi that mainly engaged in saprophytic, parasitic, and symbiotic modes (Figure 3). Consistent with previous studies (Zhang et al., 2016; Kaisermann et al., 2017; Franciska et al., 2018), fungi were more drought-resistant than bacteria (Figures 1B, 2B).

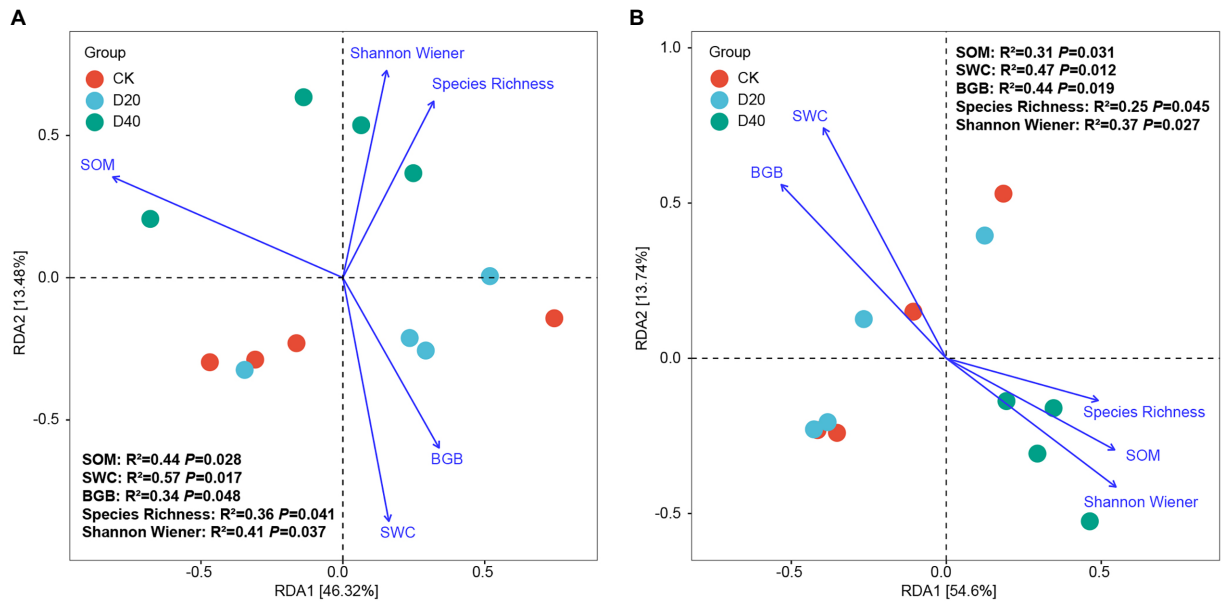


FIGURE 8
Ordination plots of RDA between predicted soil microbial functional potential and environmental factors at the AVS. **(A)** Bacterial functional potential and environmental factors, **(B)** Fungal functional potential and environmental factors.

Drought stress leads to the death of plants and bacteria, promoted the enrichment of SOM and enhanced the saprotroph function under D20 (Franciska et al., 2018; Sokol et al., 2022). However, excessive drought (D40) led to the rapid death of some fungi (i.e., Basidiomycota), changed the fungal community composition (Figure 3) and then altered the function of fungi shifting from saprotroph to pathogen–saprotroph and parasite-saprotroph symbiosis (Sokol et al., 2022). The highest positive links and smallest shortest paths in D40 of fungal networks also confirmed synergistic interaction of multiple fungal genera (Supplementary Figures S10–S12; Supplementary Table S6). Thus, the functions of fungal communities under excessive drought stress showed remarkable dependence on SOM and Shannon Wiener (Figure 8B). Drought stress changed SWC and plant diversity in the plots, altered the fungal community composition, and further indirectly affected the function shift (saprotroph, pathogen–saprotroph, endophyte–saprotroph, and parasite–saprotroph) in the fungal community. The above results also verified our third prediction. Consistent with previous findings, taxonomy and function were coupled (Fierer et al., 2012). Although the above functions shift may not necessarily simply relate to microbial community composition (such as inevitable adaptive gene loss, convergent evolution, and horizontal gene transfer; Louca et al., 2018), these results in our study indicate that the soil microbial functional potential could be predictable through taxonomic community profiles.

## 5. Conclusion

In this study, the effects of drought stress on soil microbial diversity, community composition, and predicted functional potential in alpine grasslands of Kunlun Mountains were investigated and determined. Our results showed bacteria and fungi responded differently to drought intensity, and bacteria were more sensitive to drought compared with fungi. Therefore, the diversity or structure of soil bacteria community could serve as an indicator of alpine grasslands status, with practical significance for alpine grassland ecosystem development. However, the fungal community still had difficulty maintaining resistance under excessive drought stress. Notably, soil moisture content, plant diversity (Shannon Wiener, Pieiou, and Simpson), and SOM were the main drivers affecting soil microbial community structure composition and functional potential, which provided a new perspective for the management of alpine grasslands. This work also confirmed that the soil microbial predicted functional potential could be predictable through taxonomic community profiles. Our findings improved the comprehensive understanding about the different responses of soil microbial diversity, community composition, and functional potential to drought stress in a semiarid alpine grassland and provide a theoretical basis for exploring the mechanism of microbial response to climate change in alpine grassland ecosystems.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA881479.

## Author contributions

QW analyzed data and wrote the manuscript. ZZ and YL carried out the experiments and generated the data. LB and MX analyzed the data. LL conceived the work, designed the experiment, and supervised this research. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1104944/full#supplementary-material

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# Composition and spore abundance of arbuscular mycorrhizal fungi in sweet potato producing areas in Uganda

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**Introduction:** Farming systems influence composition and abundance of microbial communities.

**Methodology:** A study was conducted using morphotyping and enumeration methods to determine the composition and spore abundance of Arbuscular Mycorrhizal Fungi (AMF) in sweet potato producing regions in eastern Uganda. Sampling was done from fields with crop types (CTs) including legumes (groundnuts, common beans, cowpea, soybeans, green grams), sorghum, sweet potato, and fallowed fields which were used as a control. Three agro-ecological zones (AEZs) i.e., Mt. Elgon High Farmlands (MEHF), Lake Victoria Crescent (LVC), and Southern and Eastern Lake Kyoga Basin (SELKB) were considered.

**Results and discussion:** A total of 6 AMF genera comprising of Glomus, *Acaulospora*, Scutellospora, Entrophospora, Archaeospora, and Gigaspora were isolated from the study sites. Agro-ecological zones had a significant ($p<0.05$) effect on Entrophospora spp. while crop types had a significant ($p<0.05$) effect on *Gigaspora* spp. although all the AMF genera were present in all AEZs and CTs. Spore abundance was similar across the AEZs except for MEHF (177) which was lower while spore abundance lowest in sweet potato (177) and largest in fallow (224), attributed to soil properties and similar crops included in the crop rotation program. The AMF can be isolated, identified, and multiplied to produce bioinoculants for the regions.

KEYWORDS

**arbuscular mycorrhiza, composition, spore abundance, agroecological zone, cropping type**

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# 1 Introduction

Arbuscular mycorrhizal fungi are obligate endosymbionts of up to 90% terrestrial plants (1) belonging to the phylum Glomeromycota (2) and sub-phylum Glomeromycotina (3). They are gaining prominence in agriculture for increasing crop production. Therefore, understanding their biogeographical patterns and drivers for maintaining ecosystem services amidst changes in farming systems aimed at agro-ecological adaptability is a prerequisite. Various biotic and abiotic factors and biological processes are reported to impact on microbial biogeographical patterns (4, 5). Several studies have identified different edaphic variables that influence soil bacterial and fungal community compositions (6). Abiotic factors include soil type (7), soil texture (8), soil acidity (9), soil temperature (10), soil moisture (11), soil available P (12) and cropping systems (tillage, crop rotation, fallowing) (13–16). Öpik et al. (17) reported that AMF communities vary in composition due to differences in ecosystems under different disturbance regimes. Other factors include organic matter (18), soil biota (19, 20), and plant communities (21, 22).

Some studies in sub-Saharan Africa have focused on the diversity and spore abundance of AMF (e.g., 23–26) but have not emphasized agricultural zoning and cropping systems moreover in Uganda. Cropping systems influence the biological, physical, and chemical properties of soils, as well as the geographical distribution of plants, which greatly impact on the diversity and spore abundance of AMF (23). Crops such as cereals, legumes, coffee, bananas, cassava, and other root and tuber crops that benefit from AMF associations dominate Africa's landscape (27). Less frequently cultivated fallow fields always have higher spore abundance than fields under frequent conventional cultivation (23). In the drier areas of the Maasai-Mara Ecosystem in Kenya, maize and wheat monocrops recorded significantly lower AMF diversity, species richness, and spore density in the wet and dry season than the maize-bean intercrops dominated by *Scutellospora* and *Acaulospora* species (28). Similarly, the dominance of Scutellospora and *Acaulospora* had been reported earlier in Western Kenya (29). Sporulation of genus *Acaulospora* was high in acidic soils (26, 30), increasing their dominance in soils of a pH range of 5.51 to 6.77 (28). Castillo et al. (31) reported high sporulation of *Acaulospora* spp. Under conventional tillage than no-tillage. On the other hand, Jansa et al. (32) reported higher sporulation of Scutellospora spp. In undisturbed and moderately disturbed soils. The limited occurrence of *Gigaspora* spp. In various ecosystems compared to other AMF genera has been confirmed by their low density reported by Schalamuk et al. (33) Jefwa et al. (34), and Muchane et al. (28).

In the study of Belay et al. (24) a total of 15 AMF genera (*Glomus*, *Acaulospora*, *Funneliformis*, *Gigaspora*, *Scutellospora*, *Septoglomus*, *Claroideoglomus*, *Entrophospora*, *Rhizophagus*, *Paraglomus*, *Diversispora*, *Pacispora*, *Racocetra*, *Sclerocytis*, and *Ambispora*) were isolated from both field and trap culture soils. A total of 31 species was observed in the study in an irrigated mixed fruit cropping system that received manure followed by 23 species in a crop rotation field with teff, sesame, and sunflower that received 50 kg Urea $ha^{-1}$ and 100 kg DAP $ha^{-1}$ (24). In the same study, 15 AMF species were noted in a 30-year-old natural forest with acacia, fig, and stinkwood trees, 14 species in an acacia plantation, and up to 11 species in fields of sorghum or maize monocrops receiving 50 to 100 kg Urea $ha^{-1}$ and 100 to 150 kg DAP $ha^{-1}$ (24). *Glomus* and *Acaulospora* AMF were the most diverse groups represented by 9 species each, followed by *Funneliformis* and *Gigaspora*. *Glomus* and *Acaulospora* spp. Produce more spores in a shorter time than *Scutellospora* and *Gigaspora* in the same environment (23, 35, 36). Seven genera comprising *Acaulospora*, *Ambispora*, *Glomus*, *Claroideoglomus*, *Pacispora*, *Gigaspora*, and *Scutellospora* were isolated from cassava cropping fields in Abengourou, East Côte d'Ivoire of which the genus Glomus was dominant (37). Earlier on in South Africa, studies had shown that the rhizosphere of cassava in Limpopo contained *Acaulospora* scrobiculata, Glomus rubiforme, and Gigaspora sp. Whereas the Mpumulanga soils had *Acaulospora* scrobiculata, *Acaulospora* mellea, *Acaulospora racticede*, *Glomus etunicatum*, *Glomus rubiforme*, *Gigaspora* sp, and *Scutellospora* sp (38).

However, no studies have been carried out on the composition and spore abundance of AMF in fields under sweet potato production. Therefore, the objective of the study was to determine the composition and spore abundance of AMF as influenced by different crop types (CTs) in three sweet potato producing AEZs in eastern Uganda.

# 2 Materials and methods

## 2.1 Study sites description

The study was conducted in eastern Uganda covering three agro-ecological zones (AEZ) namely, Mt. Elgon High Farmlands (MEHF), Lake Victoria Crescent (LVC), and Southern and Eastern Lake Kyoga Basin (SELKB). The sites were distributed, in Magola and Rubongi sub-counties of Tororo district (MEHF); Busware and Banda sub-counties of Namayingo district (LVC) and, in Bukedea, Ongi'no, Malera, Kolir, and Kachumbala sub-counties of Bukedea district (SELKB). The landscape of MEHF has steep slopes and is divided by many valleys. The climate is cool and wet with the southern part being warmer with less rain in July than the northern part. Rainfall peaks in April and May but is generally more than 100 mm per month from March to November. In the LVC, the landscape of West of the Nile River in the LVC is an old land surface marked by ridges or laterite-capped hills, long slopes, and wide, often swampy valleys while on the East of the Nile, the landscape is rolling with wide valleys and relatively less rolling. Soils are often acidic and low in K, but with moderate levels of organic matter. Southern and eastern Lake Kyoga Basin (SELKB) has a gently rolling landscape with wide valleys draining to Lake Kyoga. The soils of the western part of this zone are generally loamy on the ridges and upper slopes and sandy loam on the lower slopes. This sub-humid AEZ has two cropping seasons with almost equal average rainfall intensity, 560 mm during March-June, and 540 mm during July-November (Figure 1).

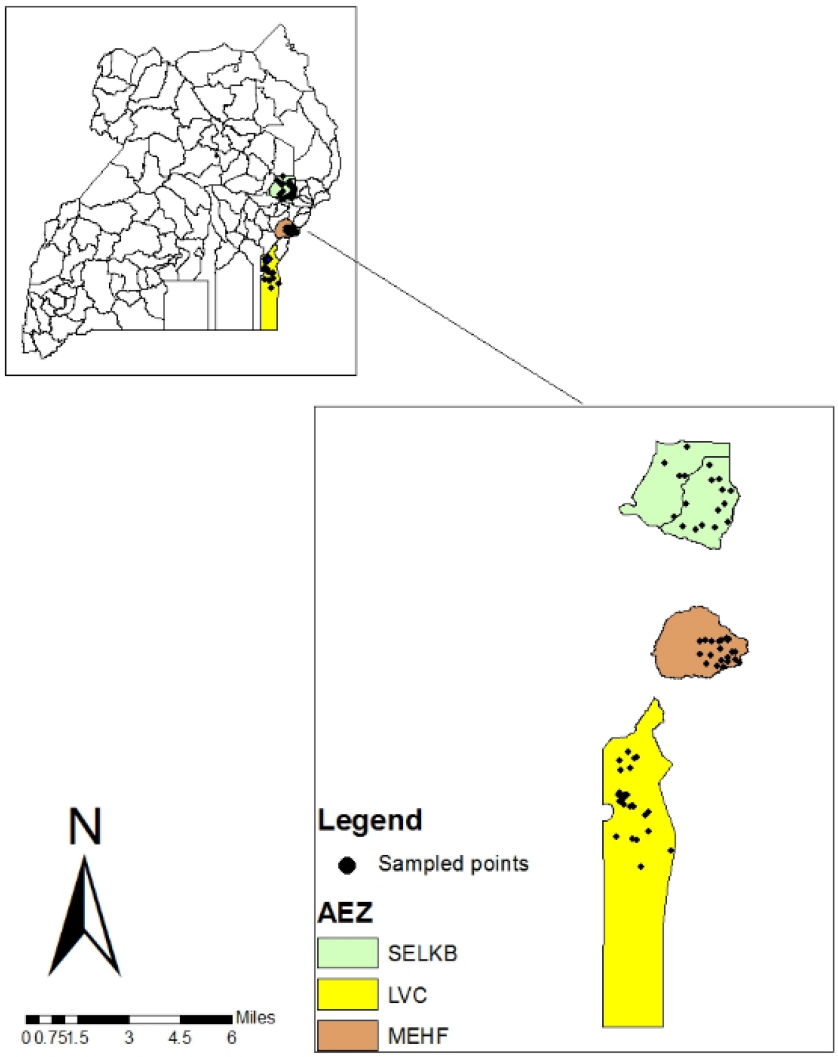


FIGURE 1
A map showing study areas in eastern Uganda. SELKB, Southern and Eastern Lake Kyoga Basin; MEHF, Mt. Elgon High Farmlands; LVC, Lake Victoria Crescent.

The area under sweet potato production in eastern Uganda is 159,948 hectares and the average yield is 5.3 t $ha^{-1}$ while in central Uganda 98,054 hectares are under sweet potato production with an average yield of 3.2 t $ha^{-1}$ (39). Further description of the AEZs is shown in Table 1.

## 2.2 Soil sampling procedure

A multistage sampling frame was used to select study sites. The study sites were selected considering the major sweet potato producing regions targeting different crop types and hence the selection of Mt. Elgon High Farmlands (MEHF), Lake Victoria Crescent (LVC), and Southern and Eastern Lake Kyoga Basin (SELKB). Districts that experience moisture stress conditions were considered and hence Tororo, Namayingo and Bukedea were selected. Occurrence of moisture stress conditions in these study sites is attributed to the high temperature recorded and rainfall that is unreliable and highly variable in terms of its onset, cessation, amount, and distribution (44). Sub counties i.e., Tororo (Magola and Rubongi sub-counties), Namayingo (Busware and Banda sub-counties) and Bukedea (Ongi'no, Malera, Kolir, and Kachumbala sub-counties) were randomly selected. In the selected sub-counties, a list of smallholder farmers (producing the targeted crops) was proposed by contact farmers in the respective sub-counties and farmers were randomly selected from the list. Farmers' fields were used as replicates and the samples were obtained following the existing crop types and soil amendments on that field. The geographical location for each field was captured using a Geographical Positioning System (GPS). Farmers provided more details on the fields that were sampled from for the previous 5 years (Table 2). Nineteen (19) samples were obtained from SELKB, 20 from MEHF and 25 from LVC. It is worth noting that the farmers had racticed mono-cropping, inter-cropping, and crop rotation including cassava, groundnuts, sweet potato, maize, soybean, green grams, millet, sesame, oranges, eggplant, green pepper,

TABLE 1 Agro-ecological characteristics of the study areas.

| Charateristic | SELKB [1] | MEHF [1] | LVC [1] |
|---|---|---|---|
| Population density (pers $km^2$) | 129 [3] | 345 [3] | 280 [3] |
| Altitude (masl) | 1143 [4] | 1400-1800 [3] | 1106 [4] |
| Mean annual temperatures (° C) | 28-31 [4] | ≤20 [1] | 22.0 [1] |
| Rainfall pattern (mm $year^{-1}$) | >1200 [1; 3;4] | >1200 [1; 3] | >1200 [1] |
| Soil textural classes and other characteristics | The Western part (loam on ridges and upper slopes, sandy loam on lower slopes); East and North West (sandy soils, occasionally acidic, often low in organic matter) [1] | North part (red clay loam, well drained, highly leached, often acid, good nutrient supply); south (surface soil-high sand content, lower nutrient supply, very low soil erosibility, moderately high rainfall erodibility[1] | High clay content, common soils (sandy-clay-loam); clay-loam, acidic, low in K with moderate levels of organic matter [1] |
| Main crops in order of priority | Finger millet, banana, maize, cotton, rice, sorghum, cassava, sweet potato, and groundnut [1] | Beans, banana, maize, groundnuts, Arabica coffee, sweet potato [1] | Banana, beans, sweet potato, cassava, maize, rice, Robusta coffee, [1] |
| Cropping practices | No inorganic fertilizer use, crop rotation, mono-cropping/inter-cropping of cassava, groundnuts, sweet potato, maize, soybean, green grams, millet, sesame, oranges, eggplant, green pepper, cabbage, mangoes, onions, cowpea, upland rice, sunflower, and watermelon, while fallowing on a few of the fields were maintained for ≤ 6 months | No inorganic fertilizer use, crop rotation, mono-cropping/inter-cropping of maize, beans, soybean, cassava, millet, cotton, tomatoes, coffee, banana, sweet potato, groundnuts, sorghum, napier grass, brinjals, eggplants, and cowpea, while fallowing on a few of the s was maintained for ≤ 6 months | No inorganic fertilizer use, crop rotation, mono-cropping/inter-cropping of banana, millet, maize, beans, cassava, soybean, sweet potato, sorghum, tomatoes, and Irish potato, while fallowing on a few of the fields was maintained for ≤ 6 months |

Source: 40[1]; 41[2]; 42[3]; 43[4].

cabbage, mangoes, onions, cowpea, upland rice, sunflower, and watermelon and, short-term fallows. A soil auger was used to obtain 12 samples from each site in a zigzag pattern at a depth of 0 – 20 cm, which were pooled together, and a 2 kg composite homogeneous sample obtained. The homogeneous sample was placed in a strong polythene bag and sealed securely to prevent further drying and labeled clearly for ease of identification. Samples were collected in a dry season, a period when sporulation increases (45). The samples were split for use in AMF identification and soil physical and chemical analysis.

## 2.3 Soil physical and chemical analysis

Each soil sample for physico-chemical analysis was air-dried, sieved through 2 mm sieve, homogenized, and analyzed for pH (1 soil:2.5 $H_2O$ ratio), soil organic carbon (SOC), total and available P, exchangeable cations ($Ca^{2+}$, $Mg^{2+}$, $K^+$, and $Na^+$) and texture at Soil, Water and Plant Analytical Laboratory of Makerere University following procedures outlined in Okalebo et al. (46). Soil pH was measured in a soil-water solution at a ratio of 1:2.5 (w/v) using a pH meter (Mettler-Toledo, AG 8603) after mixing on a rotary shaker for 30 minutes at 150 rpm (47). Total N was assessed after wet digestion of air-dried soil samples with a mixture of concentrated sulphuric acid ($H_2SO_4$) and selenium powder and salicylic acid and measured using a spectrophotometer (Jenway, 6405 UV/Vis) (48). Available P was extracted using Bray 1 method in a mixture of ammonium fluoride and hydrochloric acid, shaken for 1 minute at 150 rpm. The available P was complexed in a mixture of ascorbic acid and ammonium molybdate (49) and measured using a spectrophotometer (Jenway, 6405 UV/Vis). Exchangeable bases were extracted with ammonium acetate by shaking soil samples in ammonium solution for 20 minutes and measured using a flame-photometer ($K^+$, $Na^+$) (Jenway, Essex CM6 3LB) and atomic absorption spectrophotometer ($Ca^{2+}$, $Mg^{2+}$) (50) (Jenway, 6405 UV/Vis). Soil organic carbon was determined by wet oxidation with potassium dichromate under concentrated sulphuric acid at 150 °C for 30 minutes. The unreacted dichromate was titrated against standardized ferrous ammonium sulphate solution using ferroin indicator to determine the end point (51). Soil texture was determined using a Bouyoucos (Gallenkamp Bouyoucos) method (52).

## 2.4 Spore isolation and morphological identification of arbuscular mycorrhizal fungi genera

Arbuscular mycorrhizal fungi spores were isolated according to Jenkins (53) procedure with modifications by Ingleby (54) from 50 g of air-dried soil samples by wet sieving through 710 and 45 µm sieves, followed by sucrose gradient centrifugation. After centrifugation, spores and spore clusters were transferred into Petri dishes. The spores were distinguished into genera under the reflected light on stereomicroscope with the color of spore, spore size, hyphal attachments on spore and surface appearance of spore used as the diagnostic features and enumerated. Slide specimens were prepared for each AMF genus and further described under a

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TABLE 2 Details of the fields obtained at sampling time.

| Field no. | Site/Farmer's name | AEZ | Latitude | Longitude | CT | Fertilizer/Manure application |
|---|---|---|---|---|---|---|
| 1 | DATIC | MEHF | 0.69300 | 34.18090 | Maize, Cassava | None |
| 2 | MUARIK | LVC | 0.46370 | 32.60990 | Fallow | None |
| 3 | Margaret Ochieng' | MEHF | 0.71412 | 34.12003 | Green grams | NPK foliar feed |
| 4 | Nociata Akello | MEHF | 0.71424 | 34.12348 | Groundnuts | None |
| 5 | Samuel Sunday | MEHF | 0.71427 | 34.12454 | Groundnuts | None |
| 6 | Omita Opiyo | MEHF | 0.70722 | 34.11651 | Soyabeans | None |
| 7 | Wilson Ochieng' | MEHF | 0.62775 | 34.05937 | Groundnuts | None |
| 8 | Wilson Ochieng' | MEHF | 0.62784 | 34.05995 | Cowpeas | None |
| 9 | Betty Owour | MEHF | 0.62782 | 34.07033 | Soyabeans | None |
| 10 | Jennifer Obaa | MEHF | 0.62635 | 34.06430 | Soyabeans | Manure |
| 11 | Jennifer Obaa | MEHF | 0.62623 | 34.06410 | Groundnuts | None |
| 12 | Joseph Otieno | MEHF | 0.71429 | 34.12097 | Sorghum | None |
| 13 | DoroRoza Alowo | MEHF | 0.71447 | 34.12473 | Sorghum | None |
| 14 | Nociata Awino | MEHF | 0.71431 | 34.12348 | Sweet potato | None |
| 15 | DoroRoza Alowo | MEHF | 0.71474 | 34.12443 | Sweet potato | None |
| 16 | Betty Owour | MEHF | 0.62771 | 34.07032 | Sweet potato | None |
| 17 | Japheth Ofwono | MEHF | 0.60544 | 34.07655 | Fallow | None |
| 18 | Dennis Odoyi | MEHF | 0.71416 | 34.10313 | Fallow | None |
| 19 | Joseph Otieno | MEHF | 0.71429 | 34.12097 | Fallow | None |
| 20 | Nociata Akello | MEHF | 0.71418 | 34.12322 | Fallow | None |
| 21 | Yafesi Okoth | MEHF | 0.62561 | 34.09459 | Fallow | None |
| 22 | Emmaculate Nyapendi | MEHF | 0.63960 | 34.08994 | Fallow | None |
| 23 | Charles Ochoo | MEHF | 0.64705 | 34.08577 | Fallow | None |
| 24 | James Okumu | LVC | 0.27108 | 33.54018 | Soyabeans | Rhizobia |
| 25 | James Okumu | LVC | 0.27108 | 33.54018 | Soyabeans | Rhizobia+DAP |
| 26 | Fred Mbageya | LVC | 0.27207 | 33.54147 | Common beans | None |
| 27 | Fred Mbageya | LVC | 0.27207 | 33.54147 | Cowpeas | None |
| 28 | Simon Mbageya | LVC | 0.27190 | 33.54246 | Soyabeans | None |
| 29 | Moses Wafula | LVC | 0.27191 | 33.54250 | Common beans | None |
| 30 | Constant Wanyama | LVC | 0.27621 | 33.54440 | Groundnuts | None |
| 31 | Patrick Sifuna | LVC | 0.26278 | 33.54113 | Green grams | None |
| 32 | Contant Wandera | LVC | 0.26886 | 33.54313 | Soyabeans | None |
| 33 | Juma Nyegenye | LVC | 0.26863 | 33.54274 | Groundnuts | None |
| 34 | Frasko Maende | LVC | 0.26837 | 33.54190 | Soyabeans | None |
| 35 | Friday Mang'eni | LVC | 0.26717 | 33.54199 | Common beans | None |
| 36 | Wison Wanyama | LVC | 0.27560 | 33.54104 | Common beans | None |
| 37 | Fred Mbageya | LVC | 0.27168 | 33.54092 | Sorghum | None |
| 38 | Sam Mulino | LVC | 0.27221 | 33.54160 | Sorghum | None |
| 39 | Odinga Mbageya | LVC | 0.27190 | 33.54208 | Sorghum | None |

*(Continued)*

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TABLE 2 Continued

| Field no. | Site/Farmer's name | AEZ | Latitude | Longitude | CT | Fertilizer/Manure application |
|---|---|---|---|---|---|---|
| 40 | Constant Wanyama | LVC | 0.27257 | 33.54170 | Sorghum | None |
| 41 | Francis Hagaba | LVC | 0.27034 | 33.54238 | Sorghum | None |
| 42 | Fred Mbageya | LVC | 0.27213 | 33.54115 | Sweet potato | None |
| 43 | Sam Mulino | LVC | 0.27221 | 33.54160 | Sweet potato | None |
| 44 | Faisi Natocho | LVC | 0.26981 | 33.54166 | Sweet potato | None |
| 45 | Stephen Bwire Anania | LVC | 0.12203 | 33.53931 | Fallow | None |
| 46 | Barua Ogong'la | LVC | 0.10556 | 35.33000 | Fallow | None |
| 47 | Yohana Osenga | LVC | 0.10066 | 33.53433 | Fallow | None |
| 48 | Marsala Nafula | LVC | 0.11502 | 33.51145 | Fallow | None |
| 49 | Ochieng' Odoki | LVC | 0.11838 | 33.50678 | Fallow | None |
| 50 | Gilbert Ilomu | SELKB | 1.33543 | 34.05953 | Groundnuts | None |
| 51 | Justin Aide | SELKB | 1.49936 | 34.01362 | Cowpeas | None |
| 52 | Charles Orungo | SELKB | 1.39368 | 34.09563 | Cowpeas | None |
| 53 | Charles Orungo | SELKB | 1.39368 | 34.09563 | Green grams | None |
| 54 | Julius Ariko | SELKB | 1.39660 | 34.11750 | Common beans | None |
| 55 | Julius Ariko | SELKB | 1.39660 | 34.11750 | Green grams | None |
| 56 | Peter Okia | SELKB | 1.37978 | 34.15097 | Cowpeas | None |
| 57 | Gilbert Ilomu | SELKB | 1.33623 | 34.05870 | Sorghum | None |
| 58 | Omerisa | SELKB | 1.49332 | 34.01407 | Sorghum | None |
| 59 | John Ojakolo | SELKB | 1.34400 | 34.18990 | Sorghum | None |
| 60 | Gilbert Ilomu | SELKB | 1.38537 | 34.05912 | Sweet potato | None |
| 61 | Esugut Daniel | SELKB | 1.49795 | 34.01535 | Sweet potato | None |
| 62 | Peter Igala | SELKB | 1.25462 | 34.13432 | Sweet potato | None |
| 63 | Mary Atimong | SELKB | 1.26866 | 34.15145 | Fallow | None |
| 64 | Hellen Agoti | SELKB | 1.35250 | 34.16271 | Fallow | None |
| 65 | Joseph Akol | SELKB | 1.47300 | 34.02448 | Fallow | None |
| 66 | Anastancia Among' | SELKB | 1.37253 | 34.05537 | Fallow | None |

compound microscope at magnification x40 with spore germination characteristics, spore wall characteristics, type of spore wall, number of layers and reaction to PVLG- Polyvinyl lacto glycerin (1.66g polyvinyl alcohol 20-25 cP, 10 ml lactic acid, glycerin 1 ml and 10 ml distilled H2O) and Melzer's reagent (chloral hydrate, 1.5 g iodine, 5.0 g potassium iodide and 100 ml distilled H2O + PVLG). The spores were matched with genera described by International Culture Collection of VA Mycorrhizal Fungi (INVAM) West Virginia University Morgantown, WV, USA Website and Schenck and Perez (55, 56).

## 2.5 Data analysis

Analysis of Variance (ANOVA) using statistical analysis software (SAS), version 9.4 generalized linear model was used to determine the effects of AEZ and CT on AMF spore abundance. Spore abundance data were transformed in excel using square root pi (SQRTPI) the most preferred type of transformation for count data, to obtain symmetric distribution. Agro-ecological zone and CT were treated as fixed effects while fields were the random effects using the generalized linear model. Treatment means were separated using the least significant difference (LSD). Redundancy analysis (RDA) (57), the canonical version of principal component analysis (PCA), was used to examine the multiple correlations between soil properties (pH, OM and total N, available P, exchangeable Ca, K, Mg, and Na, sand, silt, and clay content) and genera of AMF in the sampling sites. The abundance values of AMF genera were centered and standardized in RDA using square root transformation. Soil properties were also standardized as explanatory variables using arcsine square root transformation before performing RDA. The AMF abundance data were pre-

analyzed by Detrended Correspondence Analysis (DCA) using CANOCO software 4.5 (Micro-computer Power, Ithaca, NY) to choose a linear or unimodal ordination model for analysis. As the length of the gradient (first axis) was 0.668 below 3 by DCA, the RDA (canonical correlation analysis) was applied to the data obtained in this study.

# 3 Results

## 3.1 Arbuscular mycorrhizal fungi genera and spore abundance

In this study, six AMF genera were distinguished based on morphological features. Spore features that were used to classify the AMF genera included color of spore, spore size, hyphal attachments on the spore, and surface appearance of spore (INVAM and 55). *Gigaspora* species were identified as large spores with a bulbous hyphal attachment. *Scutellospora* species were identified as large spores with bulbous hyphal attachment, germination shield, and flexible/separating walls. *Acaulospora* species were identified as spores having ornamented spore walls, presence of cicatrix/cicatrices, and spores forming on the side of the hypha. *Entrophospora* species had almost similar characteristics to those of *Acaulospora* spp. But their spores form into the neck of the hypha, and *Glomus* spp. Have either curved, straight, or gourd-like hyphal attachment (Figure 2).

Exploratory data analysis using box plots showed a variable range of distribution of spores within AEZs and CTs. The box represents the spore abundance range, the rhombus inside the box represents the mean of the spore abundance data, and the middle line represents the median, the lower and the upper bar mean the minimum and the maximum values of the data, respectively. Mean spore abundance across AEZs ranged between 48 and 58 spores while across CTs it ranged between 47 and 60 spores as shown by the medians of the box plots. Spore abundance was positively skewed across AEZs particularly in Mt. Elgon High Farmlands AEZ and in sweet potato fields because their medians are closer to the lower quartile, signifying non-normal distribution. The highest spore abundance was recorded in Southern and Eastern Lake Kyoga Basin AEZ, and in fallow fields since they had the highest mean values. The widest range of spore abundance was observed in Lake Victoria Crescent AEZ, and in legume fields since they had the longest whiskers (Figures 3A, B).

Using a generalized linear model for the presence and absence of AMF, the occurrence of AMF showed a slight variation in each AEZ and CT with two AMF genera significantly affected. The AEZs differently affected the mean spore abundance of *Entrophospora* spp. ($p < 0.05$) with SEKLB having a significantly ($p < 0.05$) higher number of spores than MEHF. The mean spore abundance of *Gigaspora* spp. ($p < 0.05$) was variable across the CTs with AMF spores dominating in fallow fields than the rest of the CTs (Table 3). T-test accepted the null hypothesis that total spore abundance between CTs in all AEZs was equal except for sorghum and sweet potato (t value=31.28; p=0.0010), sorghum and fallow (t value=0.00; p<.0001) and sweet potato and fallow (t value=9.28; p=0.0114) in MEHF and, sorghum and fallow (t value=0.00; p<.0001) in LVC.

The frequency of observation of the identified AMF genera was 100% except that of *Archaeospora* which was 86%. The dominant AMF genera across the different AEZs were Glomus spp., *Acaulospora* spp., *Scutellospora* spp., and *Entrophospora* spp., each constituting ≥ 10% of the spore abundance of the identified AMF. They accounted for 87% of the spores of the identified AMF in each AEZ while *Gigaspora* spp. And *Archaeospora* spp. Contributed only 13% of the spores. The total number of the AMF genera spores across AEZs was highest in *Glomus* followed by *Acaulospora*, *Scutellospora*, *Entrophospora*, *Archaeospora*, and *Gigaspora* except for MEHF AEZ where *Gigaspora* was more dominant than *Archaeospora* (Table 4).

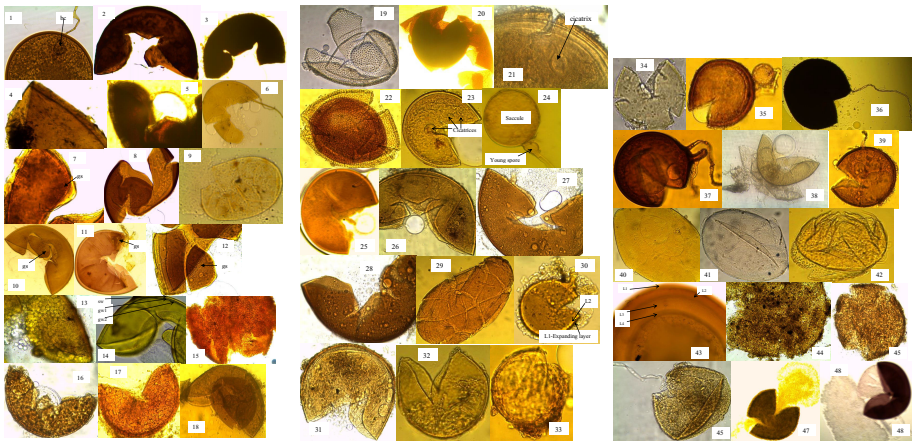

FIGURE 2
Some of the Glomeromycotan species identified from field soils across the AEZs and CTs. Magnification x40, bc, bulbous cell; gs, germination shield; sw, spore wall; gw, germination wall, l,layer, **1-6**=*Gigaspora* spp., **7-12**=*Scutellospora* spp., **13-20**=*Acaulospora* spp., **21-25**=*Entrophospora* spp., **26-39**=*Glomus* spp., **40-48**=Undefined spp.

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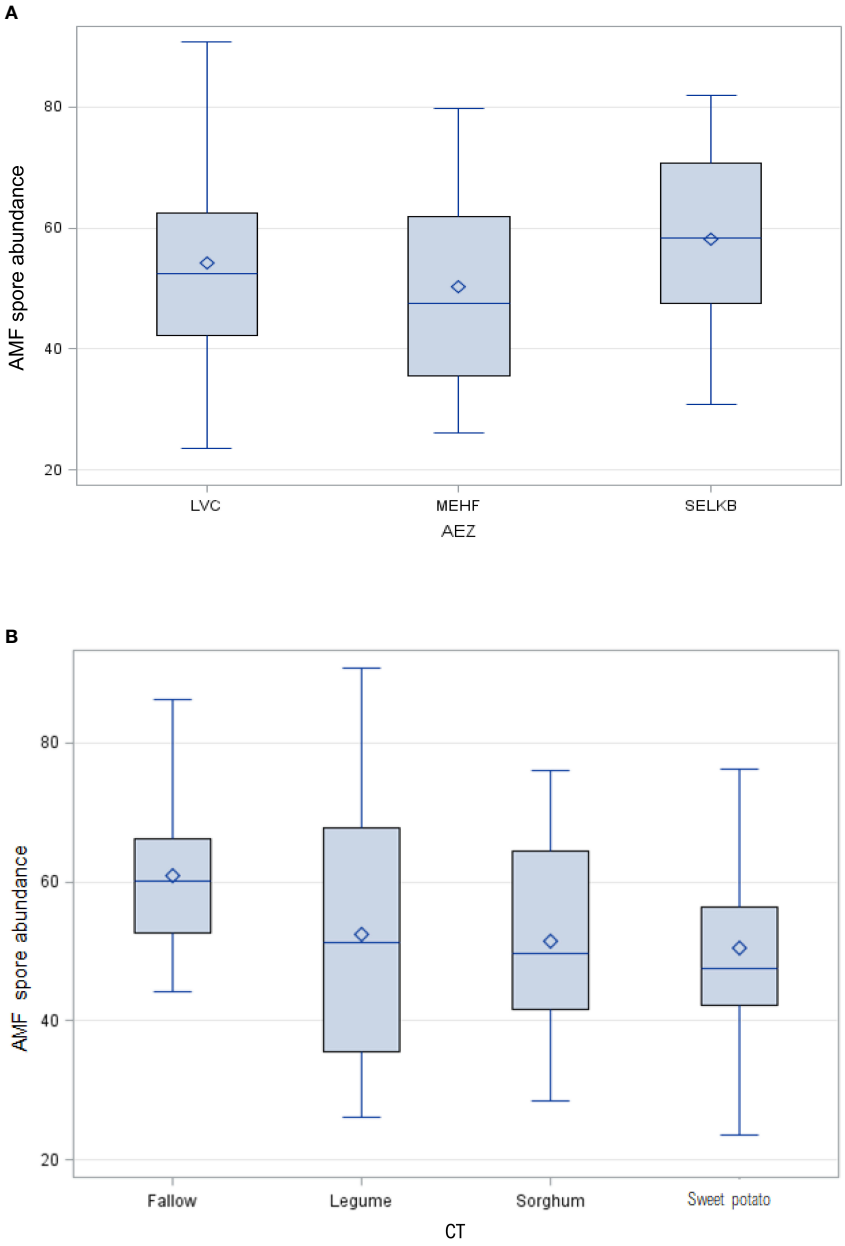


FIGURE 3
**(A, B)** Range of distribution of AMF spores within AEZs and CTs.

## 3.2 Correlation of soil properties and distribution of arbuscular mycorrhizal fungi

The soils in this study were weakly acidic but with low to moderate nutrient and OM levels. The mean values of measured soil properties were significantly ($p \leq 0.05$) different across AEZs except for P and Na. Crop types significantly ($p \leq 0.05$) influenced all soil properties except for pH, Ca, and Mg. The effect of the interaction of AEZs and CTs was only significant ($p \leq 0.05$) on P, Na, Ca, and Mg (Table 5).

The first and the second RDA axis explained variance for 71 and 18%, respectively (Figure 4). According to the lengths of the arrows and the angles among them, OM, N, and pH had a strong positive correlation with the sporulation of *Archaeospora*, *Entrophospora*, *Glomus* and *Scutellospora*, and strong negative correlation with the sporulation of *Acaulosopra* and *Gigaspora*. Clay, K, Ca, and P had slight positive effects on the sporulation of *Archaeospora*, *Entrophospora*, *Glomus* and *Scutellospora*, and slight negative effects on the sporulation of *Acaulospora* and *Gigaspora*, because the arrows representing them are relatively short. Sand also had slight positive effects on the sporulation of *Acaulospora*, *Archaeospora* and *Entrophospora*, and slight negative effects on the sporulation of *Glomus*, *Scutellospora* and *Gigaspora*. Based upon the direction of the arrows, silt and Mg had a strong positive correlation with the sporulation of *Acaulospora*, *Gigaspora*, *Scutellospora* and *Glomus*, and a strong negative correlation with the sporulation of

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TABLE 3 Mean spore abundance of arbuscular mycorrhizal fungi in 50 $g^{-1}$ air-dried soil across AEZs and CTs.

| AEZ | CT | AMF genera | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Glomus* spp. | *Acaulospora* spp. | *Scutellospora* spp. | *Archaeospora* spp. | *Entrophospora* spp. | *Gigaspora* spp. |
| Southern and Eastern Lake Kyoga Basin (SELKB) | Fallow | 58 | 45 | 31 | 23 | 35 | 15 |
| | Legumes | 104 | 51 | 43 | 17 | 33 | 8 |
| | Sorghum | 47 | 35 | 21 | 14 | 24 | 5 |
| | Sweet potato | 77 | 51 | 32 | 25 | 34 | 11 |
| | *p-value* | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* |
| Mt. Elgon High Farmlands (MEHF) | Fallow | 72 | 39 | 46 | 12 | 22 | 22 |
| | Legumes | 55 | 29 | 26 | 3 | 11 | 11 |
| | Sorghum | 117 | 38 | 47 | 1 | 20 | 15 |
| | Sweet potato | 33 | 25 | 20 | 14 | 15 | 5 |
| | *p-value* | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* |
| Lake Victoria Crescent (LVC) | Fallow | 81 | 46 | 47 | 22 | 32 | 26 |
| | Legumes | 82 | 39 | 36 | 13 | 22 | 9 |
| | Sorghum | 71 | 36 | 37 | 12 | 22 | 16 |
| | Sweet potato | 80 | 31 | 28 | 8 | 15 | 8 |
| | *p-value* | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* |
| | | F-test (*p-value*) | | | | | |
| *AEZ* | | *Ns* | *Ns* | *Ns* | *Ns* | *0.0057* | *Ns* |
| *CT* | | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* | *0.0122* |
| *AEZ*CT* | | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* | *Ns* |

Ns, not significant (p>0.05).

*Archaeospora* and *Entrophospora*. Sodium had a strong positive correlation with the sporulation of *Glomus*, *Scutellospora* and *Gigaspora*, and a strong negative correlation with the sporulation of *Acaulospora*, *Archaeospora* and *Entrophospora*.

## 4 Discussion

The high spore abundance of *Glomus* spp. And *Acaulospora* spp. Across AEZs and CTs may be attributed to their high frequency of hyphal fusions that plug into compatible extraradical networks and hence immediate access to host plants and subsequent spore formation (58). Glomus and *Acaulospora* produce more spores in a shorter time than *Scutellospora* and *Gigaspora* in the same environment (23, 35, 36). Gigasporaceae species produce large spores (260 to 440 µm for *Gigaspora margarita*) that require a longer developmental period than small spores (59). Hence, their occurrence can be further ascertained through the study of AMF diversity in the root systems since spore extraction alone may miss species that may have not sporulated.

The insignificant response of AMF genera except for Entrophospora to changes in AEZs could be due to quite similar soil properties attributed to similar agronomic practices carried out in the fields (Table 3). The total and mean spore abundance in this study (involving annual crops) were high compared to soils dominated by perennial crops and frequently supplied with inorganic fertilizers and pesticides (23, 34). The high spore abundance can be attributed to increased sporulation caused by frequent soil disturbance in annual crops fields. However, trap cultures were necessary to observe the composition of AMF as evidenced by several authors who reported fewer species from direct isolation from local soil and more species after trapping (26).

Crop type did not significantly affect AMF due to the similar conventional tillage carried out in the fields with the different crops (Table 3). The slight significant difference between fallow and sweet potato in MEHF and LVC was because fallows had been rested for an average of 6 months allowing for the recolonization and sporulation of AMF. Results showed that *Gigaspora* spp. Abundance was highest in the fallow fields (Table 3) which confirmed the results of Gai et al. (60) that *Gigasporaceae* spp. Are more often associated with wild plants than open fields. It also

TABLE 4 Rank of total number of arbuscular mycorrhizal fungi spores per genera and their proportions in 50 $g^{-1}$ air dried soil.

| Agro-ecological zone | AMF genera | Rank | Total number of spores | Proportion (%) |
|---|---|---|---|---|
| Southern and Eastern Lake Kyoga Basin (SELKB) | *Glomus* spp. | 1 | 1332 | 36 |
| | *Acaulospora* spp. | 2 | 791 | 21 |
| | *Scutellospora* spp. | 3 | 581 | 16 |
| | *Entrophospora* spp. | 4 | 541 | 14 |
| | *Archaeospora* spp. | 5 | 327 | 9 |
| | *Gigaspora* spp. | 6 | 162 | 4 |
| | *Total* | | 3734 | 100 |
| Mt. Elgon High Farmlands (MEHF) | *Glomus* spp. | 1 | 1134 | 38 |
| | *Scutellospora* spp. | 2 | 612 | 20 |
| | *Acaulospora* spp. | 3 | 571 | 19 |
| | *Entrophospora* spp. | 4 | 291 | 10 |
| | *Gigaspora* spp. | 5 | 263 | 9 |
| | *Archaeospora* spp. | 6 | 130 | 4 |
| | *Total* | | 3001 | 100 |
| Lake Victoria Crescent (LVC) | *Glomus* spp. | 1 | 2398 | 39 |
| | *Acaulospora* spp. | 2 | 1167 | 19 |
| | *Scutellospora* spp. | 3 | 1118 | 18 |
| | *Entrophospora* spp. | 4 | 673 | 11 |
| | *Archaeospora* spp. | 5 | 406 | 7 |
| | *Gigaspora* spp. | 6 | 380 | 6 |
| | Total | | 6142 | 100 |

Relative proportion (%) of the AMF across agroecological zones depended on the spore abundance of the identified genera.

TABLE 5 Selected soil physical and chemical properties across three AEZs and four CTs.

| AEZ | CT | Soil properties | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH ($H_2O$) | OM (%) | Total N (%) | Extractable P (mg $kg^{-1}$) | $K^+$ | $Na^+$ | $Ca^+$ | $Mg^+$ | Sand (%) | Clay (%) | Silt (%) |
| | | | | | | cmol(+) $kg^{-1}$ | | | | | | |
| SELKB | Fallow | 6.14± 0.17 | 1.35± 0.49 | 0.13± 0.04 | 21.21± 20.00 | 0.46± 0.12 | 0.46± $0.14^{a}$ | 7.46± 3.66 | 2.05± 0.57 | 70.00 ± 6.93 | 14.00 ± 7.12 | 16.00 ± 1.63 |
| | Legumes | 6.07± 0.58 | 3.24± 2.44 | 0.12± 0.04 | 11.83± 7.11 | 0.64± 0.36 | 0.33± $0.10^{ab}$ | 6.46± 3.98 | 1.99± 0.59 | 68.00 ± 10.30 | 19.00 ± 7.55 | 13.00 ± 5.16 |
| | Sorghum | 5.89± 0.46 | 4.12± 2.51 | 0.12± 0.03 | 7.18± 1.45 | 0.54± 0.28 | 0.26± $0.06^{b}$ | 4.02± 1.02 | 1.46± 0.54 | 69.75 ± 7.22 | 14.50 ± 5.97 | 15.75 ± 5.56 |
| | Sweet potato | 5.93± 0.41 | 2.98± 2.00 | 0.12± 0.03 | 15.11± 12.09 | 0.61± 0.31 | 0.37± $0.10^{ab}$ | 5.44± 2.06 | 1.92± 0.62 | 70.83 ± 5.31 | 16.00 ± 5.83 | 13.17 ± 4.19 |
| | *Mean* | *6.03$^{A}$* | *3.03$^{B}$* | *0.12$^{B}$* | *13.26$^{A}$* | *0.59$^{B}$* | *0.35$^{A}$* | *6.01$^{AB}$* | *1.90$^{B}$* | *69.19$^{A}$* | *16.93$^{B}$* | *13.89$^{B}$* |
| | *p-value* | *NS* | *NS* | *NS* | *NS* | *NS* | *0.0308* | *NS* | *NS* | *NS* | *NS* | *0.0158* |

*(Continued)*

TABLE 5 Continued

| AEZ | CT | Soil properties | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH ($H_2O$) | OM (%) | Total N (%) | Extractable P (mg $kg^{-1}$) | $K^+$ | $Na^+$ | $Ca^+$ | $Mg^+$ | Sand (%) | Clay (%) | Silt (%) |
| MEHF | Fallow | 6.14± 0.25 | 1.40± 0.40$^{b}$ | 0.19± 0.14 | 18.25± 10.36 | 0.44± 0.25 | 0.40± 0.16 | 6.32± 2.44 | 2.05± 0.61 | 69.43 ± 8.06 | 14.86 ± 7.20 | 15.71 ± 2.43 |
| | Legumes | 5.85± 0.36 | 3.31± 1.40$^{a}$ | 0.11± 0.03 | 19.51± 16.03 | 0.45± 0.24 | 0.22± 0.11 | 9.91± 7.30 | 3.38± 2.37 | 72.62 ± 7.23 | 12.54 ± 2.85 | 14.85 ± 6.16 |
| | Sorghum | 6.23± 0.83 | 2.65± 1.47$^{ab}$ | 0.08± 0.01 | 12.76± 9.57 | 0.56± 0.30 | 0.27± 0.17 | 8.89± 5.97 | 3.27± 1.48 | 69.33 ± 1.15 | 12.67 ± 5.77 | 18.00 ± 6.93 |
| | Sweet potato | 6.11± 0.35 | 2.73± 1.48$^{a}$ | 0.10± 0.03 | 20.42± 17.39 | 0.38± 0.15 | 0.24± 0.10 | 7.88 ± 4.12 | 2.74± 1.26 | 70.40 ± 8.17 | 12.80 ± 3.63 | 16.80 ± 5.22 |
| | *Mean* | *6.01$^{A}$* | *2.66$^{B}$* | *0.13$^{B}$* | *18.64$^{A}$* | *0.45$^{B}$* | *0.27$^{A}$* | *8.54$^{A}$* | *2.93$^{A}$* | *71.07$^{A}$* | *13.14$^{B}$* | *15.75$^{B}$* |
| | *p-value* | *NS* | *0.0016* | *NS* | *NS* | *NS* | *NS* | *NS* | *NS* | *NS* | *NS* | *NS* |
| LVC | Fallow | 6.64± 1.71 | 1.88± 0.78$^{b}$ | 0.33± 0.16$^{a}$ | 42.36± 41.61 | 0.51± 0.14 | 2.29± 2.18$^{a}$ | 13.01 ± 7.07$^{a}$ | 2.97± 0.84$^{a}$ | 52.00 ± 11.40 | 26.80 ± 10.45 | 21.20 ± 7.82 |
| | Legumes | 6.34± 0.50 | 7.13± 1.27$^{a}$ | 0.16± 0.03$^{b}$ | 11.09± 7.53 | 1.08± 0.34 | 0.19± 0.06$^{b}$ | 4.22± 1.98$^{b}$ | 1.38± 0.65$^{bc}$ | 50.31 ± 7.02 | 21.38 ± 5.11 | 28.69 ± 6.55 |
| | Sorghum | 6.32± 0.45 | 6.57± 3.06$^{a}$ | 0.14± 0.03$^{b}$ | 11.56± 6.64 | 0.96± 0.36 | 0.18± 0.15$^{b}$ | 2.59± 0.85$^{b}$ | 0.89± 0.34$^{c}$ | 53.83 ± 10.13 | 21.83 ± 7.00 | 24.33 ± 7.71 |
| | Sweet potato | 6.36± 0.32 | 4.28± 3.34$^{ab}$ | 0.18 0.02$^{b}$ | 29.55± 27.91 | 0.72± 0.34 | 0.30± 0.14$^{b}$ | 6.21± 3.80$^{b}$ | 2.06± 0.92$^{ab}$ | 51.83 ± 14.51 | 23.67 ± 14.05 | 24.50 ± 11.20 |
| | *Mean* | *6.39$^{A}$* | *5.57$^{A}$* | *0.19$^{A}$* | *19.76$^{A}$* | *0.89$^{A}$* | *0.54$^{A}$* | *5.76$^{B}$* | *1.68$^{B}$* | *51.60$^{B}$* | *22.83$^{A}$* | *25.73$^{A}$* |
| | *p-value* | *NS* | *0.0041* | *0.0146* | *NS* | *NS* | *0.0208* | *0.0044* | *0.0008* | *NS* | *NS* | *NS* |
| | | *F-test (p-value)* | | | | | | | | | | |
| AEZ | | 0.0421 | <.0001 | 0.0002 | NS | <.0001 | NS | 0.0309 | 0.0003 | <.0001 | <.0001 | <.0001 |
| CT | | NS | <.0001 | <.0001 | 0.0028 | 0.0070 | 0.0005 | NS | NS | NS | NS | NS |
| AEZ* CT | | NS | NS | NS | 0.0198 | NS | 0.0005 | 0.0154 | 0.0429 | NS | NS | NS |

Values=Mean ± standard deviation; LSD= Least significant difference; means followed by the same lower-case letters in the same column are not significantly different (p>0.05); means followed by the same upper-case letters in the same column are not significantly different (p>0.05); NS= not significant (p>0.05). The soils sampled from SELKB were sandy loam (14 fields), sandy clay loam (4 fields), and loamy fine sand (1 field); those sampled from MEHF were sandy loam (15 fields), sandy clay loam (4 fields), and loamy fine sand (1 field); while those from LVC were sandy clay loam (8 fields), loam (9 fields), sandy loam (3 fields), sandy clay (2 fields), clay (2 fields), and loamy fine sand (1 field). Soil textural classes for SELKB, MEHF and LVC were generally sandy-loam, sandy-loam and sandy-clay-loam, respectively across CTs.

confirmed that the 6 months of fallowing was enough time for the development of their large spores (59).

The varying effects of sodium and magnesium on AMF genera in the present study (Figure 4) may be attributed to varying adaptability of AMF genera to varying levels of exchangeable bases (61). The positive effect of nitrogen on the spore abundance of *Glomus*, *Scutellospora*, *Entrophospora*, and *Archaeospora* might have been indirect through the increased supply of photosynthates from the host-crop to the fungi. Availability of soil N increases mycorrhizal activity (62) and hence its positive effect on the AMF genera.

It was expected that sporulation of all AMF genera is positively affected by organic matter since it is considered a source of energy for the growth and functioning of the fungi. However, the effect of organic matter on AMF growth and sporulation depends on the efficiency of the individual species in acquiring resources from the organic matter (63). Ng et al. (64) also reported that the chemical nature of soil carbon drives the structure and functioning of soil microbial communities which may vary from one AMF type to another. For example, pure cellulose obtained after proper decomposition increases asymbiotic AMF extraradical hyphae growth and root colonization (18, 65).

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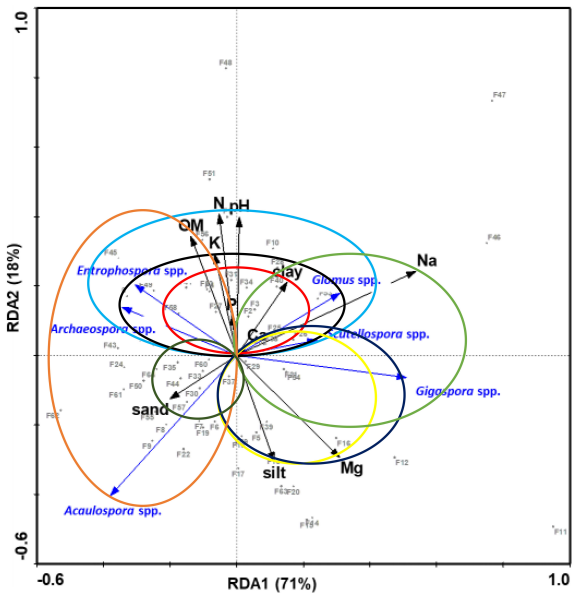


FIGURE 4
RDA (canonical correlation analysis) biplot of the 6 arbuscular mycorrhizal fungi genera (*Glomus, Scutellospora, Gigaspora, Acaulospora, Archaeospora* and *Entrophospora*) and soil properties from the sampled fields. The circle's diameter is equal to the length of the soil property's arrow. Genera lines that end in that circle have a positive regression for that soil property. The length of the arrow indicates increasing influence and the smaller the angle between the two arrows indicates closer relationships.

Soil texture has been reported to alter colonization of AMF (8, 66) depending on the sporulation patterns. The general soil textural classes in the present study were sandy clay loam and sandy loam which are reported to favor mycorrhizal development (67). Gigasporaceae (*Gigaspora* spp. And Scutellospora spp.) dominate in sandy soils (68) and they are also indicators of soils with lower clay content (69). *Glomaraceae* and other AMF families with small spores do not show any strong dependence on soil characteristics (70). However, Lekberg et al. (68) also reported that *Scutellospora cerradensis (Sc. Rtl)* was the only member of Gigasporaceae that occurred predominantly in clayey soil (containing 41:11:48 of clay, silt, and sand). From the same study they reported that Glomaraceae colonized roots well in sandy soil (containing 3:7:90 of clay, silt, and sand), clayey soil (containing 41:11:48 of clay, silt, and sand) and sand/clay mixture (containing 4:1 v/v). There's a possibility that the difference in biomass allocation and the growth patterns of extraradical hyphae of *Gigasporaceae* and *Glomaraceae* are affected by soil texture (71, 72).

Most importantly, the soil pH was within a favorable range (5.85-6.64) for fungi and supported sporulation of especially, *Glomus, Scutellospora, Entrophospora*, and *Archaeospora*, and negatively affected the sporulation of *Acaulospora* and *Gigaspora*. Earlier, Muchane et al. (28) reported that AMF were favored by pH 5.51 to 6.67, while Dobo et al. (73) reported pH 6.18 to 6.28 to increase AMF sporulation in agricultural soils. However, it was expected that *Acaulospora* spp. Would be positively affected by the present pH since Acaulosporaceae are tolerant to acidic tropical soils (26, 74) and they highly sporulate under conventional tillage (a common practice in the sampled sites) as compared to other AMF families (31). For example, *Acaulospora laevis* is predominat in low pH soils and germinates well at pH 4-5 while *Gigaspora* heterogama isolated from warm climates and maintained in tropical areas showed varying germination rates (8 to 78%) in the same environmental conditions (75).

The six AMF genera identified in this study were present in all the AEZs and CTs but differences in their spore production were not significant. The AMF genera observed in this study were lower than 12 isolated in Sudan (76), 15 in Ethiopia (24), 15 in Southern China (77), 9 in Ethiopia (73) but higher than the 4 to 5 genera isolated in Kenya (23, 28, 34) and 4 in Rwanda (78). This may be due to variances in edaphic conditions and the cropping systems of the study sites. However, *Glomus*, *Acaulospora*, *Scutellospora*, and *Gigaspora* reported in this study were also observed in the studies carried out in the Sub-Saharan Africa (SSA) countries mentioned above. Incidentally, there were no new AMF genera identified, which alludes to low composition in these AEZs with the mean spore abundance of only *Entrophospora* spp. In SEKLB being significantly higher than in MEHF.

Most of the commercial mycorrhizal bioinoculants available in SSA are imported, expensive, and majorly contain *Glomus* species. During efficacy testing of these strains, the environmental conditions of the origin of the strains and where the bioinoculant is to be used may not be compared (79) yet it is important for the adaptability of AMF in the different local SSA edaphic and climatic conditions (80). The population of the introduced strains must build up for their increased competitiveness and effectiveness. In Tchabi et al. (81), AMF from Tropical Africa (*Glomus hoi*, *Acaulospora spinosa*, *Glomus mosseae*, *Glomus etunicatum*, and *Acaulospora scrobiculata*) averagely led to increased yam tuber growth by 51, 49, 38, 38 and 31%, respectively. Whereas exotic species from Europe were less efficient except for the three isolates of *G. clarum* isolates which increased tuber yield by 8.8, 15.6 and 55.6%, respectively compared to non-mycorrhizal control. Additionally, soil (from yam field) increased tuber yield by 40, 33, and 20% compared to exotic *G. constrictum*, the non-mycorrhizal control, and the exotic *G. luteum* confirming the existence of superior native AMF species in yam producing regions. Therefore, to overcome the problem of local adaptability of imported species and cost implications, mycorrhizal bioinoculants can be produced locally from the native species observed across different locations in SSA. The physiological characteristics of the species determine to a greater extent their survival and activity in the soil. Hence, different species will show varying responses, in terms of survival and activity. The ability of AMF to enhance root surface area by hyphal growth and provide an extra route for uptake as mycorrhizal pathway (82) depends on the AMF strains colonizing the plant roots. The efficiency of AMF strains is influenced differently by their development and activity of the external hyphae, hyphal transport rates, and solute interchange at the arbuscule-host root cell interface (83, 84). Therefore, the mixing of different compatible AMF strains during bioinoculant production will promote the complementary benefits of AMF strains in crop production.

Plant-host generalist AMF strains should be considered for bioinoculant production as compared to plant-host specialist AMF strains since during fallowing the latter can colonize weeds or new

crops which eventually can serve as a source of additional spores (85). The compatibility of the sporogenous *Glomus* and *Acaulospora* species with different crops and environmental conditions require further testing for suitable local bioinoculant production. However, *Acaulospora* spp. Would greatly influence crop production in acidic tropical soils since the *Acaulosporaceae* species are tolerant to acidic soils (26, 74); highly effective in P-uptake and transfer to the host plant compared to *Glomeraceae* species (86); and they highly sporulate under conventional tillage (a common practice in sweet potato producing areas) as compared to other AMF families (31).

*Glomus* and *Acaulospora* species can be further identified using molecular techniques and their compatibility with different crops and environmental conditions in varying dosage tested for suitable local bioinoculant production. To reduce the cost of soil fertility amending inputs in crop production, bioinoculants containing the most effective species can be integrated with reduced rates of inorganic fertilizers.

## 5 Conclusion

A total of six AMF genera comprising of *Glomus, Acaulospora*, *Scutellospora*, *Entrophospora*, *Archaeospora*, and *Gigaspora* were isolated from the study sites. The composition and spore abundance of AMF recorded in the AEZs and CTs in the major sweet potato growing areas had limited significant differences due to the similar agricultural practices employed by farmers. The effect of soil parameters on AMF spore abundance varied from genera to genera, however, the strongest influence was by OC, N, pH, silt, Mg and Na. The most dominant AMF i.e., *Glomus* and *Acaulospora* species can be isolated for local bioinoculant production.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

## Author contributions

RM conceived the research idea and conducted the research, collected, and analyzed the data, and co-wrote the manuscript; JT, PE, and CM assisted with conceiving the research and critically reviewed the manuscript. All authors contributed to the article and approved the submitter version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential competing interest.

## Publisher's note



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# Differences in microbial community structure and metabolic activity among tea plantation soils under different management strategies

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**Introduction:** Microorganisms play an important role in the multifunctionality of soil ecosystems. Soil microbial diversity and functions have a great impact on plant growth and development. The interactions between tea trees and soil microbiota can be linked with planting patterns and management strategies, whose effects on soil microbial community structure and metabolites are still unclear.

**Methods:** Here we used amplicon sequencing and metabolomic analysis to investigate the differences in soil microbial composition and metabolites among three tea production systems: organic, non-organic, and intercropping.

**Results:** We detected significant differences among the three systems and found that Firmicutes, Proteobacteria, Acidobacteriota, Actinobacteriota and Chloroflexi were the main bacteria in the three soil groups, although they varied in relative abundance. *Acidobacteria* bacterium increased significantly in the organic and intercropping groups. For fungi, Ascomycota and Basidiomycota were the main differential fungal phyla. Fungi alpha-diversity in the non-organic group was significantly higher than that in the other two groups, and was correlated with multiple soil physical and chemical factors. Moreover, network analysis showed that bacteria and fungi were strongly correlated. The changes in soil microorganisms caused by management and planting patterns may affect soil quality through corresponding changes in metabolites. Metabolomic analysis showed differences in metabolite composition among different groups. It was also found that the arachidonic acid metabolic pathway was affected by changes in soil microorganisms, and may further affect soil quality in an essential manner.

**Discussion:** Planting patterns and management strategies may significantly affect soil microorganisms and therefore metabolites. Changes in soil microorganisms, especially in fungi, may alter soil quality by affecting soil physicochemical properties and metabolites. This study will provide new insights into soil quality monitoring from a microbiological perspective.

KEYWORDS

**tea plant, management strategy, soil quality, soil microbial community, soil metabolite**

# 1. Introduction

Tea (*Camellia sinensis* L.), belonging to the family Theaceae, is an evergreen shrub or small tree whose leaves and leaf buds are used to produce tea (Kui et al., 2021b). Tea has become one of the most popular beverages in the world, with multiple health benefits (Trevisanato and Kim, 2000; Perez-Burillo et al., 2021; Bag et al., 2022). Tea tree is one of the most important economic crops in China. In 2019, tea planting area in China reached approximately 3.1 million hectares, with a total yield of 2.78 million tons (Xie et al., 2022). To maintain high yield and quality, chemical fertilizers, particularly nitrogen fertilizers, have been widely used. However, the long-term excessive application of fertilizers exerts negative impacts on soil and plants, leading to soil acidification, nutrient loss, and decreased tea quality (Yang et al., 2018; Wang et al., 2020). To address these problems, the application of organic fertilizer has become one of the most important agricultural practices in tea plantations these days (Huang et al., 2022; Ye et al., 2022).

Soils are a vast reservoir of biodiversity, containing myriad life forms that are essential to the functioning of ecosystems (Nielsen et al., 2015; Mishra et al., 2023). Rapid advances in high-throughput sequencing technology have deepened our understanding of the composition and functional roles of soil microorganisms. The soil microbial community governs the biogeochemical cycling pertaining to macronutrients, micronutrients, and other elements vital for the growth of plants and animals (Jansson and Hofmockel, 2020). It is influenced by and interacts with environmental factors, such as minerals, nutrients, redox conditions, and organic carbon composition, which may alter microbial diversity and richness (Jansson and Hofmockel, 2020). Changes in the composition and function of microbial communities can also influence the biogeochemical processes of carbon flow, further accelerating or mitigating climate change (Naylor et al., 2020). Studies have shown that any loss in microbial diversity will likely reduce the multifunctionality in terrestrial ecosystems, and damage ecosystem services such as nutrient cycling, soil fertility, primary production, and climate regulation (Delgado-Baquerizo et al., 2016; Kong et al., 2023; Wang et al., 2023).

In the past few years, attention has been diverted to the effects of plant-associated microbial community on plant growth and health (Pascale et al., 2019; Rai et al., 2023). The plant rhizosphere microbiome plays an important role in plant growth, yield, and disease resistance (Qu et al., 2020). Currently, various microbial taxa including beneficial bacteria and fungi, are used as biological fertilizers. They can improve plant nutrition by mobilizing or increasing the availability of nutrients in the soil, and thus have great potential to enhance soil fertility (Singh et al., 2008, 2010; Mitter et al., 2021). Microorganisms in the soil can improve soil fertility and provide nutrients for plants by decomposing litter as well (Hattenschwiler et al., 2005).

Applying exogenous organic matter helps to improve the balance and stability of soil microorganisms (Gryta et al., 2020). Changes in the levels of soil organic matter has the potential to alter bacterial microbiome, and thereby the macrophage activation of *Echinacea purpurea* root extracts (Haron et al., 2019). It was also found that using organic fertilizers can reinforce soil ability to suppress pathogenic fungi in the peanut rhizosphere (Chen et al., 2020). Overall, the application of organic fertilizer can promote microbial activities, enhance the synergistic effect within soil microbiome, increase the availability of soil organic matter and nutrients, and improve plant biomass (Zhang et al., 2019).

Tea planting systems depend highly on soil quality. The evaluation of soil quality under different management strategies and planting patterns is important for the production of organic tea. However, variations in soil microbial composition of different types of tea plantations and their due effects on soil quality are still unclear. In this study, we explored the microbial profiles and metabonomics of three soils of tea plantations: organic, non-organic, and intercropping to clarify the unique interactions between soil microbial community and metabolites, and their influences on soil properties, such as organic matter, total nitrogen, total phosphorus, and total potassium. This research will provide valuable insights into the improvement of soil quality in tea plantations through the use of microorganisms, and finally promoting tea plant growth.

# 2. Materials and methods

## 2.1. Soil sampling

Soil samples were collected from two tea plantations in Menghai County, Yunnan Province, southwestern China in August 2022. One plantation (latitude: 22°2′56″N, longitude: 100°37′48″E) was certified organic by Controllo e Certificazione Prodotti Biologici (CCPB, a renowned and professional inspection and certification body based in Italy for accrediting organic and eco-friendly production). *Docynia delavayi* trees (a wild fruit tree distributed in southwestern China) formed a natural intercropping system with tea trees in parts of the plantation. In the other plantation (latitude: 22°2′58″N, longitude: 100°37′44″E), non-organic practices were conducted, in which chemical fertilizers and pesticides were used. The two plantations were geographically close to each other. Three groups of soil samples were collected using a stainless steel spade from the following tea production systems of the two plantations: organic, non-organic, and intercropping. Soil of 10–20 cm deep and 5–15 cm near tea tree roots were taken. Each group included 10 samples. For each sample, five subsamples were collected in a zigzag pattern and mixed thoroughly. The well-mixed soil samples were carefully transferred to aseptic sampling bags and frozen at −80°C (Tedeschi and De Paoli, 2011) for further analysis.

## 2.2. Determination of soil characteristics

Soil pH was determined in a mixture of soil and water at a ratio of 1:5 (wt/vol) using pH strips (Zhang et al., 2019). Soil ammonia nitrogen ($NH_4$) and nitrate nitrogen ($NO_3$) were extracted with a 2 M KCL solution. Available potassium (AK) was determined by the atomic absorption method (Carter and Gregorich, 2007). Available phosphorus (AP) was determined based on the OD value at 880 nm by sodium bicarbonate extration, according to the Olsen method (Olsen, 1954). Total nitrogen (TN) was analyzed by fully burning each sample in a high-temperature reactor (Ma et al., 2017). Total phosphorus (TP) and total potassium (TK) were determined by NaOH molybdenum-antimony colorimetry method (Butkhup and Samappito, 2008). Organic matter (OM) was determined by a total organic matter analyzer (multi N/C 3100, Analytik Jena, Germany).

## 2.3. DNA extraction and sequencing

DNA was extracted from 0.5 g of soil using the Magnetic Soil and Stool DNA Kit (Tiangen, China) (Zhu et al., 2021). DNA concentrations were measured using a NanoDrop 2000-UV spectrophotometer (Thermo Scientific, Waltham, MA, United States). The 341 forward (5'-CCTAYGGGRBGCASCAG-3') and 806 reverse (5'-GGACTACNNGGGTATCTAAT-3') primers (Frank et al., 2013) were used to amplify the V3–4 region of the 16S rRNA gene, while the SSU0817 forward (5'-TTAGCATGGAATAATRRAATAGGA-3') and 1,196 reverse (5'-TCTGGACCTGGTGAGTTTCC-3') primers (Borneman and Hartin, 2000) were used to amplify the ITS1-F region of the 18S rRNA gene. PCR products were detected by 2% agarose gel electrophoresis. The target strip was recovered using a glue recovery kit (Qiagen, China). The library was sequenced using the Illumina NovaSeq sequencing platform. The raw sequencing data were uploaded to the public database National Center for Biotechnology Information (NCBI), with the accession number PRJNA983565.

## 2.4. Analysis of sequencing data

Raw tags were obtained by merging pair-ended reads using FLASH (V1.2.11, http://ccb.jhu.edu/software/FLASH/). Quality control was conducted on the raw tags using the fastp program to get high-quality clean tags, from which chimeras were detected and removed with Vsearch software (2.14.1) (Rognes et al., 2016). Then the DADA2 R package (Callahan et al., 2016) was used to denoise the sequences and generate amplicon sequence variants (ASVs) for further analysis. ASVs were later classified using the Naive Bayes classifier. Alpha-diversity values of the Shannon index and Chao1 index were calculated with the QIIME2 software (Bolyen et al., 2019). Bray–Curtis dissimilarity was calculated using the R-package vegan (v4.1.1) (Dixon, 2003) while PCoA analysis was performed using the ade4 R package (Dray and Dufour, 2007). LDA EFfect Size (LEfSe) (Segata et al., 2011) was conducted to identify differential markers between sample groups.

## 2.5. Data acquisition of metabolomic study based on liquid chromatography tandem mass spectrometry (LC-MS/MS)

One hundred mg of each soil sample was transferred to an Eppendorf tube and mixed with 1,000 μL of extraction solution (methanol: water = 3:1, isotope labeled internal standard). The mixture was homogenized at 35 Hz for 4 min and sonicated in an ice-water bath for 5 min (Alseekh et al., 2021). The homogenization and sonication cycle was repeated three times. The samples were incubated for 1 h at −40°C and centrifuged at 12000 rpm (RCF = 13,800 × g, $R$ = 8.6 cm) for 15 min at 4°C (Alseekh et al., 2021). The obtained supernatant fluid was transferred to a fresh glass vial for analysis. Quality control (QC) samples were prepared by mixing an equal aliquot of the supernatants from all soil samples.

A Vanquish UHPLC system (Thermo Fisher Scientific, United States) was used for this study (Wang et al., 2016). The target compounds were separated by an Acquity™ UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm). Eluent A was water containing 5 mmol/L ammonium acetate and 5 mmol/L acetic acid, while eluent B was acetonitrile. Column temperature was at 4°C and sample volume was 2 μL.

## 2.6. Soil metabolomic analysis

The original LC–MS/MS data were converted to mzXML format by ProteoWizard. XCMS was used for peak identification, peak extraction, peak alignment, and integration (Smith et al., 2006). Then BiotreeDB (V2.1) self-built secondary mass spectrum database was applied for material annotation. The cutoff value was set at 0.3. Deviations were filtered based on relative standard deviation (RSDS), namely coefficient of variation (CV). Only peak area data with no more than 50% null value in one group or no more than 50% hollow value in all groups were retained. Missing values in the original data were simulated. The numerical simulation method was used to fill in half of the minimum value. Then, the data were normalized to the internal standard peak intensity to generate a new data matrix. Partial least squares regression was used to establish the relationship model between metabolite expression and samples. Metabolites with a variable importance in projection (VIP) value >1 in OPLS-DA analysis and $p < 0.05$ in univariate analysis were considered significantly changed (Chong and Xia, 2018).

## 2.7. Statistical analysis

R software (v4.1.1) was used for statistical analysis. The Wilcoxon rank-sum test was used to compare differences in Shannon index and Chao1 index. PERMANOVA analysis was performed to assess differences in beta diversity between soil groups. Environmental indicators were statistically analyzed by $t$-test. Spearman correlation was used to investigate microbial metabolites and environmental factors. The significance threshold was set at $|r| > 0.6$ and $p < 0.05$. Network visualization and analysis were conducted using Gephi software (v0.9.2).

# 3. Results

## 3.1. Effects of different management strategies on soil physical and chemical properties

Differences in the main physical and chemical properties of soils under different management strategies were investigated. The results showed that the levels of TN, OM, AN (alkeline-N) and pH in intercropping group were increased significantly, followed by organic and non-organic groups. TK was significantly increased in organic group compared with non-organic group soil. Although AP and AK showed no significant difference among soil samples, the lowest values were found in the non-organic group (Table 1). These results revealed that soil characteristics may be affected by different management strategies and planting methods.

TABLE 1 Physicochemical properties of soils under different management systems.

| | TN (g/kg) | TP (g/kg) | TK (g/kg) | AN (mg/kg) | AP (mg/kg) | AK (mg/kg) | OM (g/kg) | pH |
|---|---|---|---|---|---|---|---|---|
| Organic | 1.020 ± 0.25$^{c}$ | 0.95 ± 0.22 | 7.02 ± 0.069$^{b}$ | 94.26 ± 13.45$^{c}$ | 1.31 ± 0.026 | 172.97 ± 8.67 | 33.41 ± 4.29$^{c}$ | 4.94 ± 0.059$^{c}$ |
| Non-organic | 4.24 ± 0.13$^{b}$ | 0.93 ± 0.032 | 10.85 ± 0.58$^{a}$ | 240.19 ± 3.9$^{b}$ | 1.66 ± 0.43 | 208.44 ± 51.18 | 110.461 ± 4.01$^{b}$ | 5.26 ± 0.041$^{b}$ |
| Intercropping | 6.35 ± 0.25$^{a}$ | 1.16 ± 0.01 | 7.15 ± 0.098$^{b}$ | 331.73 ± 8.67$^{a}$ | 1.61 ± 0.31 | 204.18 ± 44.84 | 155.58 ± 0.90$^{a}$ | 5.78 ± 0.149$^{a}$ |

Data within a column without shared letters indicate significant differences at $p < 0.05$. Data represent the mean ± standard ($n = 3$ biological replicates). TN, total nitrogen; TP, total phosphorus; TK, total potassium; AN, alkeline-N; AK, available potassium; AP, available phosphorus; OM, organic matter.

## 3.2. Effects of different management strategies on soil microbial communities

Considering the close relationship between soil characteristics and its microbial community, bacterial and fungal compositions of the three groups of soil were analyzed (four replicates for each sample group). A total of 761,015 and 928,360 high quality sequences were obtained in bacteria and fungi, respectively. The results of microbial annotation showed that Chloroflexi, Actinobacteriota, Acidobacteriota, Proteobacteria, and Firmicutes were the main phyla of bacteria, but their proportions vary among the three soil systems. The relative abundance of Acidobacteriota in non-organic group was higher than that in organic group, while Proteobacteria and Firmicutes were higher in intercropping group. The relative abundance of Firmicutes in organic group was lowest, while that of Actinobacteriota in organic group was highest (Figure 1A). For fungal composition at the phylum level, Ascomycota was dominant with the highest abundance in all three soil systems. Basidiomycota was mostly detected in organic group, while Mortierellomycota was mostly in intercropping group (Figure 1A). At the genus level, the microbial composition showed diversity among the three groups of samples. The top 10 genera of bacteria and fungi were analyzed (Figure 1B), among which *Streptococcus*, *AD3*, *Subgroup2*, *Veillonella*, and *Rothia* were the genera of bacteria that were abundant in soil. *Streptococcus* was most abundant in intercropping group, followed by non-organic and organic groups. *AD3* was dominant in organic group, while the abundance of *Subgroup2* was highest in non-organic group. *Hygrocybe* and *Fusarium* were the two fungi genera of highest relative abundance in organic group. In contrast, non-organic and intercropping groups were mainly dominated by *Archaeorhizomyces*, which had the highest abundance in intercropping group than in the other groups of soils. Besides, a certain abundance of *Mortierella* was detected in intercropping group.

To further explore the differences in bacterial and fungal community structure among different soil groups, principal coordinate analysis (PCoA) was performed (Figure 1C). We observed significant separation of fungal composition among the three types of soil, indicating that fungal community structure might be strongly affected by different strategies of soil management. By alpha-diversity analysis, we found no significant difference in bacterial diversity among the three groups (Figure 2A). However, significant differences were detected in fungal diversity, with non-organic group displaying the highest value (Figure 2B).

The numbers of shared and unique ASVs of bacteria and fungi of different soils are demonstrated in Venn diagrams. In terms of bacteria, 779 shared ASVs were detected among the three soils, with organic group having the most unique ASVs (2474) and non-organic group the least (1633) (Figure 2C). Regarding fungi, 265 shared ASVs were detected, with the largest number of unique ASVs in non-organic group (1021) and the smallest (410) in intercropping group (Figure 2D). The higher proportions of unique bacterial and fungal ASVs in each group revealed great differences among the three soils in microbial community structure.

## 3.3. Comparative analysis of microbial biomarkers of different soils

LEfSe analysis was used to identify microbial biomarkers, which showed significant differences in the species of bacteria (Figure 3A) and fungi (Figure 3B) among soils. In organic group, *Acidobacteria bacterium*, *bacterium Ellin515*, *Paraburkholderia caledonica*, *Spartobacteria bacterium*, and *Methylobacterium oxalidis* were the most abundant bacterial species. *Bathyarchaeia* and *Rudaea* were detected to be significantly enriched in non-organic group. *Steroidobacter*, *Nitrospirae bacterium*, *Acidobacteria bacterium*, *Spirochaeta* sp., *Xanthobacteraceae bacterium*, *bacterium MI-37*, *Hyphomicrobium facile* were significantly enriched in intercropping group. For fungal biomarkers, *Saitozyma podzolica* and *Penicillium alagoense* were significantly enriched in organic group, *Beauveria australis*, *Mortierella amoeboidea*, and *Mortierella minutissim* in intercropping group, while Agaricomycetes in non-organic group. In general, these microbial biomarkers may respond to planting patterns and management strategies to varying degrees, leading to the differences among soil samples.

## 3.4. Correlation analysis between fungi, bacteria, and environmental factors

To investigate fungal-bacterial interactions in tea plantation soils, the three groups of soils were mixed, and a correlation network analysis ($|r| > 0.7$, $p < 0.05$) was performed (Supplementary Figure S1A). Overall, the network consists of 146 nodes. Fungi involved 94 nodes (64.38%) while bacteria nodes accounted for only 35.62%, indicating that the network was dominated by fungal activities. The proportion of positive correlation was 59.24%, and that of negative correlation was 40.76%, revealing predominantly synergistic interactions within the bacterial-fungal community. The topological role of each ASV in the microbial network was demonstrated in a Zi-Pi plot to investigate the bacterial and fungal co-occurrence in tea plantation soils (Supplementary Figure S1B). We found that most ASVs were categorized as connectors, indicating a high degree of connectivity in symbiotic interactions between the bacterial and fungal communities. We thus assume that there may be strongly interacted species within the co-occurrence network, which may contribute to the stability of the network itself.

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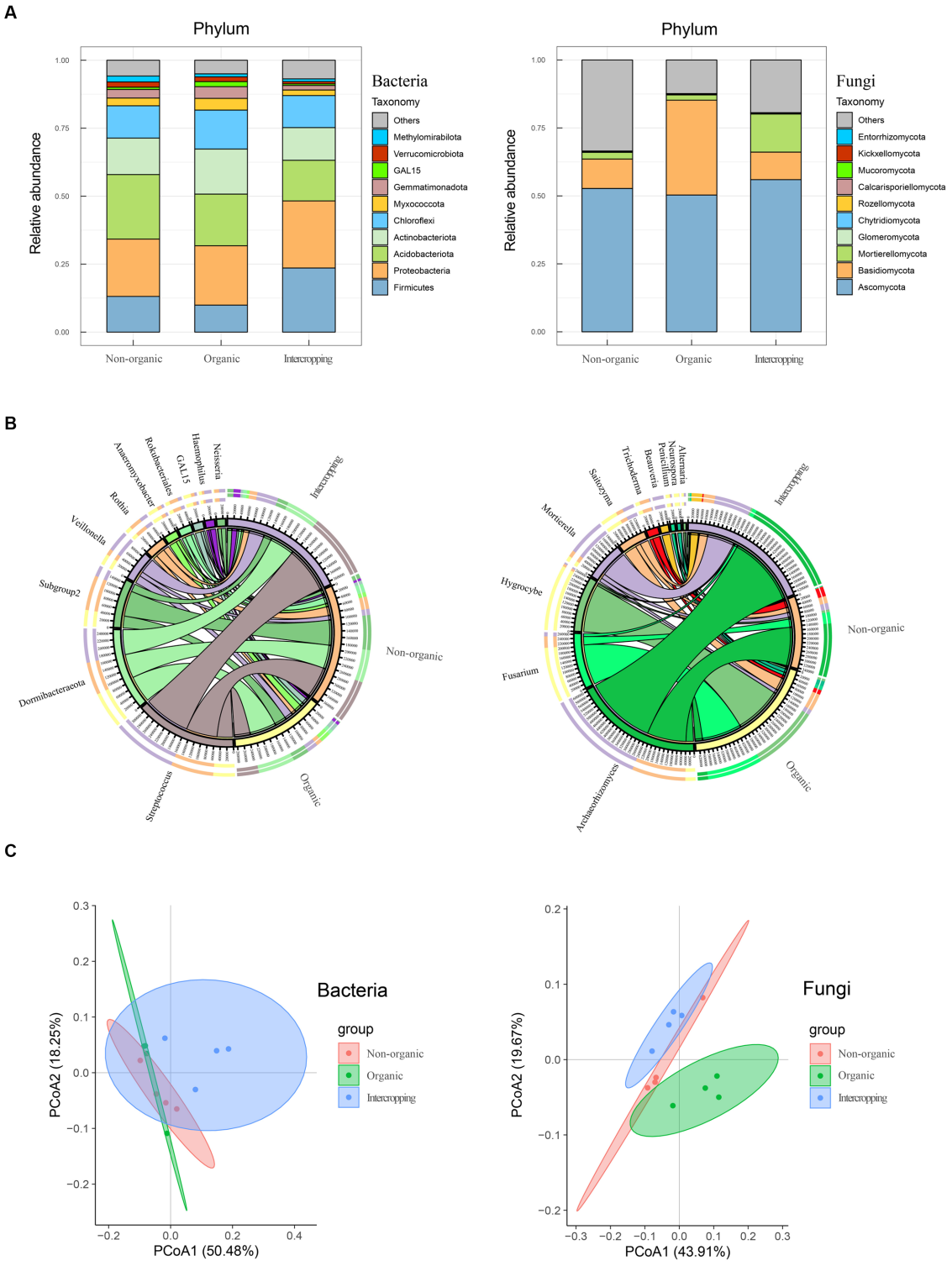


FIGURE 1
Composition analysis of soil microorganisms. Bacterial and fungal community compositions in organic, non-organic and intercropping groups at phylum **(A)** and genus **(B)** levels. Bacterial and fungal principal component analysis based on Bray–Curtis distance matrix **(C)**.

Mantel test analysis was used to explore the relationship between soil microbial community and physical and chemical parameters. The results showed that soil physical and chemical properties were mostly positively correlated with each other, which had the most significant effect on the fungal community. Bacteria, however, responded poorly to soil physical and chemical changes (Figure 4).

## 3.5. Soil metabolite patterns and differential analysis

Non-targeted metabolomic analysis was performed to unravel metabolic characteristics of different soils, and a total of 2,617 metabolites were identified. PCA was performed to establish the

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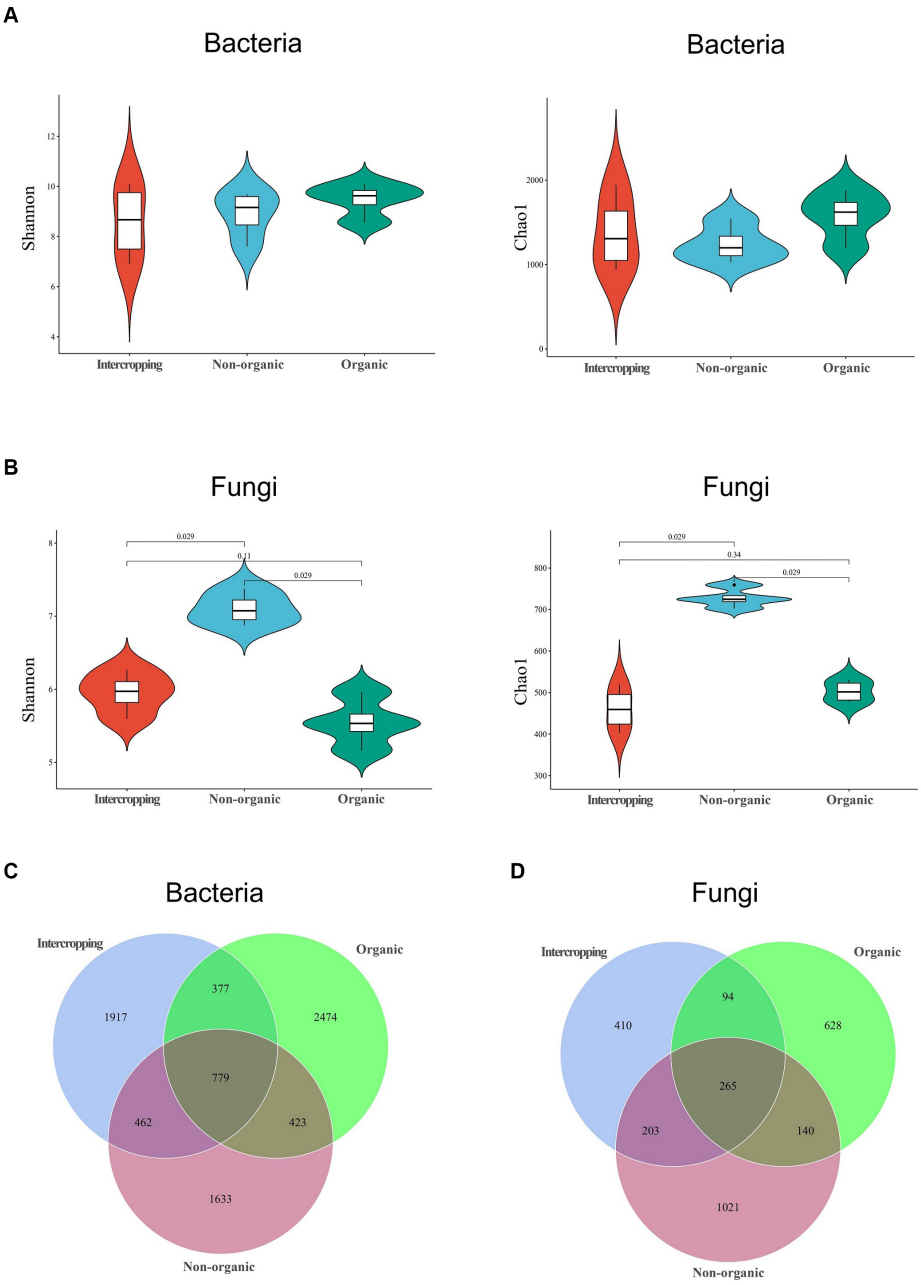


FIGURE 2
Microbial diversity in soils under different management systems. Diversity analysis of bacteria **(A)** and fungi **(B)** using the Shannon and Chao1 indices. Venn diagram analysis of bacterial **(C)** and fungal **(D)** species in the three soils.

relationship between metabolite expression and soil samples (Figure 5A). An obvious separation was observed, indicating differences in the abundances of metabolites in soils managed under different systems.

Metabolites identified in the three soils overlapped extensively. The main metabolites included lipids and lipid-like molecules, organic nitrogen compounds, organoheterocyclic compounds, organic oxygen compounds, and organic acids and derivatives, although slight differences in metabolite abundances among the soils were observed (Figure 5B). A metabolite interaction network showed that the interaction patterns of metabolites were mostly positive (78.54%), with 9,10-epoxyoctadecanoic acid, (9xi,10xi,12xi)-9, 10-dihydroxy-12-octadecenoic acid, palmitoyl serinol, sorbitol, maslinic acid, and kojibiose showing high degrees of connectivity (Figure 5C). The highest number of differential metabolites were detected between intercropping and non-organic groups, while the lowest number between intercropping and organic groups. A total of 23 overlapping metabolites were found among the three groups of soil (Figure 5D). Most metabolites were increased in organic and intercropping groups, especially acetoacetic acid, kojibiose, and deoxyguanosine, which were significantly concentrated in the two soils (Supplementary Figures S2A,B). KEGG enrichment analysis was performed on differential metabolites between organic and non-organic groups, and intercropping and non-organic groups,

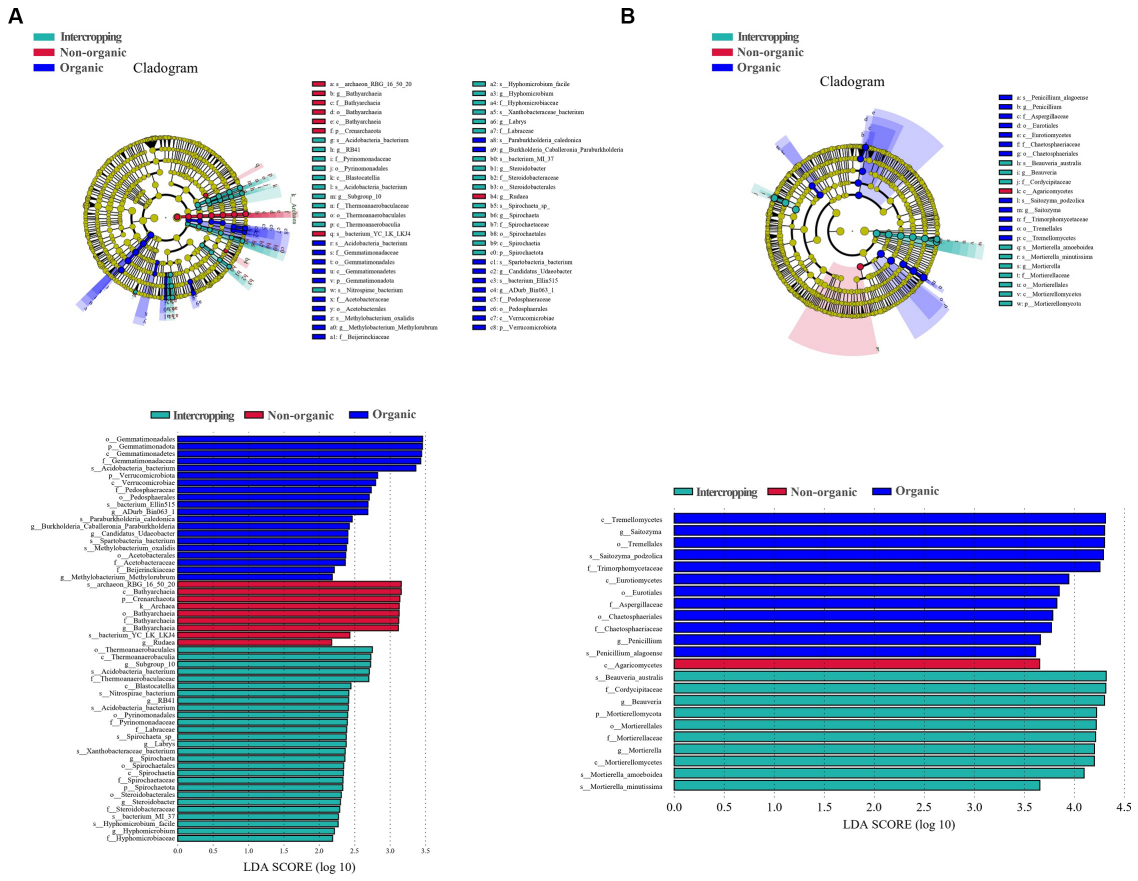


FIGURE 3
Differential microbiological analysis among different groups. LDA EFfect Size (LEfSe) analysis of soil bacteria groups **(A)**. LEfSe analysis of soil fungi groups **(B)**.

respectively. It was found that the expression of ABC transporters was higher in organic and intercropping groups than in non-organic group. We also found significant differences in arachidonic acid metabolism, linoleic acid metabolism, and other metabolic pathways (Supplementary Figures S2C,D). Changes in these metabolic pathways may be one of the factors contributing to the differences in soil fertility under different management systems.

## 3.6. Regulatory network of soil differential metabolites, microorganisms, and environmental factors

A co-occurrence network was constructed based on bacterial-fungal communities, differential metabolites, and environmental factors of the three soils (Figure 6). *Bathyarchaeia* was negatively correlated with most metabolites and environmental factors, while *Steroidobacter* was positively correlated with metabolites. The fungus *Mortierella* was positively correlated with 9,10-epoxyoctadecanoic acid, hypogeic acid, 5-KETE, trehalose-6-phosphate, and other metabolites. The metabolite alkeline-N has high connectivity in the network and is strongly correlated with most factors. Soil physical and chemical properties such as pH, TN, and OM interact with most metabolites and microorganisms, and their changes may affect the composition of soil microorganisms and metabolites.

# 4. Discussion

Soil is one of the most important assets of planet earth, encompassing a large proportion of microscopic biodiversity, including prokaryotes and microscopic eukaryotes (Mishra et al., 2023). Most of the processes of nutrient availability and loss pathways in soil are mediated by microorganisms. In this study, we collected three groups of soil samples and explored their differences in physical and chemical properties, microbiome, and metabolite composition.

Tea cultivation intensity and duration have strong impacts on microbial community structure, microbial biomass and its functioning, likely through soil acidification and fertilizer addition (Han et al., 2007). Yan et al. (2020) found that the soil of tea plantations in China tended to become acidic, and the pH value of many sites dropped to less than 4.5, which was too acidic for tea growth, and may have adverse effects on soil microorganisms. In contrast, no significant soil acidification was observed in organic tea plantations. Data from several studies showed that fungi had a higher association with pH and were more susceptible to soil pH than bacteria. An increasing soil pH will significantly affect fungal community structure and total fungal biomass (Carrino-Kyker et al., 2016; Kui et al., 2021a). Fungal alpha and beta diversity had a greater effect on tea yield and quality than bacterial diversity (Tang et al., 2022). Plant growth may therefore be affected through changes in microbial community structure by altering soil pH.

It has been reported that soil microbial community structure and biological function can be improved by organic soil management,

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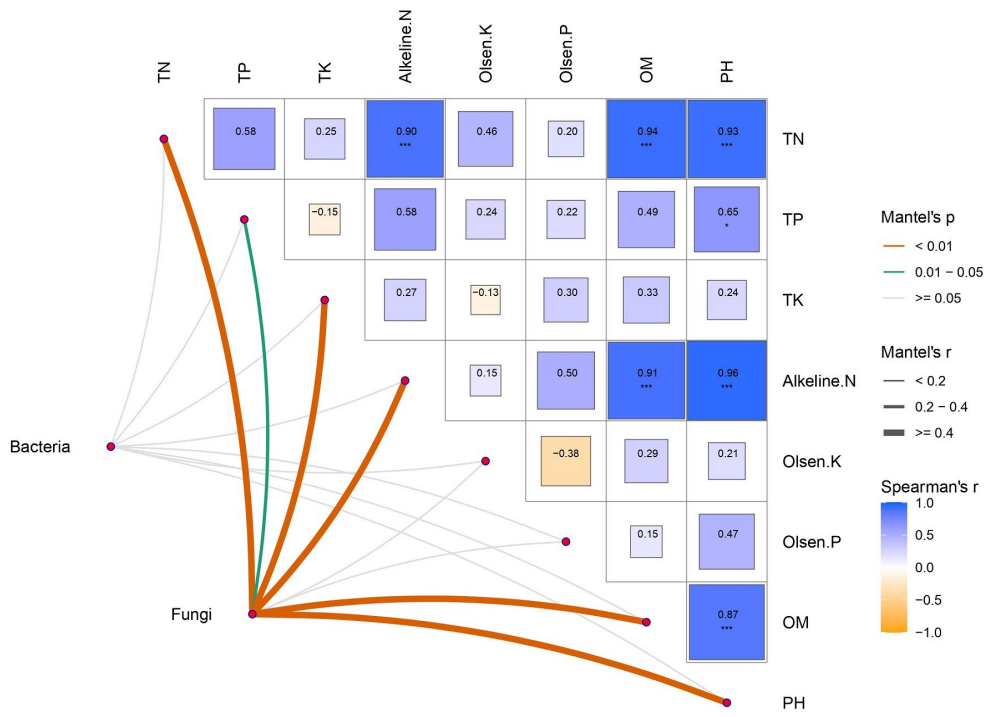


FIGURE 4
Correlations of soil microbial communities and physicochemical properties. Physicochemical properties are demonstrated in a heatmap constructed by Spearman correlation. Correlations between physicochemical properties and bacterial-fungal communities were determined using the Mantel test for correlation. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

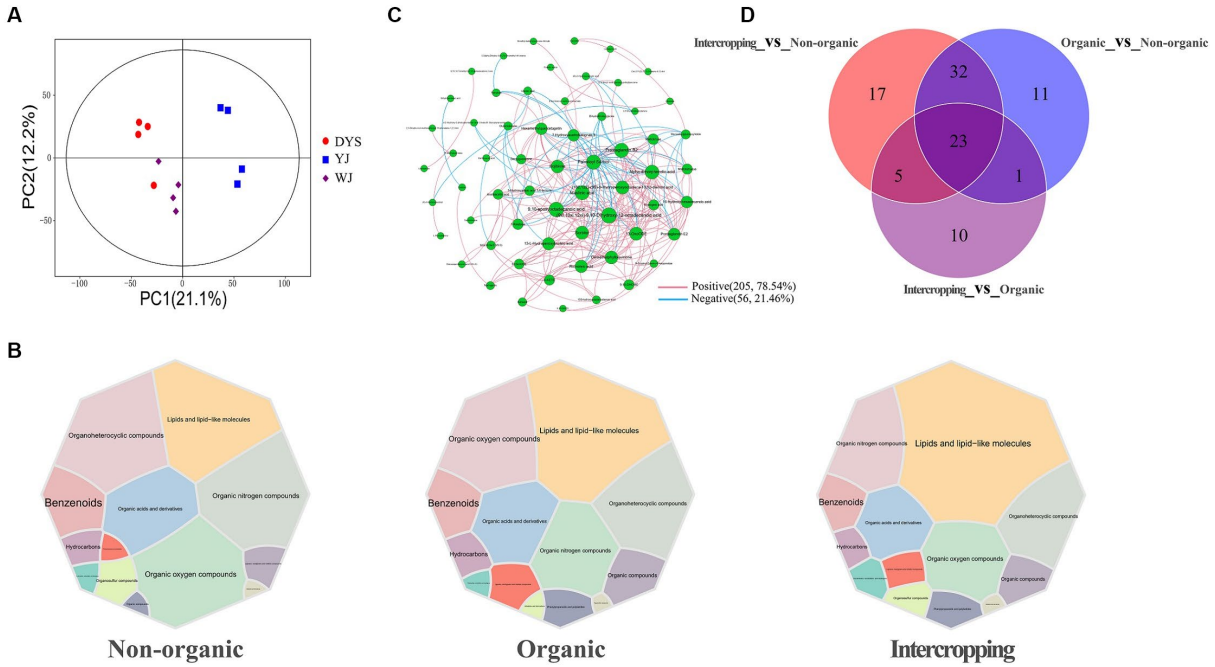


FIGURE 5
Analysis of metabolite patterns in different soils. **(A)** PCA analysis of soil metabolites in the three soils. **(B)** Main metabolites of the three groups of soils. **(C)** Correlation network analysis of soil metabolites. **(D)** Venn diagram analysis of soil differential metabolites.

such as the use of organic fertilizers (Diacono and Montemurro, 2010). In this study, the highest bacterial diversity was detected in organic soil, although not statistically significant, which indirectly reflects that organic soil management may provide a more suitable environment for bacterial reproduction, resulting in a higher bacterial diversity and abundance than other management strategies. In

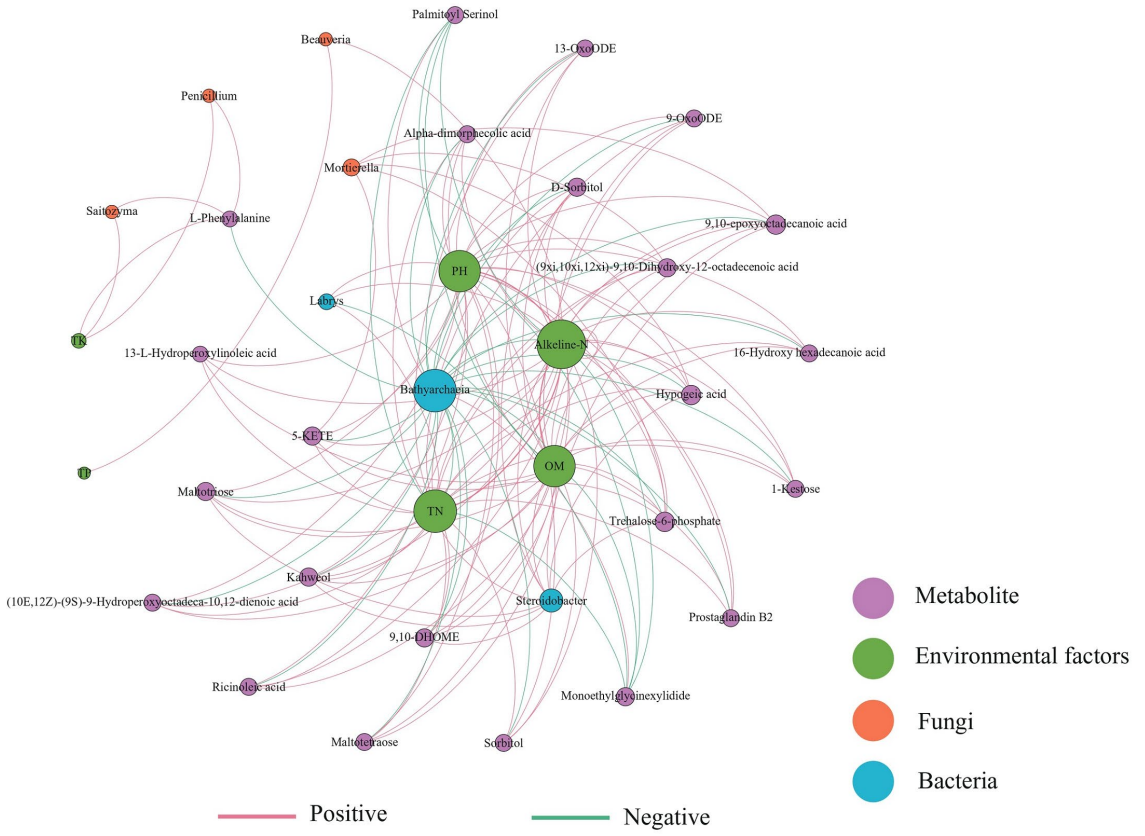


FIGURE 6
Correlation network of the soil physicochemical properties, soil differential metabolites, and microbial biomarkers (LDA score >2.0) (|Spearman's $r$| >0.6, $p < 0.05$). The red line indicates a positive correlation and the blue line indicates a negative correlation.

contrast, the diversity of fungi in non-organic group was much higher than that in organic and intercropping groups, which may be related to soil pH. Chloroflexi, Actinobacteriota, Proteobacteria, and Firmicutes were the main bacterial phylum in all the three soils, while Basidiomycota, Ascomycota were the main phylum of fungi. This result is generally consistent with previous studies (Tan et al., 2019; Naumova et al., 2021; Kui et al., 2021a; Aira et al., 2022; Liu et al., 2022). Furthermore, we detected *Streptococcus* in soil microorganisms, and its relative abundance was highest in intercropping group but lowest in organic group. *Streptococcus* is a group of pathogenic bacteria mostly detected in the intestinal tract of humans and animals and are associated with a variety of diseases (Peng et al., 2020; Zhao et al., 2022). The genera *Streptococcus* detected in soil has been reported to be heavy metal resistant, and increase with the accumulation of heavy metals (Li et al., 2020). On the other hand, *Streptococcus* has the ability to degrade hydrocarbons and improve the quality of contaminated soil (Aqeel et al., 2021). Acidobacteria *Subgroup2* was significantly positively correlated with the production of phosphatase and may be involved in the degradation of organophosphorus (Mason et al., 2021). It had a higher relative abundance in non-organic group, which may be explained by a lower abundance of organophosphorus in this soil. We assume that the relative abundance of Acidobacteria *Subgroup2* was increased to compensate for the organophosphorus loss in non-organic managed soil. In terms of fungi, higher abundances of *Fusarium* and *Hygrocybe* were identified in organic soil. The *Fusarium* genus comprises important saprophytic and phytopathogenic fungi and is widespread in nature (Zubi et al., 2021). It spends most of its life cycle in soil and interacts extensively with soil microorganisms (Mukjang et al., 2022). A higher abundance of *Fusarium* in organic group might be caused by a high carbon level in the soil, which can shelter its conidia and thus supports its growth and survival (Logrieco et al., 1995; Zubi et al., 2021). *Hygrocybe* is believed to be related to C and N cycles (Carron et al., 2020). Organic soil containing more *Hygrocybe* may be beneficial for soil carbon utilization. The relative abundance of *Archaeorhizomyces* in intercropping group was much higher than that in non-organic and organic groups. Previous studies have found that the relative abundance of *Archaeorhizomyces* in soil is positively correlated with the application of bioferfertilizer and may promote plant growth (Zhang et al., 2018). *Acidobacteria bacterium* are abundant in soil and are an important component of the soil microbial community (Kalam et al., 2020). We found that they were significantly enriched in organic and intercropping groups compared with non-organic group. Genomes of *Acidobacteria bacterium* encode a wide range of carbohydrate-active enzymes, which are involved in the decomposition, utilization, and biosynthesis of various carbohydrates (Dedysh and Sinninghe Damsté, 2018). Studies have found that the significant difference in the distribution of *Acidobacteria bacterium* among soils is mainly caused by the input of N and pH values (Liu et al., 2017). Therefore, we speculated that the enrichment of this bacterial species in organic group might be related to the high level of carbon and organic matter in the soil (Dedysh and Sinninghe Damsté, 2018). We also found that *Bathyarchaeia* was significantly enriched in non-organic soil and may be negatively correlated with a variety of soil

metabolites and environmental factors. *Bathyarchaeia* is closely related to soil pH, EC, and levels of $Na^+$and $Cl^-$ in salt-stressed soil (Wang et al., 2019). This suggests that *Bathyarchaeia* may play specific roles in regulating ecological functions in different soil environments. Some studies suggested that the improvement of soil fertility by organic fertilizer and soil regulator might decrease the relative abundance of the soil bacterium *Steroidobacter* (Wang et al., 2021). *Steroidobacter* was significantly detected in intercropping group in this study and was negatively correlated with a variety of metabolites. *Steroidobacter* may affect soil quality through the interactions with soil metabolites.

Metabolites in soil are mainly produced by plant roots and soil microorganisms (Kalu et al., 2021). A number of soil bacteria produce both volatile and soluble compounds, which likely play important roles in long-distance microbial interactions (Tyc et al., 2017). Study has found that Kojibios has a promoting effect on the growth of potential probiotic strains of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* (Garcia-Cayuela et al., 2014). It has been detected in soybean root exudates (Timotiwu and Sakurai, 2002), but its effect on plant soil remains unclear. Here we found that Kojibiose is the major differential metabolite in organic group and may be essential in the overall soil metabolic network. *Mortierella* has been reported to survive under unfavorable environmental conditions, promote plant growth, reduce chemical fertilizers and pesticides, and enhance crop yield (Ozimek and Hanaka, 2021). We found that *Mortierella* is positively correlated with the abundances of 9,10-epoxy octadecanoic acid, hypogeic acid, 5-KETE, trehalose-6-phosphate, and other metabolites. Trehalose metabolism in rhizobia is key for signaling plant growth, yield, and adaptation to abiotic stress, and its manipulation has a major agronomical impact on leguminous plants (Suarez et al., 2008). *Mortierella* has also been suggested to produce arachidonic acid (Botha et al., 1999). Organic acids and fatty acids were potential metabolites mediating the plant-bacteria interaction in the tea rhizosphere (Sun et al., 2022). The metabolic pathways of arachidonic acid and linolenic acid were detected to be different among soils. Studies have found that arachidonic acid is the main allelopathic substance affecting the interactions between the fungus Arbuscular mycorrhizal and bacteria (Lu et al., 2023). At the same time, arachidonic acid can also recruit beneficial microorganisms to the host rhizosphere to promote plant growth and soil nutrient turnover (Lu et al., 2023). Differences in the metabolic pathway of arachidonic acid among soils may be caused by varying microbial abundances, such as *Mortierella*, which may affect the growth and development of tea trees.

## 5. Conclusion

By exploring microbial and metabolite composition in soils of tea plantations under different management strategies, we detected significant differences in bacterial and fungal community compositions between organic, non-organic, and intercropping groups. Changes in soil pH might affect the composition of microorganisms, especially fungi. Soil metabolites are rich in lipids and lipid-like molecules, organic nitrogen compounds, and organoheterocyclic compounds, most of which are positively correlated. Changes in soil microbial community also affected the metabolic pathway of arachidonic acid, which is an important compound that influences soil quality. Importantly, we assume that soil quality of tea plantations may be influenced by varying microbial compositions through different metabolic pathways and their metabolites in the soil. This study will provide a basis for the improvement of soil fertility from the perspective of soil microorganisms by investigating the effects of microbial changes on soil quality and clarifying the underlying mechanisms.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA983565.

## Author contributions

GL and SZ wrote the manuscript. YH designed the experiments and revised the manuscript. JL and HM collected soil samples and assisted in interpreting results, and provided insights for writing the manuscript. GL, SZ, and YD analyzed the data and completed visualization. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

JL was employed by Xishuangbanna Luoboshanren Tea Co., Ltd.
YD was employed by Yunnan Pulis Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1219491/full#supplementary-material

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# Impact of altered groundwater depth on soil microbial diversity, network complexity and multifunctionality

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Understanding the effects of groundwater depth on soil microbiota and multiple soil functions is essential for ecological restoration and the implementation of groundwater conservation. The current impact of increased groundwater levels induced by drought on soil microbiota and multifunctionality remains ambiguous, which impedes our understanding of the sustainability of water-scarce ecosystems that heavily rely on groundwater resources. This study investigated the impacts of altered groundwater depths on soil microbiota and multifunctionality in a semi-arid region. Three groundwater depth levels were studied, with different soil quality and soil moisture at each level. The deep groundwater treatment had negative impacts on diversity, network complexity of microbiota, and the relationships among microbial phylum unites. Increasing groundwater depth also changed composition of soil microbiota, reducing the relative abundance of dominant phyla including Proteobacteria and Ascomycota. Increasing groundwater depth led to changes in microbial community characteristics, which are strongly related to alterations in soil multifunctionality. Overall, our results suggest that groundwater depth had a strongly effect on soil microbiota and functionality.

KEYWORDS

**semi-arid region, groundwater depth, soil multifunctionality, soil microbial community, relationship**

## 1. Introduction

The sufficiency of water resources is crucial for arid and semi-arid regions, as the availability of water is closely linked to vegetation productivity and energy cycling (Karthe, 2018; Ma et al., 2022). Direct and indirect accessibility of water are considered as potential mechanisms explaining plant drought tolerance (Barron-Gafford et al., 2021; Ma et al., 2022). For example, The ability of *Prosopis tamarugo Phil.* to survive in the extremely arid ecosystem of the Atacama Desert is attributed to its ability to extend roots to access groundwater (Garrido et al., 2016). Alterations of soil water resources availability is the dominant driver factor of restoration, sustainability, and interactions of soil nutrients and vegetation community (Chai et al., 2019; Condon et al., 2021; Reed et al., 2022). Drought can lead to soil fertility and nutrient loss, which reduces the support for microbial habitats and soil ecosystem services, ultimately resulting in decreased productivity (Wall et al., 2004; Schlaepfer et al., 2017; Jia et al., 2020). The simultaneous

occurrence of multiple ecological functions in an ecosystem should not be considered in isolation. Therefore, the use of integrated multifunctional indicators, such as soil multifunctionality that includes soil nutrients, soil water content, soil physical properties, soil temperature, soil pH, etc., will enhance our comprehension and prediction of the functions supplied by soils and habitats, as well as the responsiveness to biodiversity loss and ecological changes (Fanin et al., 2018; Garland et al., 2021).

Soil microorganisms play a vital role in driving nutrition utilization and biogeochemical cycling, supporting restoration of ecosystem (Bardgett and van der Putten, 2014; Delgado-Baquerizo et al., 2016; Crowther et al., 2019). Soil microbial diversity, composition, interactions, and functions are responsive to soil ecological alterations (de Vries et al., 2018; Guo et al., 2018). Desiccation induced by increasing groundwater depth has been shown to strongly modify the diversity of soil microbiota and functionality in different ecosystems (Hu et al., 2019; Zhang et al., 2020; Wu H. et al., 2021). Meanwhile, the soil microbial community is highly complex, comprising tens of thousands of species per gram of soil, and it should be noted that microbial diversity alone is insufficient to fully explain microbial functions (Van Der Heijen, 2008). Analyzing the relationships between individual microbial communities and their functional groups in co-occurring networks can reveal the interdependence arising from soil environmental heterogeneity (Banerjee et al., 2019). Network analysis can suggest whether microbial groups are more critical for maintaining network stability (Freilich et al., 2018; Zhang M. et al., 2023). The use of relevant metrics like betweenness and closeness centrality in assessing the complexity of microbial networks allows for the elucidation of the interconnectivity among operational taxonomic units (OTUs), which can be attributed to the heterogeneity of soil environmental conditions (Banerjee et al., 2019; Wagg et al., 2019; Martinez, 2023). This analysis provides insights into the influence of environmental factors on soil microorganisms and sheds light on the dynamics of microbial complexity, as well as how microbial interactions affect ecosystem function. Understanding the correlations between soil microbiota, soil multifunctionality, and drought (caused by groundwater depth increase) is crucial for the sustainability of ecosystems, as soil microbes are sensitive to soil quality.

Horqin Sandy Land, an ecologically fragile region, is one of the largest four deserts in northern China, with an area of approximately 139,300 $km^2$, of which the desertification area is as high as 71,884 $km^2$ (Wang, 2016; Luo et al., 2017; Zuo et al., 2020). The geomorphological features of the region are characterized by alternating sand dunes and slightly undulating lowlands (Luo et al., 2020). In recent years, expedited population growth and the resulting boosted requirement for food, housing, employment, and land have led to more ecological problems (Kooch et al., 2022), especially the decline of the ground water level (Maihemuti et al., 2021). As the temperature increases in the future, the potential enhancement in evapotranspiration will be more pronounced than that of precipitation, leading to more water shortage and exacerbating desertification or desertification in this region (Dai and Zhao, 2017; Su et al., 2018; Zhang Y. et al., 2023). The future changes in climate conditions may enhance the vulnerability of sandy ecosystems and strongly impact the biotic and abiotic processes in the soil environment. Groundwater depth may be a determinant regulating the changes in soil multifunctionality, soil microbial diversity and composition. Consequently, the objectives of our study were to systematically analyze the soil microbiota and soil properties in the agro-pasture crisscross region of the Horqin Sandy Land, considering variations in groundwater depth. We aimed to assess the responses of these soil factors to different groundwater depths and provide support for ecological revegetation and the implementation of groundwater conservation. Our research sought to explore the following objectives:

(1) investigate the effect of altered groundwater depth on soil multifunctionality, soil microbial diversity, and composition;
(2) analyze the co-occurrence networks and microbial functionality in different groundwater depth; and
(3) evaluate the relative contribution of different groundwater depth on soil microbiota characteristics, soil moisture, and multiple soil functions and their coupling relationship among the four.

# 2. Materials and methods

## 2.1. Study area

The study area is in the Horqin Sandy Land of eastern Inner Mongolia, China (42°55′N, 120°42′E). The elevation in our study site is 327 m. This region belongs to a typical temperate continental monsoon climate, with an annual average temperature of 5.8–6.4°C and an average annual precipitation of 351.7 mm. The spatial and temporal distribution of rainfall is uneven, with approximately 80% of the precipitation occurring from June to September (Huang et al., 2022).

The region of study is characterized by low soil nitrogen content, with levels ranging from 0.057 to 0.199 g/kg. The bulk density of the topsoil layer (0–30 cm) ranges from 1.29 to 1.59 g/$cm^3$ (Mao et al., 2012). The dominant vegetation species of this region are *Pennisetum centrasiaticum*, *Setaria viridis, Artemisia halodendron*, and *Caragana microphylla*. This study selected *Pennisetum centrasiaticum* and *Artemisia halodendron* because they are capable of surviving in various habitats, especially in areas with severe water shortage, and can help to maintain the stability of soil structure and prevent land degradation.

## 2.2. Experimental design

This experiment began on June, 2017 at the Naiman Desertification Research Station (Su et al., 2021). As shown in Figure 1, three groundwater depth levels and two main species were used in the experimental manipulations (*Pennisetum flaccidum Griseb.* and *Artemisia halodendron Turcz.et Bess.*), with six replicates per each treatment. In 1 $m^2$ concrete pools, an experimental operation was conducted, which included three treatment groups with groundwater depths of 50, 100, and 200 cm (Su et al., 2021). PVC shelters were set up to protect the cement pits from the rainfall. Establishment of a controlled experimental gradient was carried out to assess the effect of increasing groundwater depth on microbial diversity, network complexity, and multifunctionality of soil. Cement pits were buried to 1.0, 1.5, and 2.5 m according to each treatment gradient (Su et al.,

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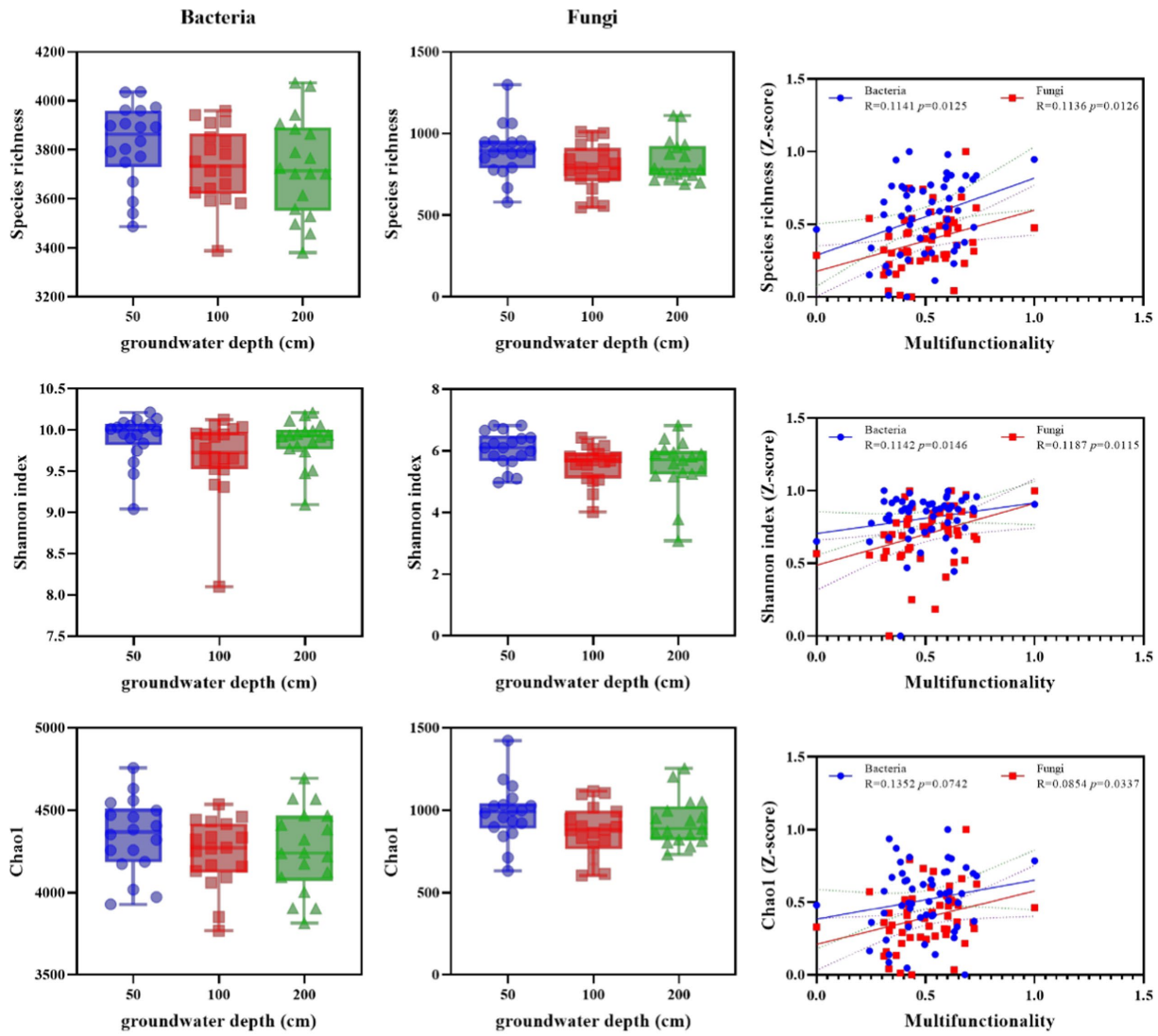


FIGURE 1
Alpha diversity of soil bacterial and fungal as altered by groundwater depth and the correlations of soil multifunctionality to alpha diversity. Diversity of bacterial and fungal communities (Observed species, Shannon index and chao1) in soils from 50, 100, and 200 cm groundwater depth plots, and the correlations of soil multifunctionality to diversity index. Box plots exhibit the first (25%) and third (75%) quartiles, and median, and the maximum and minimum observed values. Asterisks denote significant differences based on the Wilcoxon test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

2021). Stones with a diameter greater than 10 mm and a thickness of 10 cm were placed in cement pits, followed by sand to build the groundwater depth level. The bottom of each cement pit contained a 50 cm layer of stone–sand–water mixture. Adult individuals of *Pennisetum flaccidum Griseb.* and *Artemisia halodendron Turcz.et Bess.* were selected from the same sandy dune environment. The root system preserving the bud was excavated, and a root stem of equivalent diameter and length, possessing the bud, was selected. The selected rhizomes were then planted into the experimental pits.

## 2.3. Soil sampling and analyses

Sampling was conducted in August 2022. This study measured 11 soil variables, including soil moisture (SM), total nitrogen (TN), nitrate nitrogen (NN), ammonium nitrogen (AN), soil organic carbon (SOC), total phosphorus (TP), and pH in the 0–50 cm soil layer. The soil characteristics, such as nutrient availability, biogeochemical cycling, and microbial productivity, were assessed using these variables, and standard methods were employed for the measurements (Perez-Harguindeguy et al., 2013). Soil moisture (SM) was determined by oven-drying soil samples at 105°C to a constant weight. Soil organic carbon (SOC) and total nitrogen (TN) were quantified using the Walkley-Black and Kjeldahl method, respectively. total phosphorus (TP) was quantified through colorimetric analysis following digestion with sulfuric acid and perchloric acid, employing standard procedures. nitrate–nitrogen (NN) and ammonium–nitrogen (AN) were analyzed using a continuous flow analyzer (AutoAnalyzer-AA3, Seal Analytical, Norderstedt, Germany) following extraction with 2 mol/L KCl. Soil pH was measured in a soil:water extract (1:2.5) with a pH meter (Mettler Toledo, Germany). The C:N ratio was calculated as the mass ratio of SOC and TN, while the C:P ratio and N:P ratio were calculated

as the mass ratios of SOC and TP, and TN and TP, respectively. Soil multifunctionality (MF) was evaluated based on seven soil variables (TN, NN, AN, SOC, TP, SM, and pH) according to the method described by Fanin et al. (2018). This method has been widely employed in various studies (Maestre et al., 2012; Wang et al., 2019). Before analysis, the normality of the data was tested using the Shapiro–Wilk test. Non-normally distributed data was transformed by taking the logarithm or square root to approximate normality. For variables with negative values, the variable was transformed to positive values by subtracting the minimum value of the entire dataset. Variables were then standardized to a scale of 0 to 1 and the mean of these transformed values was taken as the functional value for each pit.

## 2.4. DNA extraction, illumina sequencing, and data processing

Soil microbes' DNA was extracted using FastDNA Spin Kits (Boaojingdian BiotECH Co., Beijing, China) and the primers 341F (5-CTAYGGGRBGCASCAG-3) and 806R (5-GGACTACNNGG GTATCTAAT-3) were used to amplify the V3-V4 regions of the 16S rRNA gene (Yu et al., 2005). The fungal community was targeted using the broad-spectrum primers ITS1 (5-CTTGGTCATTTAGAG GAAGTAA-3) and ITS2 (5-GCTGCGTTCTTCATCGATGC-3; Li et al., 2020a). The PCR reaction was performed using Phusion® High-Fidelity PCR Master Mix (New England Biolabs) with a total volume of 15 μL, 0.2 μM of each forward and reverse primer, and approximately 10 ng of template DNA. The PCR cycling conditions included an initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min (Liang et al., 2020). The amplicons obtained from triplicate PCR reactions were combined for each sample and analyzed by electrophoresis on a 2% (*w*/*v*) agarose gel. The PCR products were purified using a Qiagen Gel Extraction Kit (Qiagen, Germany). Libraries for sequencing, with sample index tags, were generated using the Illumina TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, United States). The quality of the library was evaluated using the Qubit® 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. The sequencing was performed on the Illumina NovaSeq platform, generating 250-bp paired-end reads at the Boaojingdian Company in Beijing, China. The Tax4Fun2 package was used for potential functional annotation and metabolic prediction. A total of 5,534,451 reads were obtained from 66 samples, ranging from 64,032 to 95,768 reads per sample.

## 2.5. Statistical analyses

The alpha diversity indices, namely the Shannon index, observed species, and Chao1, were computed using the Mothur software 8 (Montagna et al., 2018). The beta diversity among different groundwater depths was calculated using Mothur software, analyzed with Bray–Curtis dissimilarity matrix and ANOSIM function. The *p*-values were modified using Bonferroni correction in *anosim* function. The sorting of Bray-Curtis distance analysis (PCoA) was performed using the capscale function implemented in the vegan R package (Delgado-Baquerizo et al., 2019; Li et al., 2020b). Soil samples were gathered from different groundwater depths for co-occurrence network analysis. In each of the four groups, taxonomic units that constituted more than half of the samples were used to calculate Spearman correlation coefficients. Benjamini and Hochberg FDR correction was applied to control for false discovery. Statistically strong associations were detected with Spearman's $\rho > 0.8$ and FDR-adjusted $p < 0.05$ and included in the subsequent network construction (Benjamini and Hochberg, 1995). The topological parameters of the network, including the number of nodes, edges, betweenness centrality, and closeness centrality, were calculated using the igraph package (Lu et al., 2018). The impacts of soil properties on bacterial and fungal community composition and diversity at different groundwater depths were assessed using random forest and structural equation models (SEMs) in "randomForest" and "piecewise" packages, respectively (Li et al., 2020a; Kong et al., 2022). First, the relative contributions of groundwater depth, soil properties, and multifunctionality to bacterial and fungal species richness and community composition were measured using a random forest model, to identify the main response variables. Subsequently, a piecewise structural equation model was constructed to examine the direct and indirect impacts of groundwater depth, soil water content, and soil multifunctionality on the diversity and composition of bacteria and fungi. The Shipley's d-separation test was conducted to check any missing pathways in the model, and a value of p greater than 0.05 indicated no missing pathways. Standardized coefficients for each pathway in each component model were reported, as well as the Fisher's C statistic, AIC value, and BIC value for the entire model (Shipley, 2009; Kong et al., 2022).

All statistical analyses were performed using IBM SPSS Statistics (version 24) and R 4.0.5 (R Development Core Team, 2021).

# 3. Results

Significant decrease in groundwater level affected the physicochemical properties of soil and reduce soil multifunctionality (Table 1). Under deep groundwater conditions, the TN and SOC content, C:P, N:P, pH, and moisture in soil were all higher compared to shallow groundwater depths ($p < 0.05$). The content of NN increased initially and then declined as groundwater depth increased ($p < 0.05$). However, AN content was increased with the increasing groundwater depth ($p < 0.05$). Groundwater depth was negatively related to soil multifunctionality and soil multifunctionality was lower in deeper groundwater depth (Table 1).

Soil microbial diversity was significantly altered by increasing groundwater depth in bacteria and fungi (Figure 1). The observed bacterial and fungal species richness, Shannon index, and Chao1 were significantly lower in deeper groundwater depth than in shallower depth. Nevertheless, the association among soil microbial diversity and MF was significant and positive ($p < 0.05$). Thus, losses of soil microbial diversity in altered groundwater depth were related with downward transitions in soil environmental functions.

Groundwater depth had a significant effect on the structure of soil microbial communities (Figure 2). Principle coordinate analysis (PCoA) suggested that soil microbiota strongly clustered following groundwater depth at bacteria level, which explained 12.71–35.42% of the total variation (Figure 2). Our results demonstrated that groundwater depth heavily affected the soil microbiota.

TABLE 1 Effects of groundwater depth on soil properties.

| Properties | Groundwater depth | | | | | $R^2$ |
|---|---|---|---|---|---|---|
| | 50 cm | 100 cm | 200 cm | *F* | *p* | |
| TN (g/kg) | 0.3694 ± 0.0261a | 0.3633 ± 0.0161a | 0.2911 ± 0.0229b | 3.867 | 0.0273 | 0.1317 |
| NN (mg/kg) | 131.6 ± 11.07b | 145.8 ± 14.49a | 97.24 ± 12.59c | 4.979 | 0.0106 | 0.1634 |
| AN (mg/kg) | 15.68 ± 0.7663b | 16.72 ± 1.0024b | 21.47 ± 0.6641a | 14.04 | 0.001 | 0.3551 |
| SOC (g/kg) | 3.619 ± 0.2573a | 3.689 ± 0.2146a | 2.585 ± 0.2175b | 7.182 | 0.001 | 0.2198 |
| TP (g/kg) | 0.2039 ± 0.0054a | 0.2022 ± 0.0062a | 0.1967 ± 0.0074a | 0.3579 | 0.7079 | 0.0134 |
| C:N ratio | 10.68 ± 1.016a | 10.20 ± 0.4465a | 10.02 ± 1.377a | 0.1118 | 0.8944 | 0.0043 |
| C:P ratio | 17.78 ± 1.231a | 18.84 ± 1.504a | 13.24 ± 1.148b | 5.218 | 0.0087 | 0.1699 |
| N:P ratio | 1.808 ± 0.1188a | 1.843 ± 0.1065a | 1.525 ± 0.1476b | 1.843 | 0.1687 | 0.0674 |
| SM % | 4.808 ± 1.222a | 2.120 ± 0.3607b | 1.593 ± 0.1102c | 5.449 | 0.0072 | 0.1761 |
| pH | 8.329 ± 0.0485a | 8.108 ± 0.0637b | 7.861 ± 0.0686c | 14.81 | 0.001 | 0.3674 |
| MF | 0.6269 ± 0.0427a | 0.5493 ± 0.0341b | 0.3916 ± 0.0329c | 10.59 | 0.001 | 0.2935 |

Different lowercase letters in the same row suggest significant differences at $p < 0.05$. TN, total nitrogen; NN, nitrate nitrogen; AN, ammonium nitrogen; SOC, soil organic carbon; TP, total phosphorus; SM, soil moisture; MF, multifunctionality of soils.

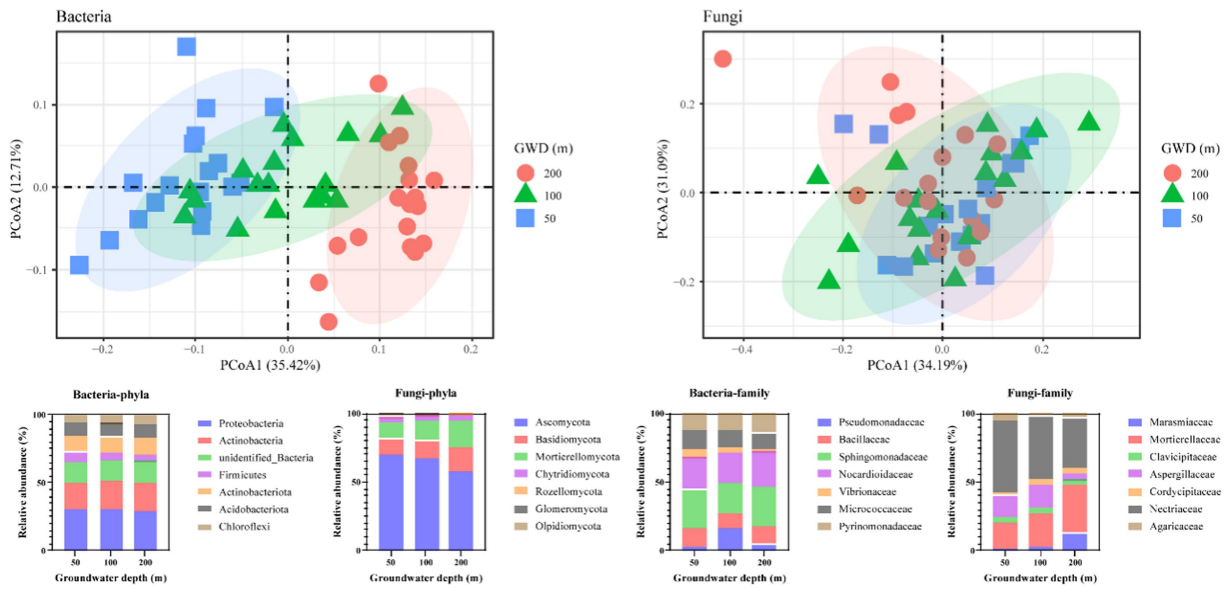


FIGURE 2
Soil microbial composition as influenced by groundwater depth on bacteria and fungi levels. Principal Coordinate Analysis (PCoA) plot of Bray–Curtis distances among groundwater depths (GWD = 50, 100, 200 cm); the relative abundance (%) of the phyla and family levels in soil dominant bacterial and fungal communities in three groundwater depth treatments.

At the phyla level, the bacterial communities in the soil were primarily composed of Proteobacteria (29.86%), Actinobacteria (20.14%), Firmicutes (5.98%), Actinobacteriota (12.04%), Acidobacteriota (9.81%), and Chloroflexi (6.18%), while about 15.42–16.57% of the bacteria were unidentified; soil fungal communities were analyzed and found to be mainly composed of members of Ascomycota (65.18%), Basidiomycota (13.85%), Mortierellomycota (15.14%), Chytridiomycota (3.77%), Rozellomycota (0.71%), Glomeromycota (1.01%), and Olpidiomycota (0.19%), and these phyla accounted for more than 85% of the relative abundance of all identified phyla (Figure 2). With increasing groundwater depth, the relative abundance of Proteobacteria decreases in bacteria, while the relative abundance of Ascomycota decreases in fungi.

At the family level, Sphingomonadaceae (26.22%), Nocardioidaceae (23.71%), Bacillaceae (12.56%), Micrococcaceae (12.28%), and Pyrinomonadaceae (13.08%) emerged as a dominant family in the bacterial communities, constituting over 85% of the total bacterial sequences (Figure 2). The relative abundance of Sphingomonadaceae, Nocardioidaceae, and Bacillaceae was declined first then increased with the increasing groundwater depth. The dominant fungal family was Nectriaceae (44.71%), which was followed by Mortierellaceae (26.29%), Aspergillaceae (12.19%), Marasmiaceae (5.78%), Clavicipitaceae (3.96%), Agaricaceae (3.61%), and Cordycipitaceae (3.44%). The relative abundance of Nectriaceae and Aspergillaceae decreased strongly with growing groundwater depth, whereas the relative abundance of

Mortierellaceae and Marasmiaceae boosted significantly with increasing groundwater depth.

The soil microbial community network exhibits apparent co-occurrence patterns (Figure 3). Groundwater depth had a negative influence on the complexity of soil bacterial co-occurring network, but increased the complexity of fungi (Figures 3C,D). For bacteria, the betweenness and closeness centrality were significantly greater in deeper depth than others, and the closeness centrality of fungi was significantly greater in 100 cm groundwater depth, indicating that the soil microbial network was less robust in the deeper groundwater depth than in the shallower (Figures 3E,F). These results suggested that increased groundwater depth impacted microbial correlations, and decreased the complication of soil bacterial community networks. Additionally, the effects of groundwater depth on soil microbial co-occurrence network were closely related to community composition and sensitive strains.

Forty-two potential metabolic functional groups of the bacterial communities were predicted using Tax4Fun2 package based on KEGG pathway (Level 2) analysis (Figure 4). Increased groundwater depth decreased the values of most bacterial metabolic functional groups. For instance, the relative abundances of groups related to Amino acid metabolism, Biosynthesis of other secondary metabolism, Energy metabolism, Nucleotide metabolism, and Xenobiotics biodegradation and metabolism decreased after

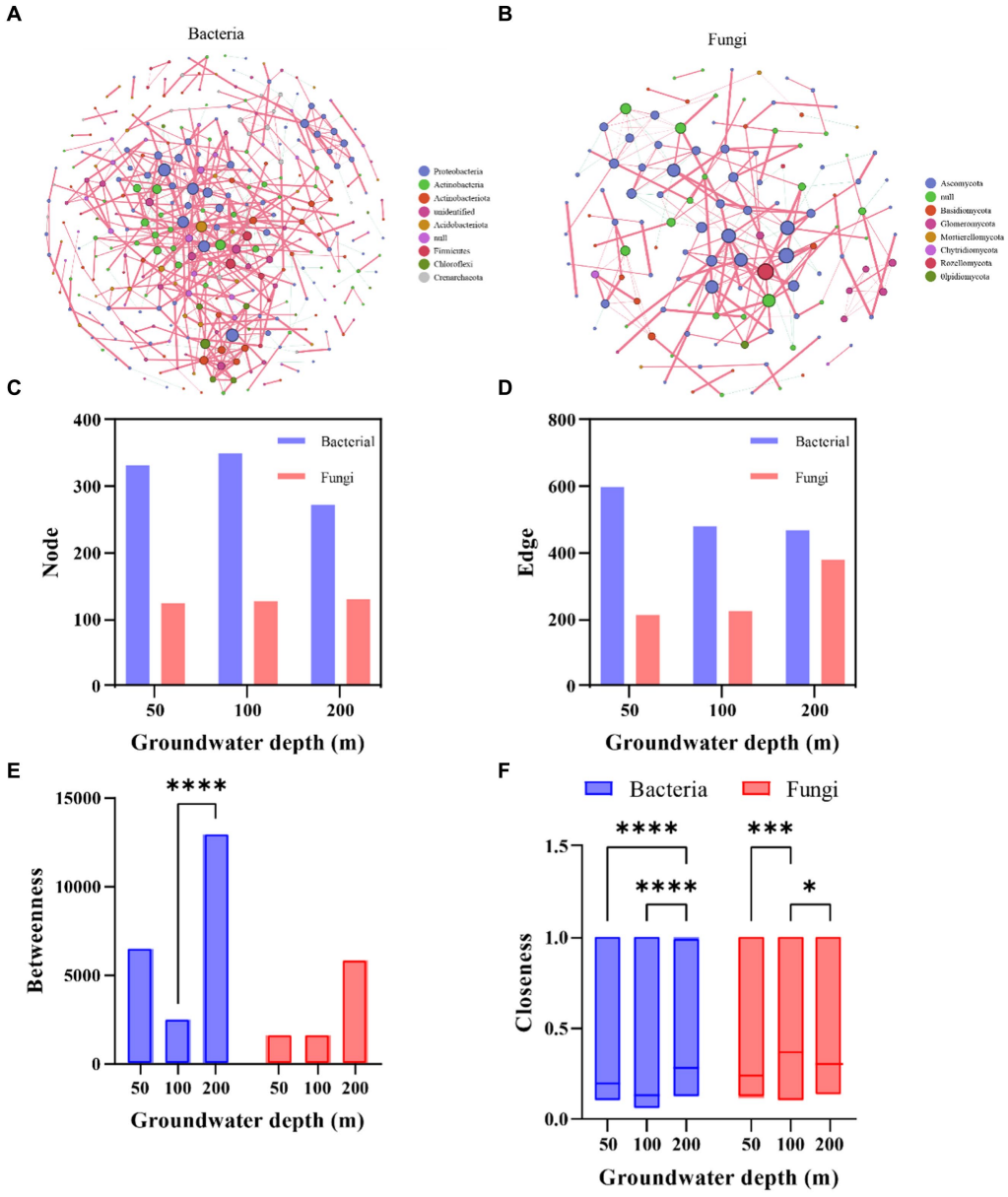


FIGURE 3
Co-occurrence networks for bacteria and fungi in different groundwater depth. Nodes in the network correspond to amplicon sequence variants (ASVs), and the color and size of each node indicate its phylum affiliation and the degree in the network. Red and green edges represent positive and negative interactions, respectively. Network properties for bacteria **(A)** and fungi **(B)**, comprised of node number **(C)**, edge number **(D)**, betweenness **(E)**, and closeness **(F)**. Asterisks denote significant differences based on the Wilcoxon test. *$p < 0.05$, **$p < 0.01$ and, ***$p < 0.001$.

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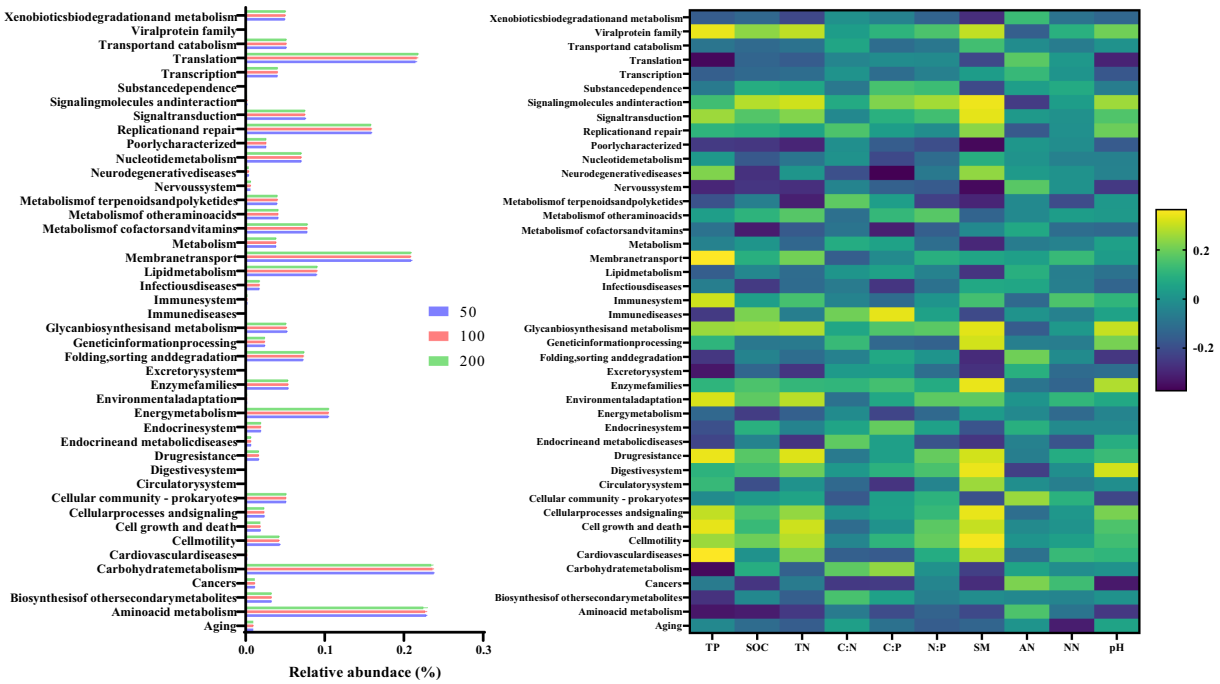


FIGURE 4
Variation in bacterial functions with altered groundwater depth. The relative abundance of the 44 bacterial functions predicted by Tax4Fun2-KEGG pathway (level 2). The Spearman's correlation between predicted bacterial functions and soil properties.

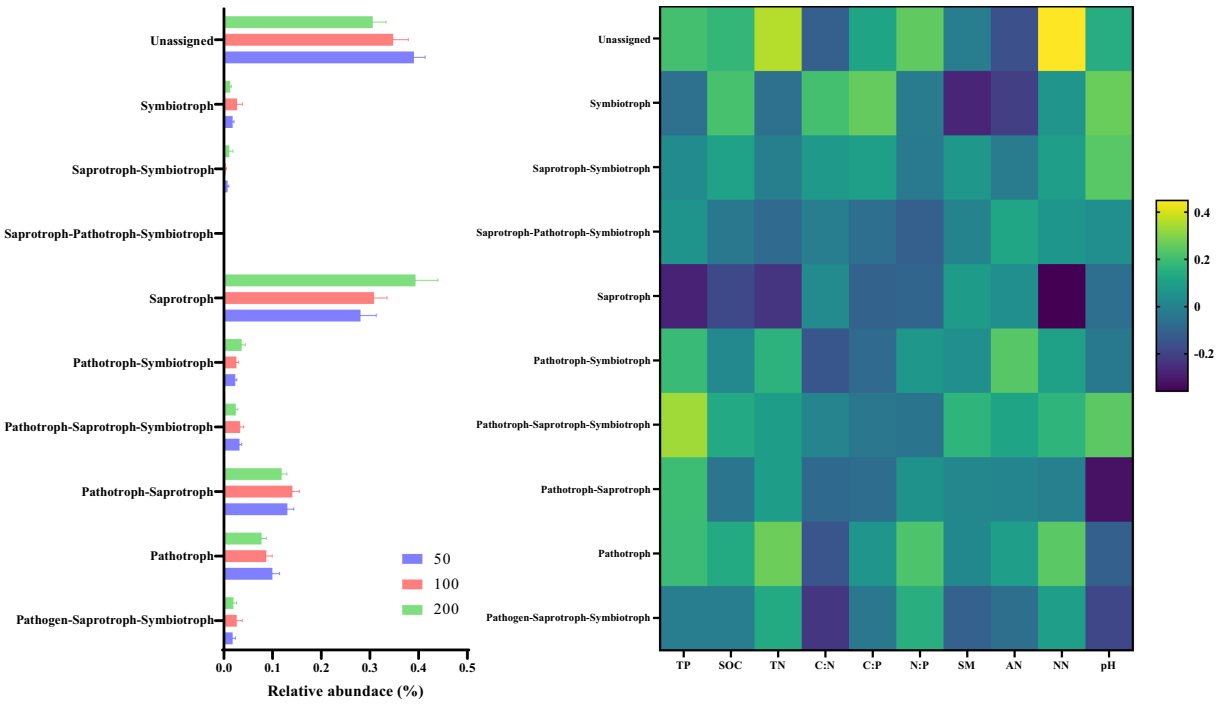


FIGURE 5
Variation in functions of fungal communities with altered groundwater depth. The relative abundance of the 10 functions predicted by FUNGuild. The Spearman's correlation between predicted fungal functions and soil properties.

increased groundwater depth ($p < 0.05$). Meanwhile, most bacterial functional groups showed a strong negative relationship with soil properties.

Derived from the results obtained from FUNGuild, the affected fungal functions are displayed in Figure 5. The relative abundance of fungal function which associated to Pathotroph decreased with

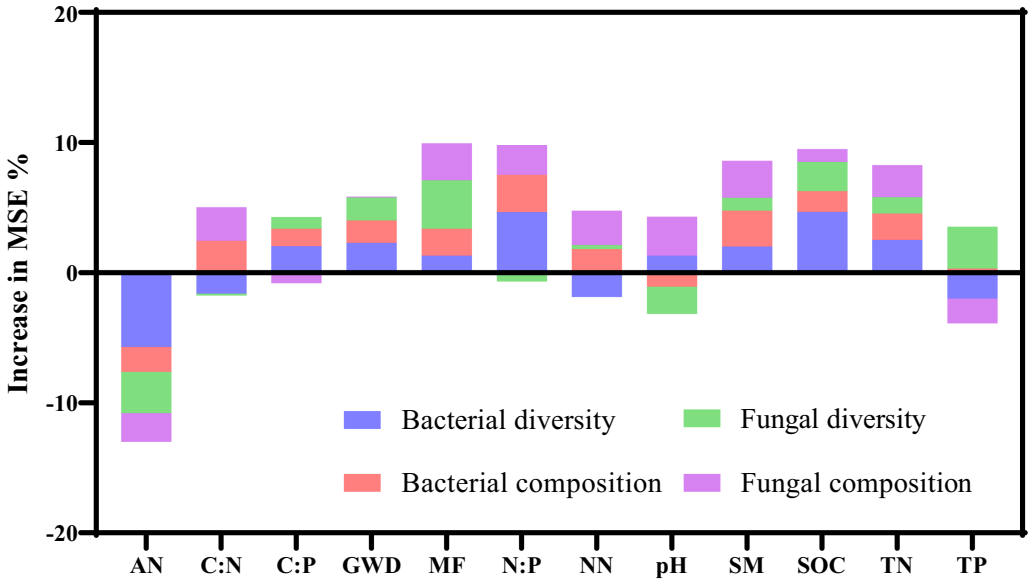


FIGURE 6
Primary predictors of the soil bacterial and fungal composition and diversity rely on random forest models. MSE, mean squared error; TN, total nitrogen; NN, nitrate nitrogen; AN, ammonium nitrogen; SOC, soil organic carbon; TP, total phosphorus; SM, soil moisture; MF, multifunctionality of soils.

increasing groundwater depth ($p < 0.05$). However, increased of groundwater depth improved the relative abundance of group associated to Saprotroph function ($p < 0.05$). Moreover, the relative abundance of fungal function related to Pathotroph was positively related to soil chemical properties ($p < 0.05$), while the relative abundance of Saprotroph group was negatively related to soil chemical properties ($p < 0.05$).

Random forest analyses were employed to identify the key properties and multifunctionality of soil measures that were critical in predicting the soil microbial composition and diversity (Figure 6). In bacteria community, random forest models explained 25.47% of the variance in diversity and 12.46% in composition. The models based on random forest analyses were able to account for 14.75 and 10.75% of the variance in diversity and fungal composition, respectively. AN, N:P, and SOC were found to be the main predictors of bacterial diversity, while N:P, C:N, SM, MF, and TN were identified as the dominant predictors of bacterial composition. The main predictors of fungal diversity were found to be AN, MF, pH, SOC, and TP, while pH, NN, SM, and TN were the main predictors of fungal composition.

Structural equation modeling demonstrated that alterations in soil properties induced by changes in groundwater depth had a primary influence on the microbial diversity and composition (Figure 7). In contrast, the microbial composition was not significantly impacted by changes in soil moisture and multifunctionality (Figures 7B,D). Moreover, soil moisture and soil multifunctionality had significant and positive effects on microbial community diversity (Figures 7A,C). Increased of groundwater depth significantly reduced soil moisture and soil multifunctionality, whereas it had not direct effects on microbial community diversity and composition. Taken together, the increase in groundwater depth, which induces soil desiccation and degradation, can contribute to a decrease in microbial community diversity and composition in this semi-arid region.

# 4. Discussion

## 4.1. microbial diversity and soil multifunctionality

Our research on soil properties and microbial diversity with contrasting three groundwater depths revealed a decline in soil biodiversity and multifunctionality induced by the decreasing groundwater level. This finding has been extensively documented that the increase in groundwater depth can result in the reduction of soil nutrients, degradation of soil structure, reduction in soil moisture content, and decline in soil functionality, all of which have a detrimental impact on soil microbial diversity and composition (Prieto et al., 2012; Sun et al., 2020; Chen et al., 2021). Whereas, the impact of rising groundwater depth on soil microbiota has been relatively overlooked (Zhang et al., 2022), and it has not been clearly defined whether the alterations in soil microbial diversity caused by increasing groundwater depth are associated with a decline in multiple soil functions. The depletion in soil total nitrogen, soil organic carbon, and soil moisture by increasing groundwater depth could directly resulted in the loss of microbial diversity because reduced soil resource availability constrains the microbial metabolism and composition, furthermore decreased their supports on soil multifunctionality (Chen et al., 2020; Ye et al., 2023). Meanwhile, a previous study suggested that decreased soil organic matter content may improve soil thermal conductivity and a decline in soil heat capacity, then increase the daily variance of soil temperature (Abu-Hamdeh and Reeder, 2000; Balashov et al., 2023). Therefore, the indirect increase in soil thermal variability due to the deficiency of microbial diversity and multifunctionality is well-established, as many soil microbes are responsive to alterations in soil temperature (Karimi et al., 2018; Li J. et al., 2023).

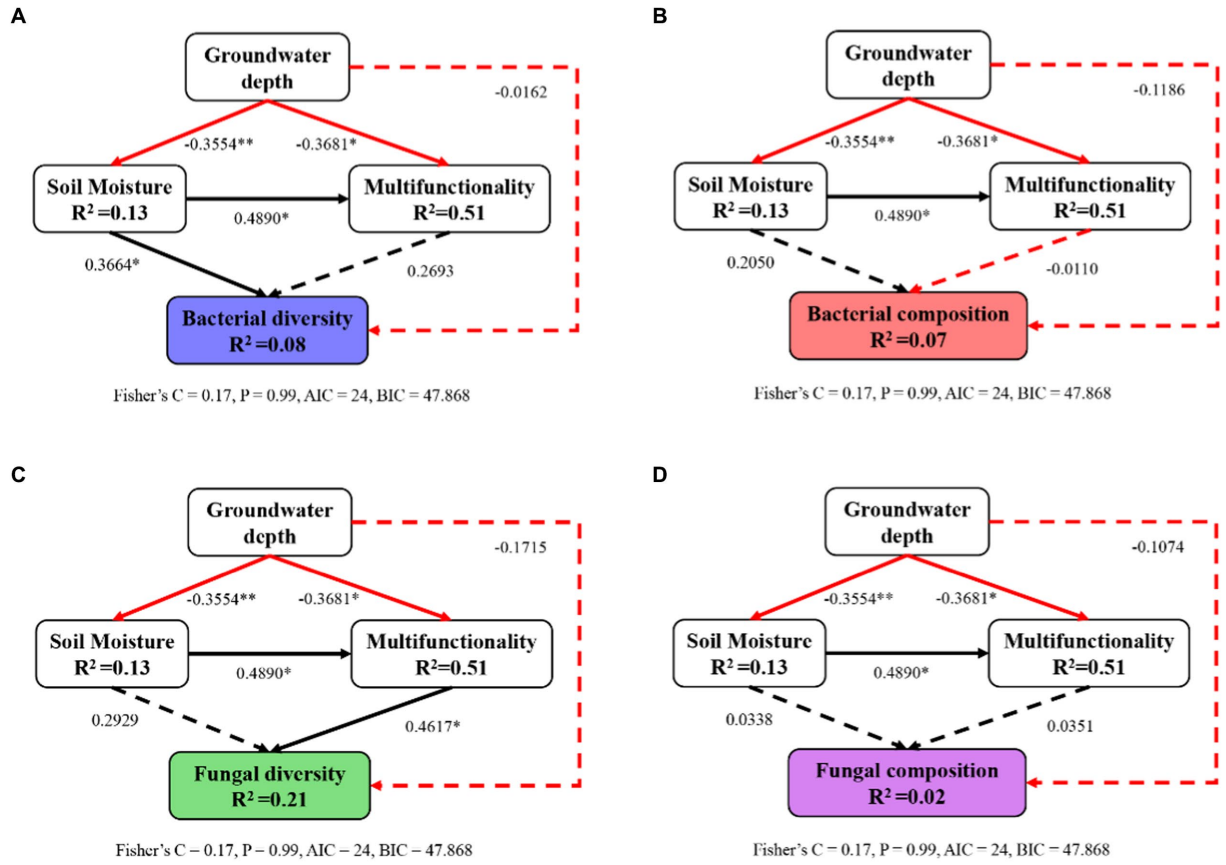


FIGURE 7
Impacts of groundwater depth, soil moisture, and soil multifunctionality on the diversity **(A,C)** and composition **(B,D)** of soil bacterial **(A,B)** and fungal **(C,D)** communities rely on a piecewise structural equation model (SEM). Solid lines indicate significant paths (*$p < 0.05$; **$p < 0.01$); dashed lines indicate nonsignificant paths ($p \geq 0.05$); black lines indicate positive effects; red lines indicate negative effects.

## 4.2. Composition of soil bacteria and fungi

At the phyla level, the result showed that the relative abundance of Proteobacteria in bacteria decreased with increasing groundwater depth. The phylum Proteobacteria was found to be the most dominant bacterial group in the majority of the soil samples collected in our study. Zhang et, al (2016) found that the Proteobacteria is highly abundant and comprises fast-growing bacteria that can use a variety of resources, which are typically considered copiotrophic (Zhang et al., 2016). Meanwhile, the results indicated that the relative abundance of Actinobacteria increased with increasing groundwater depth. We also found increasing of groundwater depth significantly reduced soil moisture of 0–50 cm layers. The relative abundance of Actinobacteria was reported that decrease by the addition of water (Van Horn et al., 2014; Monteiro et al., 2023). In the fungal composition analysis, we observed a decline in the relative abundance of Ascomycota with increasing groundwater depth. The Ascomycota had previously been reported to have a strong positive correlation with the metabolism of organic substrates in rhizodeposition (Liu et al., 2020; Zhong et al., 2020). Consequently, the loss of available substrate and nutrients reduced the relative abundances of these microbial community phyla which was associated to increasing of groundwater depth. The relative abundance of Sphingomonadaceae increased with increasing groundwater depth at the family level. Our results also suggested that soil pH was negatively correlated with groundwater depth. A previous study demonstrated that Sphingomonadaceae was able to secrete dehydrogenase to balance soil pH (Gong et al., 2021). Increasing groundwater depth improve the relative abundance of Mortierellaceae in fungal community. The observed response pattern is likely attributed to the longer plant-water transport distance associated with deeper groundwater depth, which may prompt the plant to acquire available water resources by increasing the proportion of roots. The Mortierellaceae have previously been reported to have a positive relationship with root growth (Li et al., 2017). Overall, this study suggested that the alterations in soil microbial composition following an increase in groundwater depth were closely linked to the response of soil environmental conditions.

## 4.3. Microbial co-occurrence network

Our study also showed that the decline of groundwater level reduced the complexity of microbial co-occurring network. The observed decrease in soil microbial diversity may be attributed to the increasing groundwater depth leading to soil desiccation. Drought

conditions are known to decrease microbial complexity as a result of their lower resistance to abiotic stress, and thus the loss of soil moisture caused by increasing groundwater depth could be a key driver in the observed decrease in soil microbial diversity (de Vries et al., 2018; Banerjee et al., 2019; Li Z. et al., 2023). Many studies have demonstrated that the complexity of soil microbial networks is positively associated with soil resources, such as soil moisture and fertility, and this interpretation aligns with the observed alterations in microbial composition and the response to soil conditions identified in our study (Guo et al., 2020; Hernandez et al., 2021; Kong et al., 2022; Zou et al., 2023). Besides, the robustness of microbial co-occurrence network was previously reported to be negatively characterized by betweenness and closeness (Figures 3C–F; Wu M.-H. et al., 2021). Our study showed that the responsive taxa of deeper groundwater depth had higher betweenness and closeness. This result suggested that these taxa may be responsible for driving the alterations in the microbial co-occurrence network (de Vries et al., 2018; Yang et al., 2023). Taken together, the results of our study provide additional evidence supporting the notion that deeper groundwater depth could have an indirect effect on the soil microbial co-occurrence network via soil desiccation.

### 4.4. Microbial functionality

For bacterial functionality, a decrease in the relative abundance of functional groups associated to amino acid metabolism and carbohydrate metabolism was showed in Figure 4. Many studies suggested that soil drought by increasing groundwater depth impaired these functional groups (Chen et al., 2012; Bromke, 2013; Kong et al., 2022). At a groundwater depth of 50 cm, the increase in soil moisture and soil nutrients could potentially mitigate the negative impact of drought on soil bacterial function. However, for deeper groundwater depth, drought caused by soil water stress may restrict microbial growth and hinder the availability of nutrients to bacteria by directly constraining bacterial dispersal within the pores of arid and sandy soil (Giannetta et al., 2018). We also found that the relative abundance of fungal Pathotroph decreased with increasing groundwater depth. The relative abundance of Pathotroph were suggested to be related with plant and root biomass, and deeper groundwater depth restrained growth of herb root (Levi et al., 2022). Although the relative abundance of Saprotroph was higher in the treatment with deeper groundwater depth, it is likely attributed to the increased soil heat capacity caused by drought. Consequently, the enhancement of fungal functionality, which is temperature-sensitive, may be affected (Qu et al., 2021; Li et al., 2022).

## 5. Conclusion

In summary, we explored the connections between groundwater depth, soil microbial diversity, microbial composition, soil moisture, and soil multifunctionality using the same soil and species at the same site in a semi-arid region. We found that soil moisture and multifunctionality changed by groundwater depth had different effects on soil microbial community (Figure 7). Careful consideration of such changes was necessary because in most arid and semi-arid climates, groundwater depth exceeds 50 cm (Chen et al., 2021; Trinidad Torres-Garcia et al., 2022). The results presented in our study suggest that deeper groundwater depth led to a depletion in soil moisture and soil properties, as well as a shift in microbial community structure and functionality. Essentially, soil microorganisms play a crucial role in supporting soil multifunctionality by promoting decomposition, nutrient cycling, and resource availability in different microenvironments (Delgado-Baquerizo et al., 2016, 2020). The present study highlights the significance of soil microbial communities in facilitating multifunctionality in arid environments, with diverse relationships between alterations in soil microbial diversity and composition and modifications in soil quality triggered by changes in groundwater depth. Additionally, as complex microbial communities are typically more resilient to environmental challenges than simpler ones, the observed reduction in microbial diversity and composition resulting from increased groundwater depth may have long-term undesirable impacts on soil functions (Fierer et al., 2003; Allison and Martiny, 2008; Crowther et al., 2015).

The importance of soil moisture and nutrient availability for the growth of drought-tolerant plants, especially in semi-arid and arid regions, where soil moisture plays a more significant role than rainfall, is widely recognized (McCulley et al., 2004; Li et al., 2021). Besides, the growing depth of groundwater is closely related to species mortality in response to drought (Goulden and Bales, 2019). Consequently, changes in water management may have a stronger effect on microbiota than changes in environmental factors. Furthermore, our results suggested that increasing groundwater depth which reduced soil moisture impaired soil microbial functionality. In semi-arid and arid regions, soil microbiota played a crucial role in regulating the availability of soil nutrients and water to plant roots. This study revealed that the reduction in dominant species in semi-arid and arid regions may be attributed not only to the depletion in soil water content but also to the impairment of multiple microbial functions resulting from drought (Peng et al., 2011; Fu et al., 2017; Stewart et al., 2017). Therefore, reduction of soil multifunction, soil water content, and soil microbial community caused by increasing groundwater depth together contribute to desertification. The investigation of the restoration of the soil microbiota and its functions in sandy soil under future groundwater conservation strategies is necessary.

## Data availability statement

The raw data has been successfully released with accession number PRJNA968046 and is available at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA968046.

## Author contributions

SZ: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, and writing—review and editing. XZ: funding acquisition, project administration, resources, supervision, validation, visualization, writing—original draft, and writing—review and

editing. YL and RZ: conceptualization, formal analysis, investigation, methodology, resources, supervision, validation, visualization, writing—original draft, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Discrepant diversity patterns and function of bacterial and fungal communities on an earthquake-prone mountain gradient in Northwest Sichuan, China

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Patterns of microbial diversity on elevational gradients have been extensively studied, but little is known about those patterns during the restoration of earthquake-fractured alpine ecosystems. In this study, soil properties, soil enzyme activities, abundance and diversity of soil bacterial and fungal communities at four positions along a 2.6-km elevational gradient in the Snow Treasure Summit National Nature Reserve, located in Pingwu County, Southwest China. Although there were no significant changes in the soil chemical environment, bacterial and fungal communities were significantly different at different elevations. The overall fungal community presented an N-shaped diversity pattern with increasing elevation, while bacterial diversity decreased significantly with elevation. Changes in microbial diversity were associated with soil phosphorus, plant litter, and variations in dominant microbial taxa. Differences in enzyme activities among elevations were regulated by microbial communities, with changes in catalase and acid phosphatase activities mainly controlled by *Acidobacteria* and *Planctomycetaceae* bacteria, respectively (catalase: $p < 0.001$; acid phosphatase: $p < 0.01$), and those in β-glucosidase, sucrase, and urease activities mainly controlled by fungi. The β-glucosidase and sucrase were both positively correlated with *Herpotrichiellaceae*, and urease was positively correlated with *Sebacinaceae* ($p < 0.05$). These findings contribute to the conservation and management of mountain ecosystems in the face of changing environmental conditions. Further research can delve into the specific interactions between microbial communities, soil properties, and vegetation to gain deeper insights into the intricate ecological dynamics within earthquake-prone mountain ecosystems.

KEYWORDS

**earthquake-prone areas, elevational gradient, enzyme activities, bacteria, fungi, microbial diversity**

# 1. Introduction

Mountainous areas, which is far from human interference, encompass dramatic turnover in climate and biota over relatively short elevational distances and thereby provide powerful "natural experiments" to understand how biodiversity responds to environmental change. Based on above advantage, extensive studies on biodiversity of higher organisms (e.g., vascular plants, tree and birds) demonstrate different patterns of richness with increasing elevation (Guo et al., 2013; Dhyani et al., 2019). However, the effects of environmental variability along elevational gradients on taxonomic and functional diversity of soil bacteria and fungi are ambiguous, even though soil microbial communities are critical in regulating ecosystem functions and services (Ramirez et al., 2012; Liu et al., 2021). At present, the patterns of microbial diversity along elevational gradients include monotonic decreasing (Bryant et al., 2008), increasing (Margesin et al., 2009), hump-back (Han et al., 2018), and U-shape (Wang et al., 2012) patterns, but study also showed that soil microbial diversity did not vary with elevation (Singh et al., 2014). Soil enzyme activities mediated by microbe (e.g., s phosphatase, β-glucosidase, urease, sulphatase and dehydrogenase) play important role in carbon decomposition and nutrient recycling, and easily influenced by biotic and aerobic factors (Dasila et al., 2020). So, patterns of changes in microbial enzyme activities with increasing elevation are also inconsistent. In addition, trends in microbial diversity are not always consistent with those of microbial enzyme activity along elevational gradients (Ren et al., 2018). Because of their strong and inconsistent respond to environmental changes, microbial community and enzyme activities were regarded as optimum candidate to study elevational gradients.

At present, climate, soil properties, vegetation, and historical impacts are the four main determinants of composition and diversity of soil microbial communities along elevational gradients (Praeg et al., 2020). Climatic and edaphic variables (e.g., pH and C/N ratio) are frequently reported as key factors shaping elevational patterns of microbial communities (Donhauser and Frey, 2018), although such variables can have limited explanatory power. A primary reason for limited explanatory power is that microbial diversity may be controlled by processes operating at scales that do not match the temporal and spatial scales under study (Ladau and Eloe-Fadrosh, 2019). Particularly, because of small size and specific habitat requirements, soil microbes experience strongly buffered temperature and humidity extremes compared with those of open areas, with lower seasonal and interannual variability (De Frenne et al., 2019). In such buffered conditions, soil bacteria can survive in microrefugia despite unfavorable large-scale free-air conditions (Ashcroft, 2010). Therefore, soil micro-environment and historical impacts may be the main factors affecting soil microbial diversity and function over relatively short elevational distances. Earthquakes are one example of important historical impacts. The occurrence of it not only causes the disappearance or burial of the original vegetation layer, but also changes the environment and function of vegetation and soil continuously (Cheng et al., 2012). Therefore, the vegetation and soil environment of post-earthquake damaged mountain ecosystem is more complex than that of normal mountain ecosystem. However, the effects of earthquakes on soil microorganisms along elevational gradients are poorly understood.

The Snow Treasure Summit National Nature Reserve in the Longmen Mountain seismic zone in Northwest Sichuan, China, is a nature reserve of wild creatures that mainly protects giant pandas and their habitats. The Longmen Mountain seismic zone is one of the strongest seismic zones in Sichuan. Since 1169 AD, there have been 26 destructive earthquakes, 20 of which measured 6 or higher on the Richter scale. The 8.0 magnitude Wenchuan earthquake and the 7.0 magnitude Ya'-an earthquake occurred in the Longmen Mountain seismic zone. Years of effects of geological activities on the soils and vegetation in the Snow Treasure Summit National Nature Reserve have seriously affected the survival of wild creatures. In this study, soil bacterial and fungal communities were measured at four positions along a 1.0-km elevational gradient in the Snow Treasure Summit National Nature Reserve. The gradient was used to test the following hypotheses: (i) soil bacterial and fungal communities will have unique elevational patterns of diversity, but different environmental drivers will be correlated with those patterns; and (ii) differences in bacterial communities will be the dominant factor regulating microbial enzyme activity along the elevational gradient. Testing those hypotheses will generate information to help to maintain the sustainability of ecosystems in the Snow Treasure Summit National Nature Reserve and will also provide a relevant theoretical basis for the restoration and reconstruction of damaged soil ecosystems in the reserve.

# 2. Materials and methods

## 2.1. Study area

The study was conducted in the Snow Treasure Summit National Nature Reserve (31°59′31″–33°02′41″N, 103°50′31″–104°59′13″E), which is responsible for protecting the giant panda, golden monkey, and other rare wild animals and their habitats. The nature reserve, originally established in 1993, is in Sichuan Province, China, with a total area of 63,615 ha. Because the area is in the heart of the east slope of the Minshan Mountain system and has been protected by national and local governments for over 30 years, it is characterized by natural forest ecosystems with little human disturbance but disturbance from natural hazards. The study area is in the transition from subtropical mountain humid monsoon to cold climate zones in Northwest Sichuan. According to records of the Pingwu County meteorological station (3,250 m a.s.l.), mean annual maximum and minimum air temperatures are 37°C in July and −6.6°C in January, respectively. Mean annual precipitation is 1,300 mm. The dominant soil types are yellow and yellow-brown earths (Pu, 2020).

Forest coverage in the reserve is 85.0%, and evergreen broad-leaved forest is the typical zonal vegetation. *Taxus baccata* Linn (Taxaceae), *T. chinensis* (Taxaceae), *Davidia involucrata* Baill (Nyssaceae), and *Metasequoia glyptostroboides* Hu & W. C. Cheng (Taxodiaceae) are some rare tree species in the forest. Along the elevational gradient, the vegetation types range from evergreen broad-leaved forest (<2,000 m), to mixed coniferous broad-leaved forest (2,000–2,700 m), to coniferous forests (2,700–3,400 m), to alpine irrigation meadow (3,400–3,800 m), and to the vegetation of limestone beach and snow covers (>3,800 m) (Pu, 2020). The focus in this study was on the changes in evergreen broad-leaved and mixed coniferous

broad-leaved forests, and thus, four elevations were selected: 1,600, 1,800, 2,200, and 2,600 m. A detailed description of site characteristics is provided in Supplementary Table S1.

## 2.2. Soil sampling

Soil samples were collected in July 2019. Four 20 m × 20 m permanent plots were systematically set up. Plots were roughly evenly distributed by elevation and collectively spanned 1026.19 m, from 1627.19 m to 2653.38 m a.s.l. Within each plot, six evenly distributed sampling points were set up, and six soil cores within each sampling point (10-cm depth directly below the litter layer, 5-cm diameter) were collected randomly and composited as a single sample. Visible plant roots and residues were removed before mixing. Soil samples were stored in plastic bags on ice. In the laboratory, samples were divided into two portions: one was air-dried and sieved through a 2-mm mesh screen for physicochemical analyses, and the other was stored at −80°C for DNA extraction and soil enzyme activity.

## 2.3. Chemical and enzyme activity analyses

Soil pH, soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), total potassium (TK), available nitrogen (AN), available phosphorus (AP), and available potassium (AK) were measured using standard protocols following Liu et al. (2021). Detailed edaphic properties of the soil samples are provided in Table 1.

Soil enzyme activities were measured as described by Guan (1986). Urease was determined by a colorimetric method using sodium phenol–sodium hypochlorite, and activity was expressed as mass of $NH_4^+$-N produced per unit time and per unit dry soil. Sucrase was determined by a 3,5-dinitrosalicylic acid colorimetric method, and activity was expressed in terms of glucose production per unit time and per unit dry soil mass. Catalase was determined by potassium permanganate titration, and activity was indicated by the volume of 0.02 mol/L $KMnO_4$ consumed by 1 g of dry soil filtrate. β-glucosidase was determined by nitrophenol colorimetry, and activity was expressed by the content of p-nitrophenol produced per unit time and per unit dry soil. Activity of soil extracellular acid phosphatase (ACP) was estimated by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (Tabatabai and Bremner, 1969). Potential ACP activities were expressed as μmol produced per g of soil (dry weight equivalent) within 1 h.

## 2.4. Preparation of 16S rRNA and its amplicon libraries and sequencing

The bacterial 16S rRNA gene was amplified using primers 338F/806R (Nan et al., 2016), and the fungal ITS gene was amplified using ITS1F/ITS2R (Bokulich and Mills, 2013) (ABI GeneAmp® 9700, United States). The PCR was performed in a reaction mixture containing 12.5 μL of ABI Power SybrGreen qPCR Master Mix (Applied Biosystems), 0.5 μM each primer, 1 μL of 20 ng $\mu L^{-1}$ template DNA, and sterile distilled water to make up a final volume of 25 μL. The qPCR program consisted of an initial denaturation at 95°C for 1 min and 30 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The PCR products were extracted from 2% agarose gel, further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, United States), and quantified using QuantiFluor™-ST (Promega, United States). Purified PCR products were paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform by Origingene Biotechnology Co., Ltd., Shanghai, China.

Raw sequence data were processed using the QIIME v1.9 pipeline, where sequences were quality filtered, chimera-checked, OTU-clustered, and taxonomically annotated (Caporaso et al., 2012). Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (v7.1). The taxonomy of each 16S rRNA and ITS gene sequence was analyzed using the SILVA r115 database (Quast et al., 2013) and the UNITE v5.0 database (Kõljalg et al., 2013), respectively. To eliminate the effects of different read numbers among the plots on the deduced compositions of bacterial and fungal communities, the number of sequences per soil sample was normalized to 35,100 reads for bacteria and 26,300 reads for fungi after removing the singletons. The each sample was rarefied to the identical number of 16S rRNA OTUs (3000) and ITS OTUs (600) for downstream analyses. The raw reads have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database (Accession Number: PRJNA913681).

## 2.5. Statistical analyses

Differences in soil properties and enzyme activities were tested using a random permutation test (Anderson and Walsh, 2013). Principal component analysis (PCA) was used to compare soil properties at different positions on the elevational gradient. Soil enzyme activities at different positions were compared using boxplots. Relative abundances of dominant taxa of soil bacteria and fungi in

TABLE 1 Soil parameter information of the sampling sites along mountain gradient.

| | pH | SOC (g. $kg^{-1}$) | TN (g. $kg^{-1}$) | TP (g. $kg^{-1}$) | TK (g. $kg^{-1}$) | AN (mg. $kg^{-1}$) | AP (mg. $kg^{-1}$) | AK (mg. $kg^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 1,600 | 8.16 ± 0.12a | 9.37 ± 1.97c | 1.08 ± 0.15c | 0.64 ± 0.12ab | 17.19 ± 1.36b | 105.14 ± 24.69c | 2.58 ± 0.21b | 55.76 ± 12.25c |
| 1,800 | 6.70 ± 0.10b | 34.57 ± 8.26a | 2.17 ± 0.51ab | 0.79 ± 0.06a | 15.64 ± 0.59b | 233.90 ± 35.13b | 4.70 ± 0.85a | 58.81 ± 13.33c |
| 2,200 | 5.33 ± 0.04d | 22.83 ± 1.14ab | 2.78 ± 0.14a | 0.58 ± 0.00b | 16.96 ± 0.41b | 358.34 ± 13.04a | 1.60 ± 0.16bc | 131.87 ± 24.04a |
| 2,600 | 5.68 ± 0.09c | 14.08 ± 1.53c | 1.72 ± 0.17bc | 0.66 ± 0.01ab | 23.43 ± 0.77a | 228.27 ± 20.15b | 1.14 ± 0.19c | 83.47 ± 12.86c |
| *F* | 190.253 | 6.479 | 6.133 | 1.843 | 16.342 | 17.672 | 12.177 | 4.624 |
| *P* | 0.00 | 0.003 | 0.004 | 0.172 | 0.00 | 0.00 | 0.00 | 0.013 |

Values (mean ± SE, $n = 6$) followed by different letters indicate statistically significant differences between sites (Tukey's HSD test, $p < 0.05$). SOC, soil organic carbon; TN, total N; TP, total P; TK, total K; AN, available N; AP, available P; AK, available K; 1,600, 1,600 elevation; 1,800, 1,800 elevation; 2,200, 2,200 elevation; 2,600, 2,600 elevation.

each sample were calculated and ranked. The calculation of relative abundance was based on the proportional frequencies of the DNA sequences from all samples that could be classified at the phylum level.

An OTU table was used for downstream alpha and beta analyses. Taxonomic alpha diversity of soil bacteria and fungi was estimated using OTU richness, Pielou's evenness, and the Shannon–Wiener diversity index. To calculate taxonomic beta diversity, the Bray–Curtis index for abundance-weighted dissimilarity was used. Phylogenetic beta diversity was calculated using both weighted and unweighted UniFrac distance. To examine the elevational differences in compositional dissimilarities, principal coordinates analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) were generated using "vegan" package in the R statistical software platform (v2.15.0). Differences in taxa among elevations were analyzed using linear discriminant analysis (LDA) effect size (LEfSe) analysis[1] (Segata et al., 2011) and were visualized using the R package DESeq2 (Love et al., 2014). Enriched genera (up) and depleted genera (down) were defined as genera with differences in relative abundances ($p < 0.1$) between two contiguous sites along the elevational gradient. Partial Mantel tests were used to test the correlations between environmental variables and bacterial and fungal taxa.

For the predicted functional groups, a linear model of Spearman's rank correlations was used to examine the relations between enzyme activities and relative abundances of bacterial and fungal groups, with relations visualized using the Cytoscape package v3.2.1 (Shannon et al., 2003).

# 3. Results

## 3.1. Soil properties and enzyme activities on the elevational gradient

Soil properties were significantly different along the gradient, except for total P (Table 1). Soil pH was the lowest at 2,200 m, but the contents of TN, AN, and AK were the highest. Contents of TP, AP, and SOC were the highest at 1,800 m. Although individual soil properties were significantly different among sites on the elevational gradient, overall soil environments were not clearly separated by elevation along the first principal component of the PCA, which explained over 88% of the variation in soil properties. Thus, distinct soil environments did not develop on the mountain gradient in Snow Treasure Summit National Nature Reserve (Figure 1A). Among enzyme activities (Figure 1B), sucrase and catalase activities decreased along the elevational gradient. Activities of urease and ACP were the highest at 2,200 m. The activity of β-glucosidase was slightly higher at 1,800 m than at other elevations. The changes in activities of β-glucosidase, urease, and sucrase along the gradient were not significant. In addition, catalase was negatively correlated with TK and positively correlated with pH ($p < 0.05$; Supplementary Table S2). Activity of β-glucosidase was positively correlated with SOC, TP, and AP ($p < 0.05$), and activity of ACP was negatively correlated with pH and positively correlated with SOC, TN, AP, and AK ($p < 0.05$; Supplementary Table S2).

## 3.2. Contrasting patterns with increasing elevation: N-shaped for fungal diversity, decreasing for bacterial diversity

The overall fungal community showed significant N-shaped patterns for richness, evenness, and Shannon diversity indices (Figure 2B) along the elevational gradient, whereas evenness and diversity indices of the overall bacterial community decreased significantly with elevation (Figure 2A). The Chao1 index of bacteria ranged from 3,707 to 6,153 (Supplementary Table S3), and the Shannon diversity index varied from 5.69 to 6.89 (Supplementary Table S3). Notably, the Chao1 index of bacteria increased with elevation, whereas the Shannon diversity index decreased. The lowest Shannon indices of bacteria were detected at 2,600 m, but the lowest Chao1 indices were detected at 1,600 m (Figure 2A). According to Pearson tests, the Shannon index of soil bacteria was positively correlated with AP ($p = 0.024$) and negatively correlated with TK and AK ($P_{TK} = 0.017$ and $P_{AK} = 0.037$; Supplementary Table S4). For bacteria, the phyla Proteobacteria, Acidobacteria, and Actinobacteria collectively accounted for 50.6–71.9% of the total sequences along the elevational gradient (Figure 2C). The trends in relative abundances of bacterial phyla were not similar to those in α diversity (Supplementary Figure S1A), but some bacterial phyla were correlated with soil properties (Supplementary Table S5). In addition, the dominant genera were *Massilia*, *Bradyrhizobium*, *Nitrosomonadaceae*, and *Xanthobacteraceae* in *Proteobacteria*; *Acidobacteria* and *Candidatus Solibacter* in *Acidobacteria*, and *Pseudarthrobacter*, *Gaiellales*, and *Acidimicrobiales* in *Actinobacteria* (Supplementary Figure S2).

The Chao 1 index of fungi ranged from 115 to 1,068, and the Shannon diversity index varied from 2.13 to 4.64 (Supplementary Table S3). Overall, the trends in fungal Chao1 and Shannon diversity indices were similar, with values increasing from 1,600 to 1,800 m, then declining at 2,200 m, followed by significant increases from 2,200 to 2,600 m. In Pearson tests, the Shannon index of soil fungi was positively correlated with TP ($p = 0.016$), and the Chao1 index was positively correlated with SOC ($p = 0.001$; Supplementary Table S4). In fungal communities, the phyla Ascomycota and Basidiomycota collectively accounted for 78.9–95.2% of the total sequences along the elevational gradient (Figure 2D). The trend in relative abundance of Ascomycota was similar to that of α-diversity (Supplementary Figure S1). *Ascomycota* and *Leotiomycetes* were the dominant genera in Ascomycota, and *Agaricomycetes* was dominant in Basidiomycota (Supplementary Figure S2).

## 3.3. Effect of elevation on compositional dissimilarities of bacterial and fungal communities

The composition of bacterial and fungal communities differed with elevation, as shown in PCoA plots based on Bray–Curtis distance (Figures 3A,B). According to PERMANOVA, the compositional dissimilarities among elevations were significant ($p < 0.001$; Figures 3A,B). The dissimilarity of bacterial and fungal communities significantly and unimodally increased with increasing elevation distance (Figures 3C–H). Compared with communities at 1,600 m, at 1,800 m, 66 genera of bacteria and 12 genera of fungi were enriched.

1 http://huttenhower.sph.harvard.edu/galaxy/

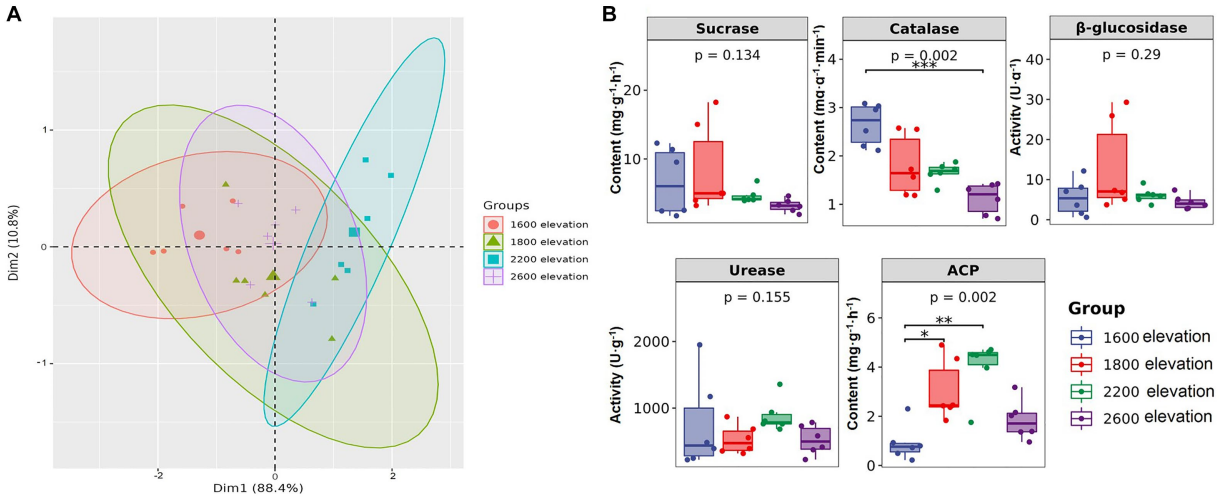


FIGURE 1
**(A)** Principal component analysis of soil properties, and **(B)** differences in soil enzyme activity along an elevational gradient in the Snow Treasure Summit National Nature Reserve. Difference significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

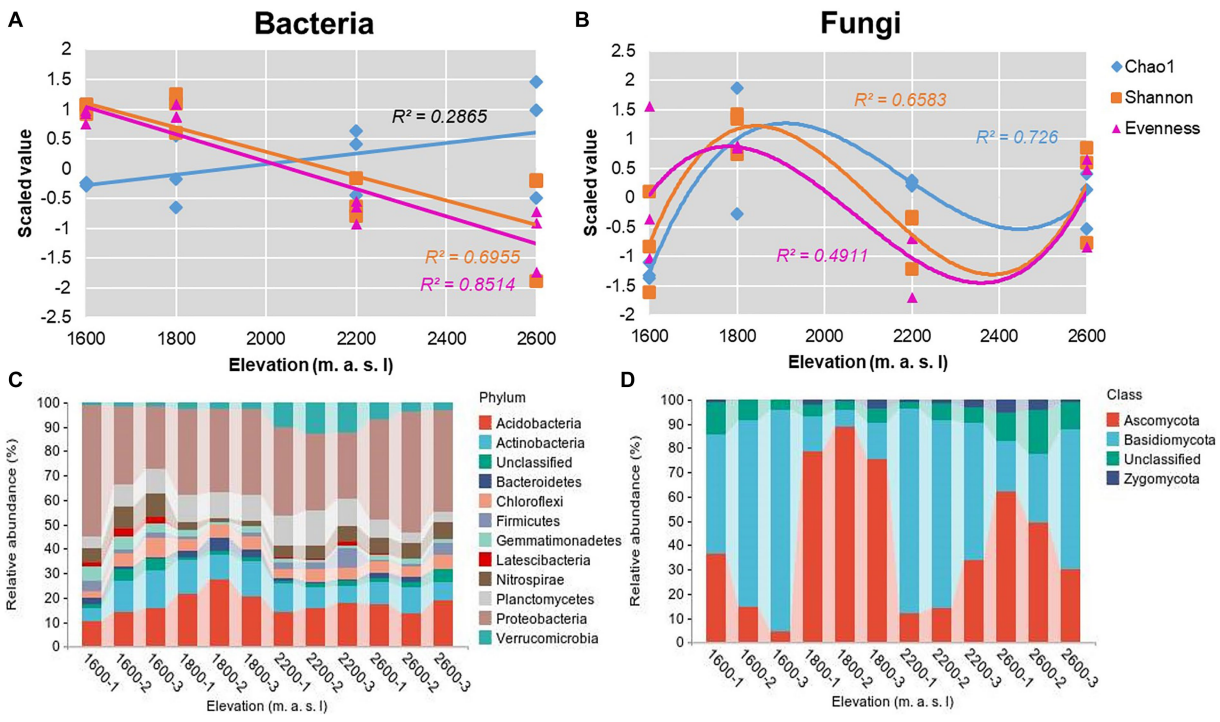


FIGURE 2
Elevational patterns for α diversity indices of overall **(A)** bacterial and **(B)** fungal communities. Scaled values were for z-transformed Chao1, evenness, and Shannon indices. Trends across elevations were determined using Gaussian process regression. **(C)** Twelve most abundant phyla in bacterial communities and **(D)** most abundant phyla in fungal communities along an elevational gradient in the Snow Treasure Summit National Nature Reserve.

Compared with 1,800 m, at 2,200 m, 112 genera of bacteria and 25 genera of fungi were depleted. Therefore, whether bacteria or fungi, the greatest differences were at 1,800 m. The bacterial genus *Phyllobacterium* increased significantly at 1,800 m (Figure 3C). The fungal families *Chaetothyriaceae* and *Hypocreaceae* also increased significantly at 1,800 m (Figure 3D). In addition, the family *Hypocreaceae* increased significantly with elevation, except at 2,600 m (Figures 3D,F,H). The number of genera with differences was similar at 2,200 and 2,600 m, but the differences were not same.

Pearson correlations indicated that TK ($p < 0.01$) and AK ($p < 0.05$) were positively correlated with elevation, whereas pH and AP were negatively correlated with elevation ($p < 0.001$; Figure 4). The Mantel test revealed that soil pH was the only determinant of differences in soil bacteria between elevations ($p < 0.05$). By contrast, differences in

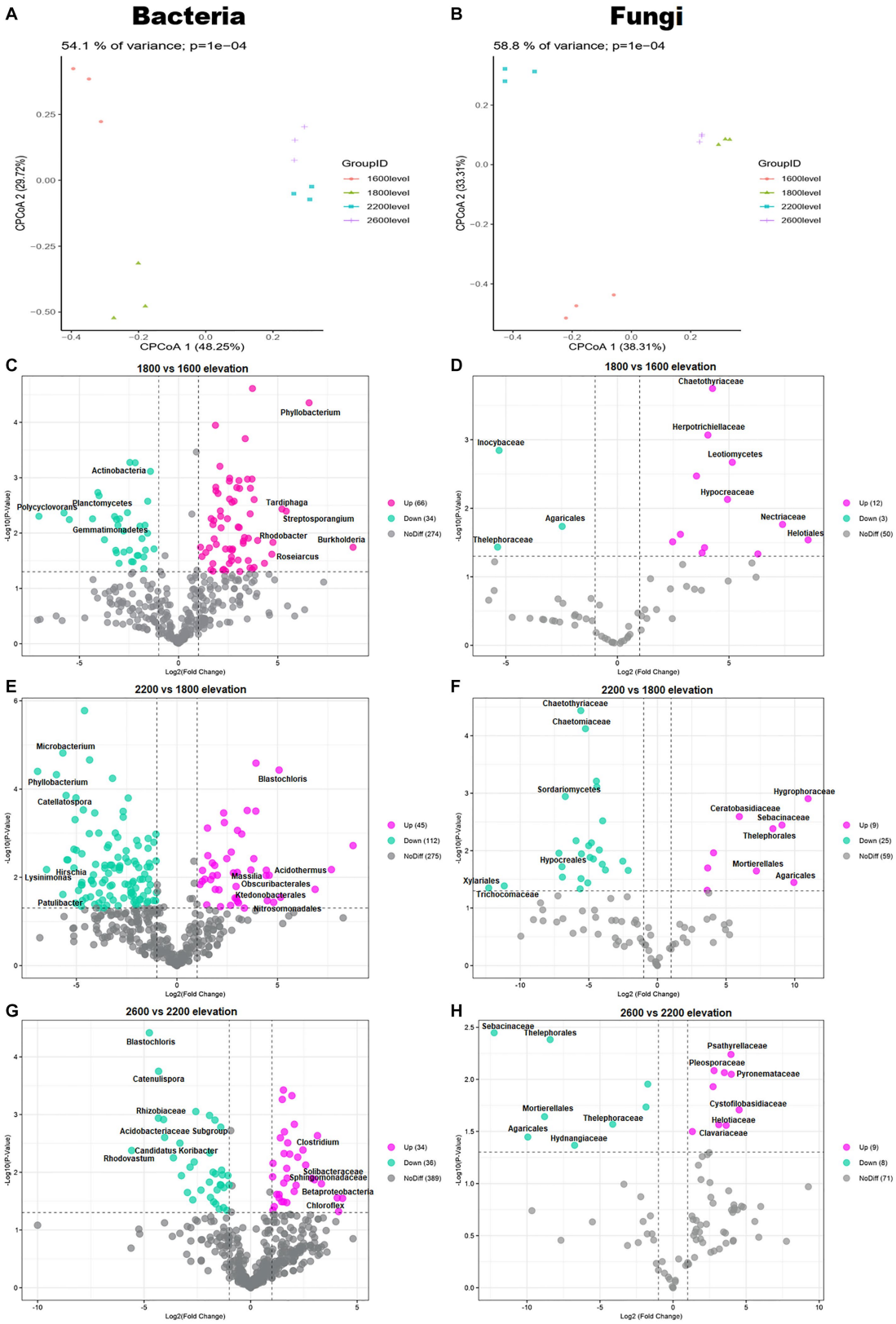


FIGURE 3
Principal coordinates analysis (PCoA) plots of trends in the composition of **(A)** bacterial and **(B)** fungal communities based on Bray–Curtis dissimilarity. Volcano plots illustrating taxa of **(C,E,G)** bacteria and **(D,F,H)** fungi with significant differences among elevations in the Snow Treasure Summit National Nature Reserve. Each point represents an individual taxon.

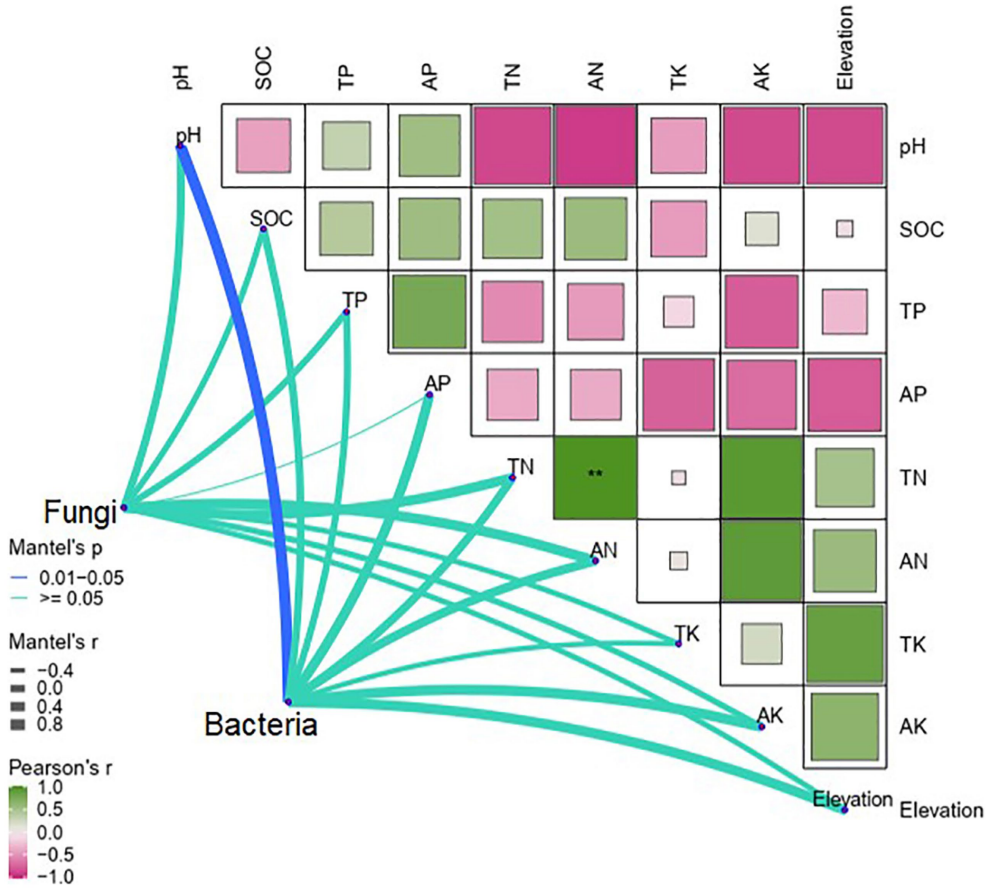


FIGURE 4
Mantel tests of differences in soil bacteria and fungi with soil properties along the elevational gradient in the Snow Treasure Summit National Nature Reserve and Pearson tests of correlations between soil bacteria and fungi and soil properties along the elevational gradient. TP, total phosphorus; AP, available phosphorus; TN, total nitrogen; AN, available nitrogen; TK, total potassium; AK, available potassium; pH, soil pH; SOC, soil organic carbon. Correlation significance: $^{**}p < 0.01$.

soil fungi were not significantly affected by elevation and soil properties (Figure 4).

## 3.4. Predicted functional groups of bacteria and fungus

Network analysis was used to determine the correlations between the top 50 genera of bacteria and fungi and activities of different enzymes (Figure 5). The network analysis showed that each enzyme had potential functional genera of bacteria and fungi. The highest number of functional genera was associated with catalase, whereas the lowest number of functional genera was associated with β-glucosidase, and all genera were fungal. Catalase was significantly positively correlated with the bacteria *Acidobacteria* and *Tepidisphaeraceae* and the fungi *Thelephoraceae* and *Chaetomiaceae* ($p < 0.001$). The enzyme ACP was significantly positively correlated with the bacteria *Planctomycetaceae* and *Isosphaera* and the fungi *Agaricales* and *Leotiomycetes* ($p < 0.01$). Urease was significantly positively correlated with only the fungal family *Sebacinaceae* ($p < 0.05$). In addition, sucrase and β-glucosidase were both positively correlated with the fungal family *Herpotrichiellaceae*. Although catalase and ACP shared some functional genera, the functional genera did not have the same effects. For example, *Gemmatimonadaceae*, a family of bacteria, was significantly positively correlated with catalase and negatively correlated with ACP.

# 4. Discussion

## 4.1. Differences in soil properties along the elevational gradient

The change in SOC along the elevational gradient in the Snow Treasure Summit National Nature Reserve was not consistent with increases in SOC with elevation in previous study (Zhang et al., 2021), which might be because of the type of the litter and other inputs into soil (Zhang et al., 2022). Compared with vegetation at other elevations, at 1,800 m, the more complex shrub community and hardwood species increased the thickness of the litter layer (Li et al., 2021) (Supplementary Table S1), which led to an increase in SOC. Additionally, the increase in SOC might be the main factor driving increases in TP and AP at 1,800 m, because organic matter formed during litter decomposition tends to fix soil P (Fekete et al.,

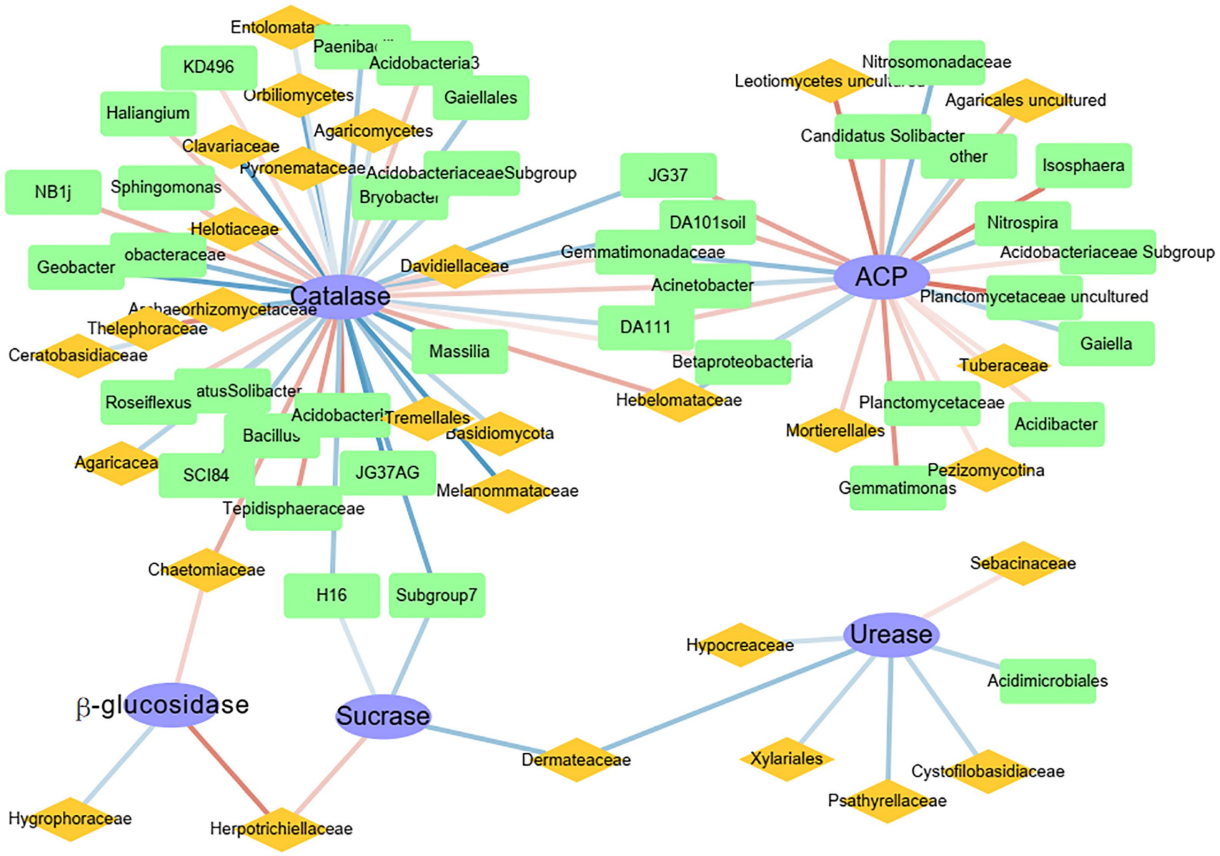


FIGURE 5
Spearman network analysis of the correlations between the 50 most abundant genera of bacteria (green squares) and fungi (yellow diamonds) and enzyme activities (blue ellipses). Blue lines, negative correlations; red lines, positive correlations. The darker the line, the greater the R-value.

2014). Simultaneously, AP is a by-product in the process of further microbial metabolism of organic matter that accumulates continuously (Luo et al., 2017). Many environmental conditions in montane ecosystems often covary with elevation (Looby and Martin, 2020). However, in this study, the soil environment at different elevations was not significantly different. It was hypothesized that the absence of differences might be associated with local, frequent seismic secondary disasters. After each secondary disaster, large areas of soil are disrupted and migrate (Cheng et al., 2012), which eliminates differences in soil environments at different elevations.

## 4.2. Soil conditions co-mediate differences in microbial communities on the elevation gradient

Along the elevational gradient in the Snow Treasure Summit National Nature Reserve, fungal communities showed N-shaped changes in diversity indices. By contrast, in bacterial communities, the richness index increased with elevation and evenness and Shannon indices decreased. The different responses of soil bacterial and fungal community diversity are consistent with results in study (Shen et al., 2020), suggesting different responses of microbial groups to environmental variations along elevation gradients. Although a global study on topsoil microorganisms demonstrated that niche differentiation between fungi and bacteria was associated with contrasting responses of diversity to soil pH (Bahram et al., 2018), in this study, the contrasting responses of fungal and bacterial diversity were associated with soil P or SOC (Supplementary Table S4). Soil texture is a key factor and can lead to an important role for P in niche differentiation between fungi and bacteria. Because the topsoil of both mountain brown soil and mountain yellow-brown soil has high levels of humus, organic P forms easily in soil (Nannipieri et al., 2011), and thus, P becomes an important factor limiting the growth and development of bacteria and fungi (Yang et al., 2022). Bacteria and fungi also have important differences in how they obtain P. Bacteria secrete alkaline or acid phosphatase to obtain P (Romanyà et al., 2017), whereas fungi obtain P by symbiosis with plants, with roots secreting acid phosphatase (Shenoy and Kalagudi, 2005). Therefore, temperature may be the main factor affecting bacterial P metabolism (Bahram et al., 2018), whereas vegetation may be the main factor affecting fungal P metabolism (Karandashov and Bucher, 2005), and the difference could lead niche differentiation between fungi and bacteria.

In this work, diversity of soil bacteria decreased with the increase in elevation, similar to results in previous study (Zhang et al., 2022). However, differences in relative abundances of bacterial phyla with elevation might not only be attributed to soil moisture and temperature (Ren et al., 2018) but also to other soil properties (Supplementary Table S5). The U-shaped relative abundance of

*Nitrospirae* with increasing elevation (Supplementary Figure S1A) might be associated with its preference for C-limited soil conditions (Feng et al., 2017) and therefore the hump-shaped soil total C content with increasing elevation (Table 1). In addition, relative abundances of *Verrucomicrobia* and *Gemmatimonadetes* were both correlated with soil pH and N, but the correlations were the opposite. According to Xu et al. (2020), the *Verrucomicrobia* are considered an oligotrophic group, but moderate increases in N can boost its abundance at low soil pH (Ramirez et al., 2012). The ratio between abundances of *Proteobacteria* and *Acidobacteria* (P/A) can indicate soil nutrient status, with higher values indicating more nutrient-rich soils. Therefore, changes in relative abundances of bacterial phyla with elevation may be attributed to differences in ecological strategies (Ivanova et al., 2016). Although decreases in diversity of soil bacteria with elevation are well known, increases in the Shannon–Wiener index of diversity of soil fungi with elevation are rarely reported. Theoretically, for microbial populations at high elevations, inhabitable environments due to desiccation and severely nutrient-limited soils at low temperatures can pose extreme stresses, which lead to reductions in activities and diversity (Donhauser and Frey, 2018). However, Miyamoto et al. (2014) reported that fungal diversity showed a hump-shaped pattern with increasing elevation from 1,100 to 2,250 m on Mount Fuji, which is similar to the pattern in this work (Figure 2B). Therefore, there may be environmental factors affecting fungal diversity other than desiccation and low temperatures. In further analysis, relative abundance of the dominant fungal phylum Ascomycota showed an N-shaped response to increasing elevation (Figure 2D), which contributed to the overall fungal community pattern (Figure 2B). The response was generally consistent with the changes in SOC and P resulting from abundant vegetation. By contrast, relative abundance of the second most dominant phylum, Basidiomycota (which contains abundant mycorrhizal fungal taxa), was inversely correlated with those changes. The response of Basidiomycota might be because plants rely on the mycorrhizal fungus symbiotic exchange system for nutrient uptake. However, when levels of soil nutrients, such as N and P, are adequate, plants are less dependent on mycorrhizal fungi and reduce the quantity of C flowing into the rhizosphere, thus initiating a decrease in mycorrhizal fungal taxa (Eisenlord and Zak, 2010). Therefore, the most important influence of vegetation on fungal diversity may be determining the quantity and quality of litter substrates.

## 4.3. Dissimilarities of microbial communities respond to different ecological drivers

In general, the dissimilarities in both bacterial and fungal communities unimodally increased with elevation, but the increases were influenced by different ecological drivers. Many studies show that soil pH and soil organic matter have significant effects on soil microbial communities (Wang et al., 2015; Shen et al., 2019). The results in this study are consistent with that conclusion, and soil pH significantly influenced the relative abundance of bacteria between elevations (Figure 4). Generally, bacteria have a relatively narrow pH tolerance range for growth. However, the sites on the gradient in this study had a wide range of soil pH values, from 5.33 to 8.16 (Table 1), resulting in a strong correlation between bacteria and soil pH. A similar correlation is observed in other elevational studies (Wang et al., 2015; Shen et al., 2019). Additionally, soil pH was highly correlated with the relative abundance of two dominant bacterial phyla, *Verrucomicrobia* and *Gemmatimonadetes*. According to difference analysis, the bacterial genus *Phyllobacterium* increased significantly at 1,800 m, which was associated with increased richness in plant coverage. The association was likely because *Phyllobacterium* transiently or continuously inhabits plant leaves, and its numbers vary among plant species (Coutinho and Bophela, 2021). Thus, the thick litter layer provides an excellent environment for the genus. Although there was dissimilarity in fungal communities among elevations, the dissimilarities were not controlled by single soil property. Fungi have important ecological roles as decomposers, mutualists, and pathogens of plants (Shen et al., 2020). Thus, because of the many differences in fungi, such as *Chaetothyriaceae* and *Hypocreaceae*, that occurred at 1,800 m where the vegetation was most diverse, it was hypothesized that there was a significant difference in the plant type-fungal community along the elevational gradient in this study. Members of *Chaetothyriaceae* have been reported since the 19th century as sooty molds, which gain nutrients from sugary exudates, after their ascomata were found adpressed to the surface of leaves and stems (Tian et al., 2021). Culture and sequence data are available for only a fraction of the *Chaetothyriaceae*. Appropriate description of the *Chaetothyriaceae* is therefore not yet possible. However, life cycles of the family have been described in relation to plants, especially lichens, which led to the expansion of cytochromes, providing windows of opportunity for diversification (Quan et al., 2020). Similarly, most *Hypocreaceae* are free-living, avirulent plant symbionts, commonly in all types of soils inhabiting root ecosystems (Barman et al., 2021). The fungi can protect plants by improving vegetative growth and stimulating natural resistance (Barman et al., 2021). Therefore, both *Chaetothyriaceae* and *Hypocreaceae* were strongly influenced by vegetation type and litter. However, the type of vegetation involved is uncertain, and that determination will be part of future research.

## 4.4. Linkages between soil microbial communities and enzyme activity

In this study, sucrase and catalase activities decreased with elevation, but those of urease, ACP, and β-glucosidase did not (Figure 1B). It was hypothesized that in addition to soil chemical factors, soil microbiological factors also had important effects on enzyme activities (Salazar et al., 2011; Jain and Pandey, 2016; Looby and Treseder, 2018). Each soil enzyme is regulated by different soil nutrients (Salazar et al., 2011; Jain and Pandey, 2016), which is consistent with the results in this study. Soil chemical factors associated with catalase were consistent with those affecting bacterial community diversity (Supplementary Tables S3, S5), that is, catalase activity was regulated by the indirect influence of the soil environment on bacterial communities. In addition, the correlation between catalase and dominant microbes (Figure 5) also supported that conclusion, because catalase was almost entirely regulated by bacteria. In previous studies characterizing humus intensity and organic matter conversion rate in soil, catalase has indeed been shown to have a close relation with bacteria (Zhu et al., 2018). Notably, *Acidobacteria* and *Tepidisphaeraceae* were significantly positively correlated with catalase (Figure 5). In a recent report, *Acidobacteria* were responsible for a

substantial fraction of catalase transcripts to increase $H_2O_2$ detoxification in a western Lake Erie *Microcystis* bloom community, despite relatively low abundance (Smith et al., 2022). Therefore, combined with results in this study, *Acidobacteria* express catalase well in both terrestrial and aquatic ecosystems. *Thelephoraceae* is a new family in the phylum *Planctomycetes*, and therefore, sufficient reports about its correlation with catalase are not yet available.

In contrast to catalase, ACP did not decrease with elevation but showed a hump-shaped pattern (Figure 1), which was possibly related to its complex sources. First of all, plant roots, microorganisms, and fauna all secrete ACP to increase soil P availability (Spohn and Kuzyakov, 2013). However, soil ACP was mainly regulated by bacteria in this study (Figure 5). Because bacteria are most likely to secrete ACP in neutral or acidic soil environments (Fraser et al., 2017), which could be why acid phosphatase was negatively correlated with soil pH value (Supplementary Table S2). In addition, bacteria secrete ACP as a by-product of C metabolism (Luo et al., 2017). So, ACP was significantly positively correlated with organic matter (Supplementary Table S2). Although fungi in the form of mycorrhizae promote the secretion of ACP by plant roots (Eisenlord and Zak, 2010), the highest content of ACP was not at 1,800 m, which had the highest vegetation richness, indicating that fungi were not the dominant source of ACP in this region. The *Planctomycetaceae* includes major bacteria in global N and C cycles (Strous et al., 1999). In addition, the *Planctomycetaceae* are also typical phosphate-solubilizing bacteria, which contain *phoD* or *phoC* genes (Zhao et al., 2022). Therefore, the *Planctomycetaceae* have important roles in biogeochemical cycles.

Except for ACP and catalase, the other enzymes were most influenced by fungi. The enzyme β-glucosidase was only controlled by fungi, such as *Hygroraceae*, *Herpotrichiellaceae*, and *Chaetomiaceae* (Figure 5), which might have been influenced by plant litter. Because β-glucosidase activity was correlated with SOC, TP, and AP (Supplementary Table S2), those fungi were controlled by those nutrients. As noted in 4.1, there were relations between plant litter and SOC and P. *Herpotrichiellaceae* were important fungi in this ecosystem, because the family was positively correlated with β-glucosidase and sucrase (Figure 5). However, the *Herpotrichiellaceae* includes well known pathogens, with several species associated with infections in humans and animals (Yang et al., 2021). In fact, according to metagenomic data analysis, the largest number of species in the *Herpotrichiellaceae* are found in soil-associated material and in plants, because the oligotrophic nature of these fungi enables them to survive in adverse environments where common saprobes are absent (Costa et al., 2020). Unfortunately, research on the family remains in its infancy, and little is known about its various metabolic capabilities. Urease is responsible for decomposition of N compounds. In this study, urease was not correlated with soil properties (Supplementary Table S2) but was significantly positively correlated with *Sebacinaceae* (Figure 5). Molecular and ultrastructural studies reveal a broad diversity of mycorrhizal associations involving members of the heterobasidiomycetous *Sebacinaceae*, which are fungi, owing to the inconspicuous basidiomes, have been often overlooked. As mycobionts, the fungi in the family have important roles in N accumulation in soils (Yang et al., 2022). Fungi in the *Sebacinaceae* are enriched at the early climax forest stage and decrease soil organic N accumulation by expediting the decomposition of soil organic matter. As a result, the *Sebacinaceae* use C to convert organic N for use by plant or microbes. Thus, a significant positive correlation between *Sebacinaceae* and urease is plausible. To conclude, the significant differences in soil enzyme activities among elevations were caused by differences in microbial communities.

## 5. Conclusion

In this study, although the soil chemical environment was not significantly affected by elevation, bacterial and fungal communities were significantly different at different elevations. Diversity of overall fungal community showed a significant N-shaped pattern with an increase in elevation, whereas diversity of the overall bacterial community decreased significantly with an increase in elevation. Differences were correlated not only with soil P and plant litter but also with changes in dominant microbes. The taxa of bacteria and fungi with differences were the richest at 1,800 m, but only bacteria with differences were significantly correlated with soil pH. Soil enzyme activities were different among elevations and were controlled by different microbes. The enzymes catalase and ACP were mainly controlled by *Acidobacteria* and *Planctomycetaceae* bacteria, respectively, whereas β-glucosidase, sucrase, and urease were mainly controlled by fungi. β-glucosidase and sucrase were both positively correlated with *Herpotrichiellaceae*, and urease was positively correlated with *Sebacinaceae*. Thus, the changes in microbial diversity and soil enzyme activities along the elevational gradient were associated with changes in the soil chemical environment and plant litter.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

## Author contributions

TH proposed the study, designed the experiments, and participated in supervision. YW analyzed data and wrote the manuscript. XW and LM conducted experiments. XW and XY participated in field investigation. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1217925/full#supplementary-material

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# Responses of a soil fungal community to severe windstorm damages in an old silver fir stand

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Forests are increasingly threatened by climate change and the Anthropocene seems to have favored the emergence and adaptation of pathogens. Robust monitoring methods are required to prevent biodiversity and ecosystems losses, and this imposes the choice of bioindicators of habitat health. Fungal communities are increasingly recognized as fundamental components in nearly all natural and artificial environments, and their ecosystem services have a huge impact in maintaining and restoring the functionality of ecosystems. We coupled metabarcoding and soil analyses to infer the dynamics of a fungal community inhabiting the old silver fir stand in Vallombrosa (Italy), which is known to be afflicted by both *Armillaria* and *Annosum* root rot. The forest was affected in 2015, by a windstorm which caused a partial falling and uprooting of trees. The remaining stand, not affected by the windstorm, was used as a comparison to infer the consequences of the ecosystem disturbance. We demonstrated that the abundance of pathogens alone is not able to explain the soil fungal differences shown by the two areas. The fungal community as a whole was equally rich in the two areas, even if a reduction of the core ectomycorrhizal mycobiome was observed in the wind-damaged area, accompanied by the increase of wood saprotrophs and arbuscular mycorrhizas. We hypothesize a reshaping of the fungal community and a potentially ongoing re-generation of its functionalities. Our hypothesis is driven by the evidence that key symbiotic, endophytic, and saprotrophic guilds are still present and diversified in the wind-damaged area, and that dominance of single taxa or biodiversity loss was not observed from a mycological point of view. With the present study, we aim at providing evidence that fungal communities are fundamental for the monitoring and the conservation of threatened forest ecosystems.

KEYWORDS

**fungal community ITS, forest disturbance, *Armillaria* and *Annosum* root rot, Vallombrosa forest, *Abies alba* ecosystem**

## 1. Introduction

Forest disturbances associated with extreme events and natural disasters are important drivers of forest ecosystem development. Furthermore, they are expected to continuously increase in intensity, quantity, and frequency in the coming years, seriously threatening the world's forest (Masiero et al., 2019). Strong winds, one of the major natural disturbances for European forests (Seidl et al., 2011; Forzieri et al., 2020), have intensified over the last decades

globally and their natural and socio-economic consequences can be especially critical (Romagnoli et al., 2023). Climate change will make these destructive windstorm events more frequent and more intense with damage and destruction of thousands of hectares of forests (uprooting and breaking trees) and millions of cubic meters of timber lost (Patacca et al., 2023). Many destructive severe windstorm were recorded in Europe in recent years: windstorm Lothar (1999) caused the loss of 165 million $m^3$ of timber in France, Germany, and Switzerland; windstorm Gudrun (2005) 75 million $m^3$ in Sweden; windstorm Kyrill (2007) 49 million $m^3$ in Germany and the Czech Republic; windstorms Klaus (2009) in France and Xynthia (2010) in Spain a total of 45 million $m^3$; windstorm Vaia (2018) in Italy 8.5 million $m^3$ (Forzieri et al., 2020).

An extreme wind event, also affected in 2015 the Vallombrosa forest, one of the most famous and studied forests in Italy (northern Apennines), the birthplace of the Italian Forestry School in the late 19th century, and today a teaching forest for students of Forestry and Environmental Sciences at the University of Florence. On the night of March 5, 2015, between 15 and 20 thousand trees (about 50 ha) were blown down in the forest by a hurricane, with wind gusts reaching 150–160 kilometers per hour (Chirici et al., 2019; Dálya et al., 2019).

The forest of Vallombrosa, today a Biogenetic State Nature Reserve and a Natura 2000 Site, is also famous because the monks of the Vallombrosa Abbey (Benedictine order) in the XVII century began a centuries-old tradition, widespread to much of Central Europe, of growing pure and coetaneous silver fir (*Abies alba* Mill.) stand that still characterizes part of the forest landscape (Ciancio and Nocentini, 2011) that is now managed by the State body Carabinieri Forestali.

During an investigation in this forest Farina et al. (1990) observed the massive presence of *Heterobasidion abietinum,* which is among the most destructive forest pathogens commonly associated with European silver fir, and other species of the genus Abies (Gonthier and Thor, 2013). *H. abietinum* causes root rot and decay of the stem, which typically leads to decrease of the tree stability and uprooting under certain stressful conditions (Honkaniemi et al., 2017). A severe windstorm occurred in April 2015 at the Nature Reserve of Vallombrosa and, as a consequence, about 50 ha of forest were destroyed. In an investigation after the severe windstorm damage in the forest of Vallombrosa, Dálya et al. (2019) assessed the distribution of *Heterobasidion abietinum*, the presence of which had been already reported by Farina et al. (1990), and *Armillaria* spp. which had only been observed sporadically and are among the most destructive forest pathogens in the world. In this research, *H. abietinum* presence was confirmed and extended at two new localities at upward elevation, an occurrence probably favored by climate change. Four species of *Armillaria* (*A. cepistipes*, *A. ostoyae*, *A. gallica*, *A. mellea*) were found in the area of the Nature Reserve of Vallombrosa, among which the most frequent species was *A. cepistipes*, followed by *A. ostoyae*, which was often detected just in soil samples from plots cultivated with conifers (Dálya et al., 2019).

Both *H. abietinum* and *A. ostoyae* are known as fearsome silver fir pathogens, typically show higher incidence in artificial stands derived from monospecific and coetaneous plantations exposed to climatic stress (La Porta et al., 2008). An additive effect to the high presence of *H. abietinum* is also the 'history' probably related to the development of the existing microbial community. In fact, a higher presence of this pathogen has been observed in former cropland or former pastureland than in forest soils (Puddu et al., 2003), condition very frequent in some areas of the Vallombrosa forest (Galipò et al., 2017; Caramalli et al., 2020).

The role played by the telluric pathogens described above in the event that led to the uprooting of many silver firs in Vallombrosa is still much debated. In areas with such a disturbance the status and resilience of the soil microbial community, which is fundamental for the forest ecosystems functioning, are little known. Identifying the drivers of microbial community stability is crucial for predicting community response to disturbance. These drivers are defined as 'keystone taxa', capable of influencing the community structure through strong interactions with the environment or with other members of the microbiome in co-occurrence networks (Trivedi et al., 2020). Among forest microorganisms, fungi have several key roles as decomposers of the organic matter and as plant symbionts, and support numerous ecosystem services, acting as a crucial tool for the adaptation of forests to climate change (Van der Heijden et al., 2015; Mello and Balestrini, 2018; Sapsford et al., 2021). Recent metabarcoding studies targeting the rDNA Internal Transcribed Spacer (ITS) have been shown to be the best tools to describe fungal communities (Nilsson et al., 2019) and to identify keystone taxa.

In order to assess the soil microbial community in the silver fir stand at 'Metato' (Natural Reserve of Vallombrosa, Reggello, Florence), affected by *H. abietinum* and *Armillaria* spp. and characterized by both presence of undamaged and wind-damaged areas (stumps), we profiled soil fungal communities by using metabarcoding, and combined sequencing results with soil physico-chemical parameters. The objectives of this work were the following: to investigate relations in the soil fungal community associated with *A. alba* undamaged and wind-damaged areas. We hypothesize that an ecological succession has occurred since the windstorm event, and hence that each condition is associated to a peculiar functional guild: for instance *A. alba* undamaged trees associate with ectomycorrhizal fungi, while stumps and the surrounding vegetation cover associate with Mortierellomycetes, arbuscular mycorrhizal fungi, and lignicolous fungi.

## 2. Materials and methods

### 2.1. Description of the site, soil sampling and soil physico-chemical analyses

The investigated mature and pure silver fir stand of approximately 11 ha called 'Metato', a stone hut in a chestnut grove, for drying chestnuts, which, piled on mats, are subjected to moderate heat, known as a former farm until the late 1800s (Galipò et al., 2017), is located in the Vallombrosa Biogenic reserve (Tuscan Apennines in Florence district, Italy). The climate is characterized by a mean annual temperature of 9.8°C and a mean annual precipitation of 1,275 mm (thermopluviometric station of Vallombrosa, 980 m a.s.l.; Dálya et al., 2019). The trees in the area are known to be affected by *Annosum* and *Armillaria* root rot, which may have been a possible contributing factor of tree uprooting as a consequence of a windstorm in 2015, therefore segmenting the forest in two areas 'undamaged' and 'wind-damaged' (Figure 1) where trees were felled down by the hurricane and an extensive gap in forest cover was created. According to the


FIGURE 1
Wind-damaged **(A)** and undamaged **(B)** areas at the "Metato" site. The gaps in the forest caused by the windstorm allowed an increase of grasses and undergrowth. By contrast, the dense canopy cover in the undamaged area impedes this phenomenon.

pedological map of Tuscany Region, the soil is mainly classified as partially humic dystrudepts, coarse-loamy, mixed, mesic.

Soil collection was carried out in two areas located at least 20 m apart from each other: one of 59,400 $m^2$ characterized by undamaged trees and the other of 50,600 $m^2$ by wind-damaged stumps. Sampling was done under six silver fir trees and six stumps: five soil cores (200 $cm^3$ each) of topsoil from underneath the litter layer were collected around two meters from each tree or stumps in opposite directions, and at least two meters from each other to avoid sampling of the same genet. Each biological replicate consisted in a pool of five soil samples (coarse roots and stones were removed), resulting in a composite soil sample for each tree. A subset of each sieved, composite soil (500–750 g; mesh size 1 mm) was used for physico-chemical analyses, and another for microbial community analysis by metabarcoding.

Soil samples were heated overnight at 105°C to determine their water content (ISO 11465:1993), while sieving and sedimentation were used for texture evaluation (ISO/FDIS 11277). A potentiometric evaluation was performed to determine the pH of each sample, using a mix of one part air-dried soil and 2.5 parts deionized water (1:2.5; samples were left to equilibrate overnight). $BaCl_2$-based compulsive exchange method (ISO 11260:2018) was used to quantify cation-exchange capacity (CEC) and soil exchange acidity. Exchangeable cations $K^+$, $Na^+$, $Mg^{2+}$, $Ca^{2+}$ were determined by a 1100B Atomic Absorption Spectrometer (Perkin Elmer, United Kingdom). Total C and N were determined using a NA 1500 CHNS Analyzer (Carlo Erba, Italy). Phosphate was extracted from soil samples using HCl and $NH_4F$, therefore removing acid-soluble P forms (Bray and Kurtz, 1945). The R package ggridges v0.5.3[1] was used for density plots showing the results of all the physico-chemical analyses. Significant differences between undamaged and wind-damaged samples were determined with Kruskal-Wallis test in R at a 0.05 value of *p* threshold.

1 https://CRAN.R-project.org/package=ggridges

## 2.2. DNA extraction, sequencing, and OTU table generation

Twelve silver fir trees and stumps (6 vs. 6) were considered for the analysis. Three DNA extractions (technical replicates) have been performed for each composite soil sample with the FastDNATM SPIN Kit for Soil (MP Biomedicals, Europe). A total of 36 DNA samples were therefore diluted 1:10 (5–10 ng/μl) and amplified with a nested PCR approach targeting the Internal Transcribed Spacer 2 (ITS2) (Nilsson et al., 2019). A first amplification was done with the ITS1/ITS4 primers couple (5'-TCCGTAGGTGAACCTGCGG-3′ and 5'TCCTCCGCTTATTGATATGC-3′, respectively), for 25 cycles at 52°C annealing temperature. The PCR products were again amplified in a second round with the ITS9f/ITS4r primers (5'-GAACGCAGC RAAIIGYGA-3′; 5'-TCCTSCGCTTATTGATATGC-3′) to which Illumina adapters have been added (TCGTCGGCAGCGTCAG ATGTGTATAAGAGACAG and GTCTCGTGGGCTCGGAGAT GTGTATAAGAGACAG, respectively), for 35 cycles at 54°C annealing temperature. The libraries have been sequenced at IGA technologies (Italy) using Illumina MiSeqTM with a paired-end strategy (2×300 bp, NexteraXT index kit) producing 10 million reads in output. FastQC (Andrews et al., 2012) was used for quality assessment of the libraries, and primers were removed with Cutadapt v3.4 (Martin, 2011). The trimmed libraries were processed within the DADA2 pipeline v1.18.0 (Callahan et al., 2016). Quality trimming was achieved with the "filterAndTrim "function ["maxEE(2,7)"], setting a minimum length threshold of 165 bp for trimmed reads. Error models were produced through the evaluation of 1E8 bases, and errors were removed from de-replicated reads using pseudo-pooling with the "dada" function. Merged forward and reverse reads were subjected to *de novo* and reference-based chimera screening using DADA2 and VSEARCH v2.17.0 (Rognes et al., 2016), respectively. For the latter case, the UNITE v8.3 fungal ITS database (Nilsson et al., 2019) was used as reference. ITS2 sequences were extracted from the dataset with ITSX v1.1.3 (Bengtsson-Palme et al., 2013). The dataset was then processed

with QIIME2 v2020.11 (Bolyen et al., 2019) clustering the extracted sequences into OTUs at 97% identity.

## 2.3. Taxonomy assignment and validation

Centroids were used for a BLASTn v2.11 (Camacho et al., 2009) search against the nt database. Taxonomy annotation was achieved with the "Assign-Taxonomy-with-BLAST" scripts,[2] using the following criterion: a maximum of 10 BLAST hits were considered if they fell in a 0.5% identity interval based on the best BLAST hit. For example, if an OTU had 100% identity with its best BLAST hit, other BLAST hits were considered only if they had at least 95% identity with the OTU. Species-level annotations were considered valid based on a 97% identity threshold. Family- or phylum-level annotations were instead assigned based on 80 and 75% identity thresholds, respectively. OTUs with divergent taxonomy assignment were resolved with a phylogenetic approach. For each of these OTUs, the whole sequence cluster and its best BLAST hits were aligned with MUSCLE v3.8.31 (Edgar, 2004). The alignments were processed with Gblocks v0.91b (Talavera and Castresana, 2007) with relaxed parameters, and phylogenetic trees were produced with FastTree v2.1 (Price et al., 2010). Trees were inspected to manually curate the inferred phylogenetic relationships, allowing to deal with "unidentified" taxonomies which are abundant in the NT, and that were the most common cause of taxonomic inconsistency in OTUs. After taxonomic assignment, OTUs that did not belong to the Fungal Kingdom were discarded. Functional guilds were assigned to each OTU with FungalTraits v0.0.3 (Põlme et al., 2020), and manually curated by an expert mycologist.

## 2.4. Core community identification, alpha and beta diversity measures, and correlation analyses

Prior to fungal community analyses, the OTUs abundances were normalized using median library size, according to the protocol proposed in the phyloseq v3.12 package (McMurdie and Holmes, 2013). Taxonomy plots were generated combining the "subset_taxa" phyloseq function and the "ggstripchart" function of the ggpubr v0.4.0 package (Kassambara, 2020). The R packages phyloseq, microbiome v1.13.3 (Lahti and Shetty, 2012), and ggplot2 v3.3.3 (Wickham, 2009) were used for the core community analysis in accordance with Shetty et al. (2017). In this case, the ratio between the abundance of each family and the abundances of all other taxa (relative abundance) was plotted. Taxa with a relative abundance <0.01%, or that were above this threshold in less than 60% of samples were discarded. Statistical significance of core relative abundances between healthy and diseased samples was calculated with Kruskall-Wallis test ($p < 0.05$). Alpha- and beta-diversity values were computed with phyloseq and QIIME2, respectively. The correlation between the abundance of *Armillaria* and *Heterobasidion* pathogens, and soil physico-chemical parameters was calculated with the base "cor" R function and plotted with ggplot2. The correlation threshold for the analysis was set to 70%.

2 https://github.com/Joseph7e/Assign-Taxonomy-with-BLAST.git

## 2.5. Differential taxa abundances

DESeq2 v1.30.1 (Love et al., 2014) was used for the final estimation of differentially abundant OTUs, starting from raw abundances. The DESeq2 pipeline involved physico-chemical soil parameters measured for each sample, and the included variables were selected as follows: at first, to avoid multicollinearity among covariates, the correlation between these variables was calculated with the "cor" base R function, and a 70% threshold was set to discard highly correlated variables. The correlation plot was generated with the pheatmap package v1.0.12 (Kolde, 2019). A Redundancy Analysis (RDA) plot was then produced using the RAM 1.2.1.7 package (Chen et al., 2018) to screen for variables influencing the distribution of samples. The RDA analysis was coupled with a variance partitioning analysis in Vegan v2.5–7 (Oksanen et al., 2020). Statistical support was calculated with ANOVA ($p < 0.05$). Uncorrelated variables that significantly impacted the variance in the dataset were finally used as covariates in the DESeq2 design formula. The dataset was further filtered to include only OTUs that had a summed abundance >10. DESeq2 was run with the "betaprior" option, and the "contrast" function has been used to calculate differential abundances (the wind-damaged condition was used as numerator), at an adjusted value of $p$ threshold of 0.05. The results were plotted using metacoder v0.3.4 (Foster et al., 2017).

# 3. Results

## 3.1. Soil analyses and taxonomy overview

The soil samples obtained from the surroundings of stumps from the wind-damaged area had higher humidity, pH, and phosphate content (Figure 2). The other analyzed soil parameters did not differ between the two conditions (wind-damaged and undamaged). The final OTU table obtained from the fungal sequences extracted from the whole silver fir stand soil is shown in Supplementary Table S1. The composition of the fungal community in the two areas showed the overall prevalence of the phylum Basidiomycota (Figure 3). However, this dominance was attenuated in soils sampled in the wind-damaged area, where Mucoromycota showed quite high relative abundance. Inside the Basidiomycota phylum, Agaricomycota were the dominant class in both areas. Mucoromycota were mostly represented by Mortierellomycetes in the undamaged area; by contrast, Glomeromycetes and Endogonomycetes contributed to the increase in Mucoromycota observed in the wind-damaged area. Ascomycota were well-represented in both sites, with an even distribution of abundances in Dothideomycetes, Eurotiomycetes, Pezizomycetes, and Sordariomycetes.

## 3.2. Pathogen detection and core community analysis

At first, we manually searched for the presence of the two root-rot agents, *Armillaria* and *Heterobasidion*, in the dataset: both were present irrespective of the undamaged and wind-damaged conditions, even if absent from several samples (Supplementary Table S1). We could not obtain a species-level classification for the two OTUs corresponding to *Armillaria* and *Heterobasidion* but, according to

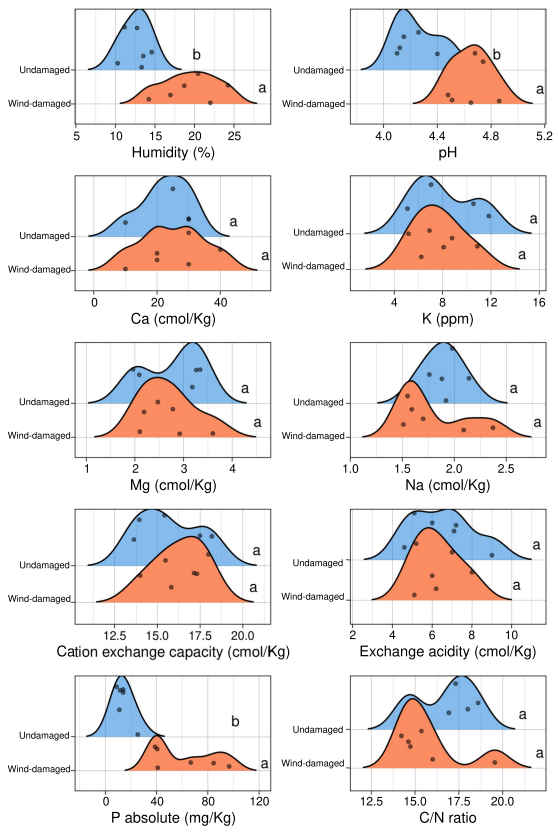


FIGURE 2
Physico-chemical characteristics of soils collected near the same stumps and undamaged trees that were selected for metabarcoding. For each measurement, the density plots show the number of samples (represented by dots) having a specific value in the wind-damaged or undamaged area. Statistical significance is shown in letters (Kruskall-Wallis test 0.05 threshold).

literature data, the most abundant species found were *A. ostoyae* and *H. abietinum* (Dálya et al., 2019). We also checked whether the presence of *Armillaria* and *Heterobasidion* was correlated with any of the measured soil properties (Figure 2), irrespective of the sampling area, but found that none of the measured parameters influenced the abundance of the pathogens (Supplementary Figures S1).

At the family-level, the core microbiome of the whole site was represented, after unknown families, by Mortierellaceae (with at least 50% relative abundance in 10% of the samples) followed by families such as Cortinariaceae (mostly ectomychorrhizal), Russulaceae, and Hydnaceae (Mycorrhizal; Figure 4A). This core community was overall less represented in soil samples from the wind-damaged area (Figure 4B).

## 3.3. Alpha diversity analyses

With alpha diversity calculations, we aimed at investigating a potential impact on fungal biodiversity in soil as a consequence of the fall of the trees, and of the subsequent creation of gaps in the forest. At first, we used all the taxa in the dataset to obtain different alpha diversity measures (Figure 5). Only Faith's phylogenetic diversity (PD) index allowed us to detect a higher biodiversity in the wind-damaged area. Simpson and Shannon indices are preferentially used to calculate dominance and diversity, respectively, while Faith's PD has been frequently used to measure the diversity of fungal successions (Zhang et al., 2018; Matsuoka et al., 2019; Adamo et al., 2021). The latter is fitted on a phylogenetic backbone, and it does not consider OTUs individually, but also as a function of their phylogenetic distance. Indeed, the index is based on the sum of all branch lengths observed in the OTUs tree. The generation of OTUs (and their number) is biased by the low taxonomic resolution typical of fungal amplicons, which makes OTUs richness a weak measure for fungal biodiversity (Song, 2023). By contrast, phylogenetic reconstructions might help mitigating such phenomenon: indeed, two OTUs that were erroneously considered as different taxonomic entities would be very closely related in a phylogenetic tree, lowering their overall impact on biodiversity measures. PD has been indeed noted to be more sensitive in investigating biodiversity data (Armstrong et al., 2021). Therefore, we decided to use this index for further analyses, even if it introduces an evolutionary point of view that does not fit the present investigation.

To further investigate the impact of fall of the trees on the alpha diversity of specific functional guilds, we measured the same alpha diversity indices on the ectomycorrhizal and wood-decay communities separately (Supplementary Figures S2, S3). Faith's PD was higher for the wood-decay community in the wind-damaged area; by contrast, all alpha diversity indices indicated a strong reduction of the ectomycorrhizal diversity as a consequence of fall of the trees.

## 3.4. Beta diversity analysis and differential taxa abundance

To further highlight compositional differences of the mycobiota between soils sampled in the wind-damaged and undamaged area, we used both unweighted (phylogeny-based) and weighted (which further adds abundance data to the phylogeny-based method) UniFrac indices (Lozupone and Knight, 2005). In both cases beta diversity revealed a clear separation between soils from the wind-damaged area *vs* those sampled in the undamaged area (Figure 6).

Next, to identify the OTUs driving these differences, a differential taxa abundance analysis was performed. Starting from the physico-chemical soil parameters that significantly differed between the two areas (Figure 2), we checked whether some of them influenced the ordination of the different samples. We removed pH, which was highly correlated with P content and humidity (Supplementary Figures S4): the choice of removing pH was due to the fact that P content and humidity were not significantly correlated, and therefore this allowed to remove only one variable. An RDA plot showed that exchange acidity, C/N ratio, cation exchange capacity, and humidity might have an influence on taxa composition (Supplementary Figures S5). Therefore, we included the above-mentioned soil parameters in the statistical model to infer differential taxa abundances. Confirming the overall screening of the OTU table, *Armillaria* and *Heterobasidion* OTUs were not among the differentially abundant taxa (Figure 7). By contrast, several dead wood-associated fungi such as *Pluteus*, *Mycena*, *Byssocorticium,* and Meripilaceae were more abundant in the area affected by falling of the trees, whereas ectomycorrhizal fungi such as *Amanita,*

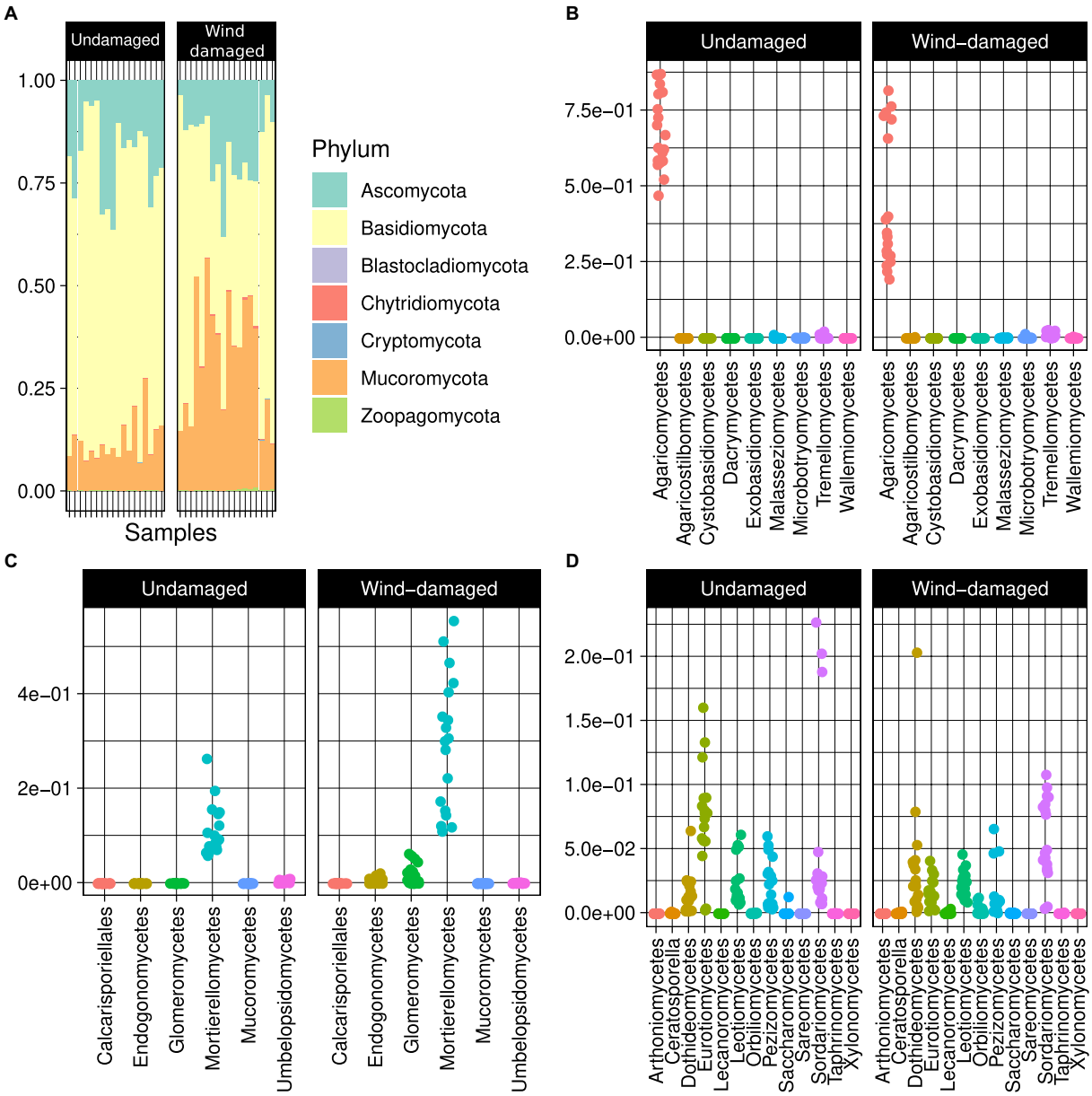


FIGURE 3
Overall composition of the fungal community in Vallombrosa. **(A)** Basidiomycota were predominant overall in terms of relative abundance (i.e., their abundance over the abundance of all OTUs). The classes Agaricomycetes **(B)** and Mortierellomycetes **(C)** were predominant in Basidiomycota and Mucoromycota, respectively, while Ascomycota **(D)** had a more even class distribution. Dots in **(B)**, **(C)**, and **(D)** represent samples and are distributed according to the relative abundances of each class (y axis).

Boletaceae, *Cortinarius*, *Lactarius*, *Leucogaster*, *Tomentella*, and *Tricholoma* mostly underwent a depletion in such area. By contrast, *Hygrophorus*, *Pseudosperma*, *Sebacina* were more abundant in the wind-damaged area, compared to the undamaged one. In addition, *Tephrocybe* was more abundant in the wind-damaged area. Among Ascomycota, *Trichoderma* was more abundant in the wind-damaged area, while the ectomycorrhizal *Otidea*, *Tuber*, and *Trichophaea* were depleted (Supplementary Figures S6). Finally, the Arbuscular Mycorrhizal (AM) fungus *Paraglomus laccatum* and Endogonales, preferentially associated with herbaceous plants or hornworts and liverworts (Van der Heijden et al., 2015), were more abundant in the wind-damaged area (Supplementary Figures S7).

# 4. Discussion

## 4.1. Are root rot pathogens responsible for habitat disruption in Vallombrosa?

The effort of the scientific community in understanding the weakening of forests led to the conclusions that (a) the fitness of major plant pathogens increased in the Antrophocene, mainly due to climate change (Stenlid et al., 2011; Hessenauer et al., 2021), (b) the presence of these pathosystems is deeply integrated in habitat ecology, eventually contributing to biodiversity shifts (Winder and Shamoun, 2006; Gomdola et al., 2022), and (c) extreme meteorological events

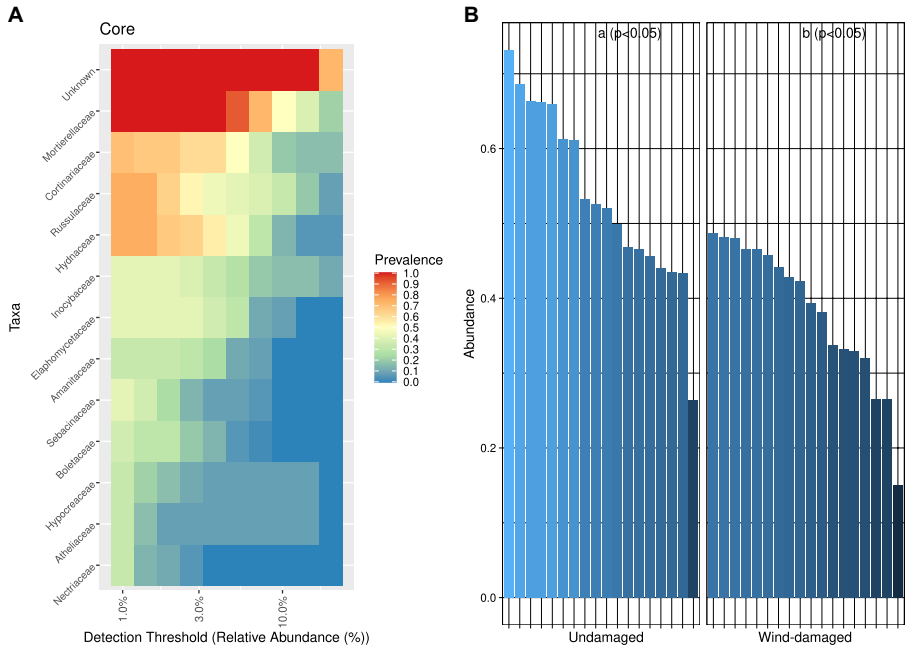


FIGURE 4
Core fungal community analysis. **(A)** Core community composition in terms of predominance, i.e., the fraction of samples (1 = all samples and 0 = no sample) in which a specific family had at least the relative abundance defined on the x axis. For example, based on the representation, Mortierellaceae were present at 3% relative abundance in all samples, and at 10% ~50% of samples. **(B)** For each sample and sub-area, we divided the abundances of OTUs representing the core mycobiome, by the abundance of all OTUs (relative abundance). The core fungal community was more abundant in the undamaged area (Kruskall-Wallis test at $p < 0.05$).

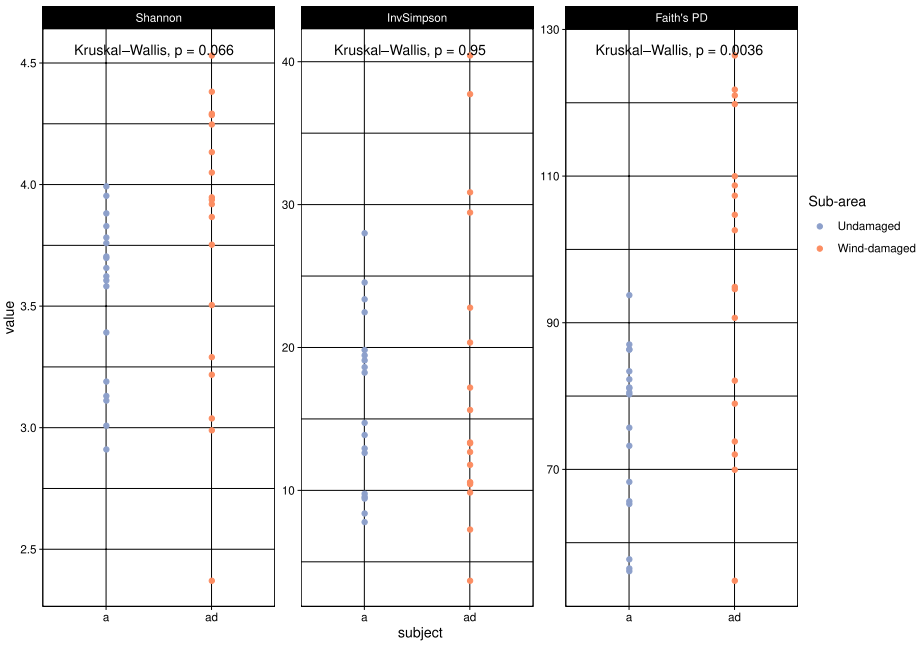


FIGURE 5
Alpha diversity calculations. The color codes represent undamaged (a) vs wind-damaged (ad) samples, and are placed on the vertical axis based on their alpha diversity values according to each index. Significance values were calculated with ANOVA, at $p < 0.05$.

due to climate change are main contributors in habitat destruction. If root rot pathogens were the only determinants of tree damages, then we hypothesize that a higher abundance of *Armillaria* and *Heterobasidion* should have been detected in the wind-damaged area, a situation that we did not observe. In addition, the investigated site (Metato) is a silver fir stand whose origin dates back to the end of XIX

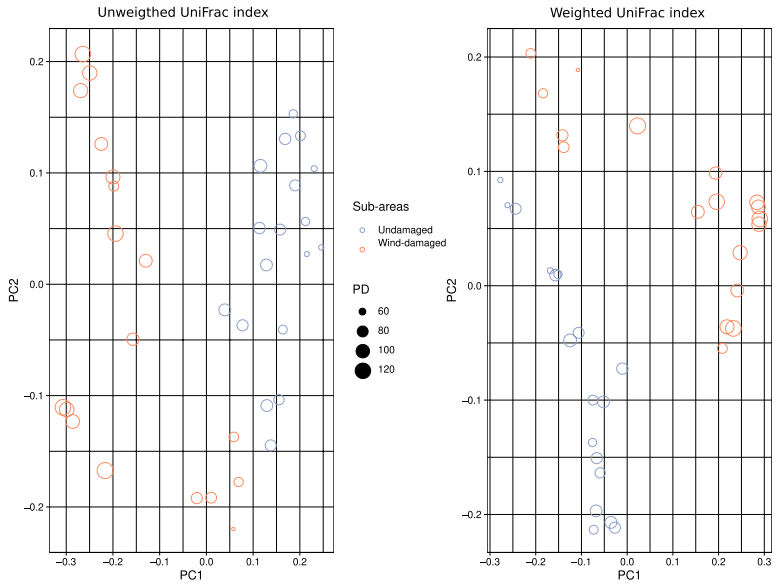


FIGURE 6
Beta diversity calculations. The color and shapes of the dots represent undamaged vs. wind-damaged samples, and both weighted and unweighted UniFrac indices were calculated. The size of each dot (sample) is related to the Faith's PD of that sample. Both indices highlight a separation of the two areas in terms of mycobiota composition.

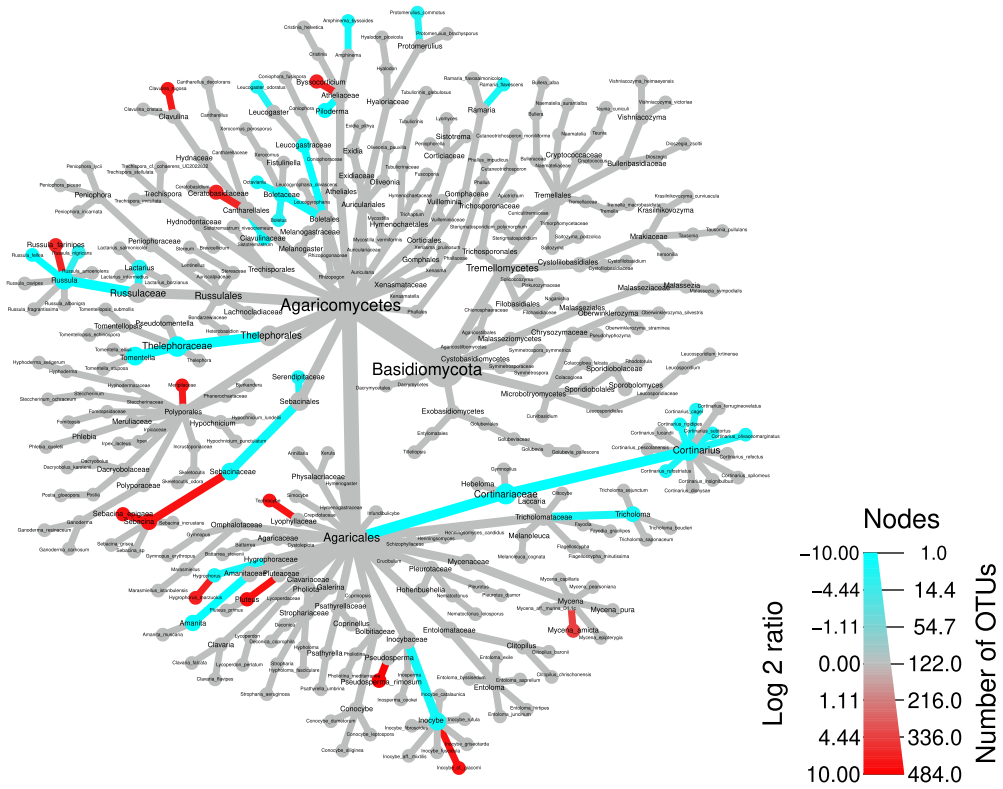


FIGURE 7
Differential abundance tree showing differentially-abundant Basidiomycota in the wind-damaged *vs* undamaged areas. Red color for nodes and edges indicates over-representation in the wind-damaged condition, while blue indicates the opposite. The color intensities were calculated by summing all log2 fold changes of OTUs gathered at each node and branch.

century; the presence of *Armillaria* and *Heterobasidion* spp. was reported in this artificial and coetaneous stand since the early 1900s, while the extreme event that led to falling of the trees and uprooting in a large part of the site took place in 2015 (Dálya et al., 2019). Therefore, the history of co-existence between these pathogens and the residing tree is long, and the wind damages should likely have

manifested earlier. Indeed, the incidence of both diseases is known to be extremely severe in pure and coetaneous stands planted in former agricultural land and in localities where summer drought is exacerbated by climate change (Puddu et al., 2003) as in this case.

We observed a depletion of several ectomycorrhizal taxa in the wind-damaged area, while the same taxa were abundant in the undamaged area. This result leads to the quandary of whether such depletion contributed to the localized wind damages (due to a loss of fitness of the host trees), or whether certain taxa were depleted because of the disappearance of their hosts after the fall of the trees. Evidence collected worldwide agrees that ectomycorrhizal fungi can increase forest resistance to abiotic and biotic stressors, such as fungal pathogens (Suz et al., 2015; Anthony et al., 2022; Authier et al., 2022). However, ectomycorrhizal communities are strongly impacted by climate change (Fernandez et al., 2023), and this could have been one cause of the decline of tree health in Vallombrosa as well. We therefore suggest that a depletion of ectomycorrhizal taxa preceded the observed damages. At the same time, different soil properties such as pH, humidity, and C/N content were altered in the wind-damaged area. Other authors have suggested that soil characteristics can greatly boost the spreading of *Armillaria* and *Heterobasidion* (Singh, 1983; Kubiak et al., 2017; Bruna et al., 2019; Piri et al., 2021). We found no correlation between soil parameters and the abundance of *Armillaria* or *Heterobasidion:* it is possible that, while not altering pathogen abundances, these potential abiotic stressors may have a negative impact on tree fitness and ectomycorrhizal communities. Finally, it is likely that parameters such as C/N ratio and mineral content are directly influenced by the increased amount of dead plant material accumulated after the falling of the trees.

## 4.2. Ectomycorrhizal *Agaricomycetes* and *Mortierellomycetes* as primary components of the mycobiota

Several authors have previously investigated the prominent role of Basidiomycota as biomarkers in forests (Richard et al., 2011; Ruiz Gómez et al., 2019; Venice et al., 2021). Since forests are increasingly threatened by extreme meteorological events due to climate change, studying the impact of these fungi on plant health is crucial for the conservation of biodiversity and for forest management. It has been demonstrated that ectomycorrhizal communities are sensible to habitat fragmentation caused by fires and windstorms (Sapsford et al., 2020; Idbella et al., 2023), even if there is evidence that, despite severe disturbances, the ectomycorrhizal network is affected in its composition but not disrupted (Veselá et al., 2021). The investigated site makes no exception, and a key result in our analyses was that, besides an overall reduction, the ectomycorrhizal community is still detectable in the wind-damaged area, and specific genera are core components of the "Metato" mycobiome. Ectomycorrhizal Ascomycota were also present but, however, were relatively less abundant in the investigated soils. Overall, this diversity and specificity may lead to the hypothesis that an ectomycorrhizal succession is supporting habitat restoration in Vallombrosa. Ectomycorrhizal fungi have different hyphal exploration types that vary from long-range to contact-range. It is possible that, thanks to a long-range hyphal exploration type, they maintain symbiotic associations with living trees in the undamaged area, which is close to the wind-damaged one, while extending their mycelial network in the proximity of fallen trees.

*Mortierella* is a ubiquitous group of soil saprotrophs and endophytes, and is often found as key component of conifer forests mycobiota (Allmér et al., 2006; Wagner et al., 2013; Mikryukov et al., 2021), even if its prominent role was also highlighted in other forests (Venice et al., 2021; Kalntremtziou et al., 2023). The abundance of several, specific *Mortierella* (and higher taxonomic ranks) indicates a major contribution of this fungal group in maintaining the "Metato" ecosystem functioning. Indeed, the presence of some species from this clade was preferentially observed in "sheltering" dead conifer wood (Mikryukov et al., 2021) while others were preferentially found as root endophytes (Summerbell, 2005), pointing out to different fundamental environmental services.

## 4.3. A biodiversity succession, rather than a reduction, characterizes the wind-damaged area

Our analyses indicate that fungal diversity is maintained also in the wind-damaged area By splitting the dataset, focusing the analysis on either ectomycorrhizal or wood-decay taxa, we demonstrated that tree symbionts are indeed depleted in the wind-damaged area, while wood-decayers are likely sustaining alpha diversity in this area. As for concerns the contrasting results obtained with different alpha diversity indices, a possible explanation could reside in the fact that PD is an evolutionary measure that evaluates the phylogenetic distance between the amplicon sequences of each sample. However, if PD shows significant differences while other diversity indices do not, this may be due to the history of the species library, according to the species pool hypothesis: indeed, biodiversity might not only be related with environmental or ecological factors, as it can be strongly limited by the regional species pool. In this context, Shannon and Simpson would be more appropriate indices, and they should not be overlooked also in the present study. However, irrespective of the fact that alpha diversity is equal or lower in the undamaged area, compared to the wind-damaged area, we hypothesize that the whole fungal community in the undamaged area is well-established and specialized. Therefore, redundancy in its taxonomic and functional composition might be expected. By contrast we advocate that, after a relatively recent perturbation, the wind-damaged area might constitute a less-established, re-generating habitat with a larger quantity of dead plant material and a higher degree of invasiveness by both saprotrophs and symbiotrophs. This hypothesis is also in line with the beta diversity results, which demonstrate a different composition of the communities detected with both weighted and unweighted UniFrac indices. For example, the wind-damaged area showed a strong increase in taxa such as *Mycena*, *Byssocorticium*, and *Pluteus*, all able to feed on dead wood, together with the presence of *Tephrocybe*, that grows preferentially in re-generating forest soils after disturbances that include wildfires and chemical treatments (Yamanaka, 2001; Ratkowsky and Gates, 2009; Pulido-Chavez et al., 2023). Finally, we found that the falling of the trees was associated with a higher colonization of soil by AM fungi and Endogonales. This is in line with the fact that tree falling, and the consequent absence of crown (Figure 1), allowed the growth of many grasses and hornworts, which are the preferred hosts for these fungi (Van der Heijden et al., 2015; Chang et al., 2019). As already observed for other forests (Song et al., 2020), the plant succession in the wind-damaged area could stimulate a parallel microbial succession, with

rapid replacement of taxa with increasing adaptability to the rhizosphere.

## 5. Conclusion

In this study, we investigated a portion of a mature silver fir stand that was subjected to trees uprooting after a windstorm and compared it with a close area which was not visibly damaged by the storm. Due to the proximity of the two areas, we exclude the possibility that microclimatic factors drove the differentiation of the two sites in terms of damages caused by meteorological events. Our investigation was also driven by the evidence that the whole site is affected by the root rot agents *A. ostoyae and H. abietinum*, which we excluded as the only cause for the damages. We observed an increase in alpha diversity consequent to tree uprooting, an increase that was driven by wood-decay fungi and likely depends on the increase of dead plant material in the area. Besides saprotrophic fungi, specific mycorrhizal taxa seemed to respond to the plant succession, which involved the increase of grasses and the consequent shift in plant symbionts. From a mycological point of view, the investigated ecosystem resulted to be resilient, rather than resistant (insensitive) to the disturbance. We do not have enough data to infer the outcome of the above-mentioned succession, but we argue that the described fungal community is complete in its trophic components, plastic and not affected by dominance of few species, and that could support the repopulation of the habitat. Any naturalistic forestry management activity, which will promote this natural trend, will certainly favor the resilience of these forest formations to extreme events exacerbated by the climate changes underway, especially with regard to drought and windstorms (Ciancio and Nocentini, 2011). Future development of this approach would arguably require monitoring of the fungal community over time, which would help to identify when adverse conditions take place, and which actors are at play before and after a disturbance.

## Data availability statement

The data presented in the study are deposited in the NCBI database, accession number PRJNA1009257.

## Author contributions

FV: formal analysis, writing–original draft, and investigation. AV: validation and data curation. RD: Resources. GDR: conceptualization and funding acquisition. AM: conceptualization, writing–original draft, and supervision. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1246874/full#supplementary-material

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# Assessing the structure and diversity of fungal community in plant soil under different climatic and vegetation conditions

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**Introduction:** Understanding microbial communities in diverse ecosystems is crucial for unraveling the intricate relationships among microorganisms, their environment, and ecosystem processes. In this study, we investigated differences in the fungal community structure and diversity in soils from two contrasting climatic and vegetation conditions: the Xinjiang western China plateau and the Fujian southeastern coastal province.

**Methods:** A total of 36 soil samples collected from two climatic regions were subjected to high-throughput ITS gene sequencing for fungal community analysis. In conjunction soil physicochemical properties were assessed and compared. Analyses included an examination of the relationship of fungal community structure to environmental factors and functional profiling of the community structure was using the FUNGuild pipeline.

**Results:** Our data revealed rich fungal diversity, with a total of 11 fungal phyla, 31 classes, 86 orders, 200 families, 388 genera, and 515 species identified in the soil samples. Distinct variations in the physicochemical properties of the soil and fungal community structure were seen in relation to climate and surface vegetation. Notably, despite a colder climate, the rhizosphere soil of Xinjiang exhibited higher fungal (α-)diversity compared to the rhizosphere soil of Fujian. β-diversity analyses indicated that soil heterogeneity and differences in fungal community structure were primarily influenced by spatial distance limitations and vegetation type. Furthermore, we identified dominant fungal phyla with significant roles in energy cycling and organic matter degradation, including members of the *Sordariomycetes*, *Leotiomycetes*, *Archaeosporomycetes*, and *Agaricomycetes*. Functional analyses of soil fungal communities highlighted distinct microbial ecological functions in Xinjiang and Fujian soils. Xinjiang soil was characterized by a focus on wood and plant *saprotrophy*, and endophytes, whereas in Fujian soil the fungal community was mainly associated with ectomycorrhizal interactions, fungal parasitism, and wood *saprotrophy*.

**Discussion:** Our findings suggest fungal communities in different climatic conditions adapt along distinct patterns with, plants to cope with environmental stress and contribute significantly to energy metabolism and material cycling within soil-plant systems. This study provides valuable insights into the ecological diversity of fungal communities driven by geological and environmental factors.

KEYWORDS

**ecological diversity, fungal communities, geological and environmental factors, functional and structural traits, fungal diversity**

## 1 Introduction

The diversity of terrestrial ecosystems is correlated with the diversity of organismal adaptations (Lu et al., 2020), with broadleaf forests, coniferous forests, and grassland three of the main vegetation types found in most ecosystems (Geng et al., 2019). These differing vegetation types present significantly varied litter and understory environments. For example, soil organic carbon and soil nutrients in coniferous plantations are significantly lower than those in broadleaf plantations in subtropical China, while soil environmental factors in grasslands are also significantly different from those in coniferous forests (Wang et al., 2010; Zhang et al., 2021). Litter differences between coniferous forests, broadleaf forests and grasslands have been show to reflect differences in microbial diversity (Rime et al., 2015). Many studies have focused on aboveground plant communities in terrestrial ecosystems, examining plant diversity (Isbell et al., 2011), spatial organization and structure of plant communities (Nakagawa et al., 2013), and ecological service functions (Peng et al., 2023). Aboveground plant communities and underground microorganisms are interrelated and interact with each other (Singh and Gupta, 2018). Although soil microorganisms play an important role in the stability and function of terrestrial ecosystems (Singavarapu et al., 2023), our understanding of the relationship between plants and soil microorganisms, particularly fungi remains limited. In addition, the spatial distribution characteristics of soil fungi in terrestrial ecosystems, comparing different climatic regions, has been poorly examined.

Fungi are components of terrestrial biodiversity and play important roles in ecosystem processes including energy cycling, remediation and in-/organic matter turnover, nutrient (e.g., carbon, nitrogen, phosphorus, and water) availability, and soil and mineral formation (Averill et al., 2014; Rusterholz et al., 2023). They play a role in plant and animal growth, development, and parasitism, helping to maintain the stability of ecosystem functions (Chen et al., 2020). Soil fungi, both free living and ecto-/endophytic can affect plant roots through the underground food chain, including altering the physical and chemical properties of the soil (Ding et al., 2019). In turn, plants can affect the surrounding microbial communities through the microenvironment of their roots (Berg and Smalla, 2009). Corresponding soil fungi can also affect aboveground plant communities by changing soil nutrient composition, by altering physicochemical properties of soils, and/or by regulating plant coexistence (van der Heijden et al., 2008). Studying the relationship between aboveground plants and underground soil fungal diversity can help in the development of strategies to improve conservation, maintenance of the stability of terrestrial ecosystems, and stress resistance. Previous studies have shown that soil fungi in different habitats have significantly different growth characteristics and transmission capabilities (Han W. et al., 2021), and fungi are usually highly sensitive to environmental changes (He et al., 2022). However, little is known concerning the extent to which spatial distribution characteristics of soil fungal communities are consistent in ecosystems of the same vegetation type but in significantly different climatic regions.

Soil fungal and vegetation species composition of different habitats depends on the spatial distribution of environmental requirements and conditions, resulting in unique biodiversity patterns (Legendre et al., 2009). Although it is well known that different climatic environments with different vegetation types have different associated microbiomes (Han X. et al., 2021; Fu et al., 2022), far less is known concerning correlations to fungal diversity. Fujian and Xinjiang represent two very different climatic regions, with consequent geographical environments deferring, however, they share some similarity in vegetation type (broad leaved forests), and thus provides a unique opportunity for comparison of soil fungal diversity. Fujian is located in the subtropical monsoon climate zone (coastal Southeast China), has a warm and humid climate, and is rich in vegetation types that include evergreen broadleaf forests and temperate coniferous forests. Xinjiang is in an arid continental climate zone (Western plateau China), where the climate is dry and cold, and the vegetation mainly includes grassland, deciduous broadleaf forests and evergreen coniferous forests. Albeit significantly different, both regions contain broadleaf and coniferous forests, thus providing a unique comparative context to study the structure and diversity of soil fungal communities.

We hypothesized that: (1) differences in microbial diversity and community composition between similar forest types in the two climate zones would correlate with soil physiological parameters, and (2) the relationship between soil microbial communities and surface plant communities would vary between the same vegetation types in the two climatic zones. To address these hypotheses, we used high-throughput sequencing to characterize the microbial community of soil samples in Fujian and Xinjiang. We defined the types of vegetation habitats according to the similarity/differences of vegetation conditions and analyzed the relationship between soil fungal community structure and diversity, plants and abiotic factors in the defined microbial habitats.

# 2 Materials and methods

## 2.1 Site description

This study was conducted in Fujian Province (115° 50′ E-120° 43′ E, 23° 31′ N-28° 18′ N) and Xinjiang Autonomous Region (73° 40′ E-96° 23′ E, 34° 22′ N-49° 10′ N, **Figure 1**). The study area Fujian belongs to one of the five major climate types found in China (National Meteorological Administration [NMA], 1979). The study area of the Xinjiang Autonomous Region belongs to the temperate continental climate type, and the linear distance between the two is 4,283 km. The annual average temperature of Fujian Province in 2021 was 20.8°C, and the annual average temperature of each county/city was between 16.3 and 23.3°C, increasing from north to south. The average precipitation of the province was 1477.1 mm, and the maximum annual precipitation was 1860.7 mm, and the minimum was 1058.5 mm. In 2021, the average temperature in Xinjiang was 8.9°C, with extreme weather patterns that included four defined blizzards, three extreme rainstorms, seven cold waves and two low temperature events within the year, and an average precipitation of 162.2 mm (data from China Meteorological Data Service Center).

## 2.2 Experimental design and sampling

In July 2021, according to the different vegetation types in the study area, evergreen coniferous forests (XZ), deciduous broadleaf forests (XK), grasslands (XC) in Xinjiang and temperate coniferous forests (FZ), evergreen broadleaf forests (FK) and grasslands (FC) in Fujian were selected as sampling areas. Six 10 × 10 m repeated standard plots were established in each vegetation type plot. Each plot followed a five-point sampling method in which five soil samples were collected from the 0–15 cm soil layer near the plant roots. Soil samples from the same grid were thoroughly mixed into a sterile bag. A total of 36 soil samples were collected and divided into two parts, one of which was stored in a refrigerator at −80°C. The other portion of the soil sample was air-dried to constant weight at 30°C. The roots, stones and other debris in the soil samples were removed, and then the soil samples were sieved by 5 and 2 mm for soil physical and chemical properties analysis (Qi et al., 2018). Soil pH value was measured by a glass electrode pH meter, total nitrogen content was determined through the potassium dichromate-sulfuric acid digestion method, total phosphorus was determined using the sulfuric acid-perchloric acid digestion method, total potassium was determined by using a NaOH melting-flame photometer. $Ca^{2+}$ and $Mg^{2+}$ were determined through complex ometric titration, and $Mn^{2+}$ was determined using atomic absorption spectrophotometer. Total phosphorus and total potassium contents were determined as described by Hengl et al. (2021).

## 2.3 DNA Extraction, polymerase chain reaction

Fungal DNA was extracted from 1 g of soil samples using the Fast DNA Spin Kit for Soil kit method. PCR amplification was performed using ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) as fungal universal primers (Zhu et al., 2019). The PCR reaction system included in a 50 μL reaction mixture was: 25 μL 2 × San Taq PCR Mix, 2 μL upstream and downstream primers (10 μmoL/L), 2 μL DNA template, and $ddH_2O$ to 50 μL. The PCR amplification procedure was as follows: pre-denaturation at 95°C for 3 min, followed by 34 cycles, each cycle including 98°C for 1 min, 55°C for 30 s, 72°C for 30 s, and finally 72°C for 10 min.

## 2.4 Bioinformatic analysis

PCR amplification products were sequenced using the PacBio sequencing platform (Beijing Baimaike Biotechnology Co., Ltd). The resultant ITS gene sequences were processed by Lima v1.7.0 software, with barcode CCS identified to obtain the original Raw-CCS sequence data. Primer sequences were identified and removed by Cutadapt 1.9.1 software and filtered to obtain clean CCS sequences without primers. Next, UCHIME v4.2 software was used to identify and remove any chimera sequences to obtain an Effective-CCS sequence, and reads were clustered to obtain OTUs at a similarity level of 97.0% using the Usearch software. Taxonomic annotations were assigned to the feature sequences utilizing the naive Bayes classifier in conjunction with sequence alignment against the UNITE reference database, providing species classification information for each feature. Subsequently, community composition at various taxonomic levels (phylum, class, order, family, genus, species) were determined. Species abundance tables at different taxonomic levels were generated using the QIIME software.

Unless otherwise specified, all data were processed using the R programming language. The significance level for all tests was set at $p < 0.05$. We conducted data normality tests using independent sample *t*-tests. For non-normally distributed data, we applied log10, square root, or sine transformations to achieve a normal distribution. Parametric or non-parametric tests were used for normally or non-normally distributed data, respectively. The correlation analysis between soil physicochemical data in Fujian and Xinjiang was performed using independent sample *t*-tests for normally distributed data or Wilcoxon tests for non-normally distributed data.

Canonical Correspondence Analysis (CCA) was conducted using the vegan package in RStudio to explore the relationship between community structure and environmental factors. In addition, we used the vegan package in RStudio to visualize the similarity and dissimilarity of fungal community structures between the two regions through Non-Metric Multidimensional Scaling (NMDS) plots and Principal Coordinates Analysis (PCoA). Heatmaps depicting the relationship between species and environmental factors were generated using the pheatmap package in RStudio. FUNGuild was employed for predicting the functional profiles of fungal communities. Finally, CCA in R software was employed to reveal the physicochemical parameters that best explained variations in microbial community composition.

To assess the relative importance of stochasticity and determinism in the assembly of fungal communities in two regions, we evaluated the goodness of fit of the Sloan neutral community

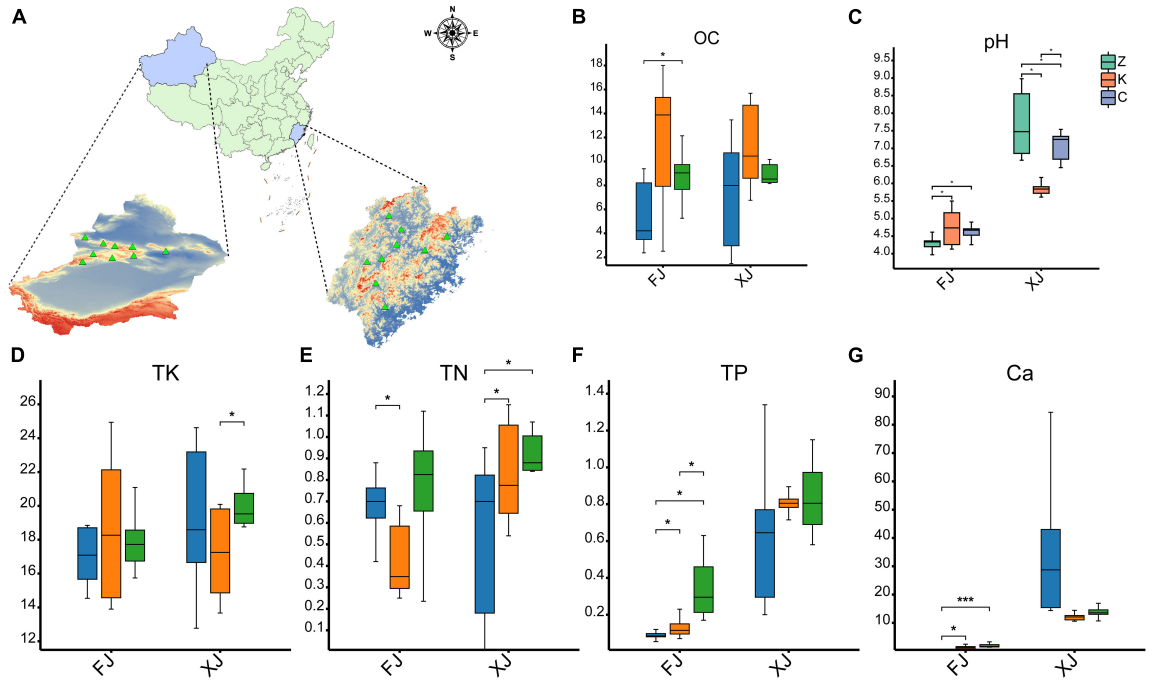


FIGURE 1
Comparison of physical and chemical properties of soil samples. **(A)** Map of location of isolation of soil samples from different provinces/regions in China ($n$ = 36). **(B–F)** Soil physical and chemical properties as follows: **(B)** soil organic matter, **(C)** pH, **(D)** total potassium (TK), **(E)** total nitrogen (TN), **(F)** total phosphorus (TP). **(G)** the metal ion $Ca2^{+}$ in the soil. The symbol * indicates $p < 0.05$, while the symbol *** indicates $p < 0.001$. FJ: soil samples from Fujian province, XJ: soil samples from Xinjiang Autonomous Region.

model (Sloan et al., 2006; Rosindell et al., 2011). The output plots of the neutral community model (NCM) primarily display the goodness of fit of the neutral model ($R^2$) and the migration rate (m), as well as the predictions of the neutral model and their corresponding 95% confidence intervals (lines).

# 3 Results

## 3.1 Analysis of physicochemical properties

Comparing the soil samples derived from Fujian and Xinjiang revealed that the pH of the soil in the two climatic zones was significantly different ($P < 0.01$). The soil pH of Fujian averaged 4.56 ± 0.41, while the soil pH of Xinjiang averaged 6.87 ± 1.00. There were no significant differences in soil pH values among the three different vegetation types in the Fujian province. However, a significant difference in soil pH was observed between the deciduous broadleaf forests and evergreen coniferous forests and grasslands in Xinjiang ($P < 0.01$). In contrast, no significant difference in soil pH was found between the evergreen coniferous forest and grassland in the Fujian province. There were also no significant differences in soil organic matter between the sampled habitats of the two provinces ($P > 0.05$), but the soil organic matter content in the broadleaf forests in Fujian and Xinjiang was higher than for evergreen coniferous forests and grasslands. The phosphorus (P) content in Xinjiang soil samples was significantly higher than that in Fujian soil samples ($P < 0.01$). There was no significant difference in the P content of soil samples in the different regions within Xinjiang, but there was a significant difference in the P content between the soils in three habitats in Fujian, with the P content in grassland soil samples the highest ($P < 0.05$). There was no significant difference in total nitrogen (TN) and total potassium (TK) between the two regions.

A total of 196,419 high-quality sequences were obtained from the 18 soil samples obtained in the Fujian province by high-throughput sequencing after filtration, splicing and chimera removal. The sequence length ranged from 569 to 629 bp. A total of 754 fungal OTUs were detected, involving 11 phyla, 30 classes, 61 orders, 111 families and 192 genera. A total of 118,550 high-throughput sequences were obtained from the 18 Xinjiang derived soil samples after quality control, with sequence lengths ranging from 550 to 657 bp. A total of 917 fungal OTUs were detected, that were dispersed within10 phyla, 29 classes, 70 orders, 134 families and 266 genera. Differences in OTU levels in these samples are shown in the Upset plot (Figure 2A). The number of OTUs unique to deciduous broadleaf forests, evergreen coniferous forests, and grasslands in Xinjiang was 88, 88, and 37, respectively. The number of unique OTUs of soil fungi in the three different vegetation regions samples in Fujian was lower than that seen for the in Xinjiang samples. For Fujian province samples, there were 41 unique OTUs in the evergreen broadleaf forest, 24 unique OTUs in the coniferous forest and 11 unique OTUs in the grassland sampled regions. The number of fungal OTUs shared by the three different vegetation regions of Fujian and Xinjiang was only 82, accounting for only 10.9% of the number of OTUs in Fujian and 8.9% of the total number of OTUs in Xinjiang. OTUs distributions for each

sample at different classification levels including phylum, class, order, family, genus, and species were determined (Figure 2B). The number of OTUs in samples at each classification level was relatively uniform and showed no significant increase. The variation trend of species richness with sequencing depth was sparse (Figures 2C, D). When the sequencing depth increased to 4000, all fungal curves for the Xinjiang soil samples reached a plateau, and similarly for the Fujian soil samples as the sequencing depth increased to 6000 for the fungal curves. The dilution curve of Fujian soil fungal samples was more deformed than that of Xinjiang soil samples, indicating that the community diversity of Fujian soil fungi was higher than that of Xinjiang soil fungi. As the dataset was increased in size, only a small number of low abundance species were detected, indicating that the sequencing depth gave good coverage of the species in the sample.

## 3.2 Alpha diversity analysis of fungal communities

The species richness and diversity indices of the 36 soil samples were determined at 97% similarity level (Table 1). α-Analyses of the soil fungal communities of Xinjiang and Fujian samples revealed similar trends in ACE and Chao1 indices for the Fujian and Xinjiang samples, with Fujian samples significantly higher than those sampled from Xinjiang. The Simpson index and Shannon index were used to estimate the species diversity of the six samples locations (3 each in Fujian and Xinjiang). These results showed that soil fungal species abundance in the Fujian sampled locations were significantly higher than that in Xinjiang, but the diversity of soil fungi in Fujian was not significantly different from that in Xinjiang.

## 3.3 Comparative analysis of beta diversity

Principal Co-ordinates Analysis (PCoA) was used to examine the similarity/dissimilarity of sample community composition (Figure 3A). The results showed that the microbial community structure in the same sampling area was similar. Principal component 1 (PCoA1) and principal component 2 (PCoA2) explained 31.1% of the total variation and could be identified as the main source of variation. The samples along the first axis were clearly separated, indicating that the largest variation between samples come from soil samples from different habitats in the two regions (PCoA1 contribution rate was 17.6%). The analysis of the second principal component (PCoA2) showed that it explained 13.5% of the soil heterogeneity, likely due to variation in the number of sample repeats within the group.

Similar to PCoA, non-metric multidimensional scaling analysis (NMDS) was also applied to reveal the effects of soil at different sampling locations on fungal community composition (Figure 3B). These analyses indicated significant differences in fungal communities between coniferous forests, broadleaf forests, and grasslands between the two climate zones of Xinjiang and Fujian. The distribution of the 36 samples was relatively scattered, but samples from the same area clustered, indicating that fungal community composition was significantly different between soils from different regions and different habitats in the same region.

## 3.4 Analysis of taxonomic composition of dominant fungal populations between samples

Analysis of the sequencing results indicated that within the Fujian samples a total of 11 phyla and 192 genera of fungi were identified, with three phyla (the Entorrhizomycota, Kickxellomycota, and Zoopagomycota) being unique to Fujian. In Xinjiang, 10 phyla with 267 genera of fungi were identified, with two phyla (the Blastocladiomycota and Calcarisporiellomycota) uniquely to the Xinjiang soil samples. Significant differences in the dominant fungal phyla between the soil samples from the two regions were seen, with Fujian soil samples having higher species abundance at the phylum level compared to Xinjiang samples (Figure 4A). The top four dominant fungal phyla in both regions were the same (in order of proportion): Ascomycota, Basidiomycota, Mortierellomycota, and Rozellomycota. The Ascomycota dominated in Xinjiang grasslands, deciduous broadleaf forests, and in Fujian evergreen broadleaf forests and temperate coniferous forests. However, in Fujian grasslands and Xinjiang evergreen coniferous forests, there was no significant difference between the Ascomycota and Basidiomycota, with the Basidiomycota more prevalent than the Ascomycota in the Xinjiang evergreen coniferous forest soil samples examined.

At the genus level, the relative abundance analyses revealed that *Trichoderma*, *Mortierella*, and *Hygrocybe* were the dominant genera in Fujian grasslands (Figures 4B, C). *Chloridium*, *Trichoderma*, and *Mortierella* were the most dominant genera in Fujian coniferous forests, whereas *Phlebia*, *Entoloma*, *Trichoderma*, and *Mortierella* were found to be abundant in Fujian broadleaf forests. *Chaetomium*, *Fusarium*, and *Archaeorhizomyces* were the dominant genera found in Xinjiang grassland soil samples, and the dominant genera in Xinjiang deciduous broadleaf forests were *Preussia*, *Chaetomium*, *Fusarium*, and *Penicillium*, while the dominant genera in Xinjiang evergreen coniferous forests were different from those found in broadleaf forests and more similar to those identified in Fujian grasslands, with *Inocybe*, *Hygrocybe*, and *Mortierella* being dominant.

A comparison between the abundance differences between multiple samples and the compositional differences at each taxonomic level was performed with statistical tests. By selecting the top 10 genera with the highest abundance in Xinjiang and Fujian samples, a total of 20 genera were analyzed (Figures 4D–W). These data revealed that *Inocybe* was significantly distributed in Xinjiang coniferous forests, while the abundance of *Hygrocybe* in Fujian grasslands was significantly higher than in the other five soil samples. Additionally, these analyses indicated that *Mortierella*, unlike other genera, was widely present in soil samples from both regions.

## 3.5 Correlations between dominant fungal communities and soil factors

Spearman correlation analyses were employed to explore the relationship between soil physicochemical parameters and fungal community structure in two climatic regions (Figures 5A, B).

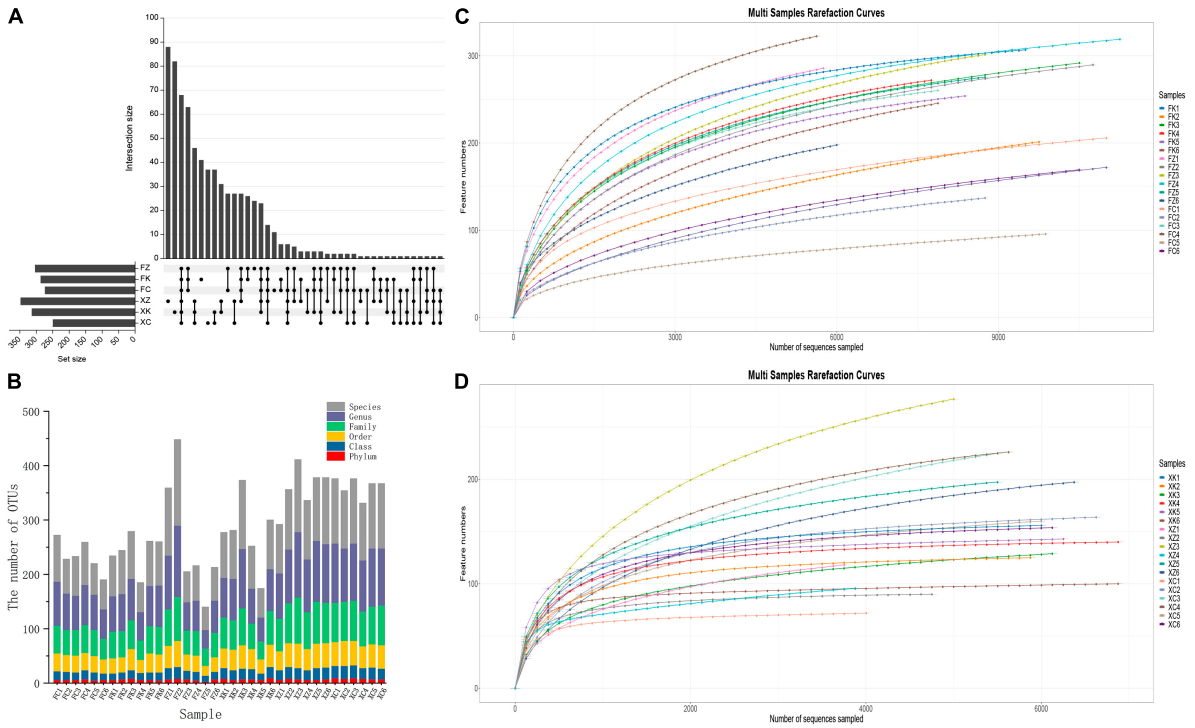


FIGURE 2
Operation taxonomic units (OTU) status identification and cluster analysis. **(A)** OTU (UpSet) analyses of soil samples from the different Fujian and Xinjiang regions. **(B)** OTU division and classification status identification. *X*-axis = sample ID, *Y*-axis = number of OTUs at each classification level (Phylum, Class, Order, Family, Genus, and Species. **(C)** Fujian and **(D)** Xinjiang dilution curves: *X*-axis = randomly selected ordinal number, *Y*-axis = corresponding number of species, with curves of different colors representing different samples.

TABLE 1 α-diversity analysis of different soil type samples.

| Soil types | ACE | Chao1 | Simpson | Shannon |
|---|---|---|---|---|
| Coniferous forests in Xinjiang Province (XZ) | 200.62 ± 110.08bc | 176.29 ± 100.38cd | 0.91 ± 0.09ab | 4.96 ± 0.68a |
| Broadleaf Forests in Xinjiang Autonomous Region (XK) | 141.4 ± 19.2c | 141.48 ± 19.09d | 0.95 ± 0.02a | 5.33 ± 0.54a |
| Grasslands of Xinjiang autonomous Region (XC) | 216.5 ± 54.49b | 217.63 ± 50.6bc | 0.88 ± 0.09abc | 4.62 ± 0.87a |
| Coniferous forests in Fujian Province(FZ) | 348.44 ± 48.55a | 327.79 ± 36.23a | 0.9 ± 0.05ab | 4.85 ± 0.85a |
| Broadleaf forests in Fujian Province (FK) | 265.05 ± 43.16b | 254.96 ± 36.07b | 0.79 ± 0.11c | 3.3 ± 0.63b |
| Grasslands in Fujian Province (FC) | 336.82 ± 24.01a | 331.33 ± 26.14a | 0.84 ± 0.09bc | 4.53 ± 0.68a |

ACE, abundance-based Coverage Estimator represents the richness index used to assess the diversity of species in the samples; Chao1 stands for the Chao1 index in richness estimation, used to estimate the number of unobserved species and is one of the diversity metrics; Simpson, signifies the Simpson index, which measures the dominance of species in species diversity; higher values indicate lower diversity; Shannon denotes the Shannon diversity index, used to measure both species diversity and evenness in the sample; higher values indicate higher diversity. Means with different superscript letters in the same column are significantly different ($P < 0.05$).

The results indicated significant variations in fungal species composition due to different soil nutrient levels. Notably, the genus *Mortierella* exhibited no significant correlations with the measured factors in both climatic regions, suggesting potential environmental adaptability and tolerance of this genus. *Hygrocybe* showed consistent correlations with environmental factors, exhibiting significant positive correlations with pH in both regions. In addition to *Hygrocybe*, pH in Xinjiang soil also displayed a significant positive correlation with the genus *Sebacina* but a highly significant negative correlation with the dominant genera *Preussia* and *Penicillium* in Xinjiang deciduous broadleaf forest soil. *Archaeorhizomyces* in Fujian soil showed a highly significant positive correlation with elevation and a significant correlation with $Mg^{2+}$, while in Xinjiang soil, *Archaeorhizomyces* exhibited no significant correlations with environmental factors. Furthermore, elevation in Xinjiang soil was significantly positively correlated with the dominant genera *Preussia* and *Fusarium* but significantly negatively correlated with *Sebacina*. It also displayed a highly significant negative correlation with the dominant genera *Phlebia* and *Trichoderma* in Fujian soil. In Xinjiang soil, with the exception of the correlation between TP and the genus *Sebacina*, total nitrogen (TN), total phosphorus (TP), and total potassium (TK) did not exhibit significant correlations with the dominant genera associated with three environmental factors, which differed from the microbiota in Fujian soil. In Fujian soil, TN exhibited a highly significant positive correlation with *Phlebia* and a significant correlation with *Entoloma*, while *Lycoperdon* showed a significant positive correlation with TP.

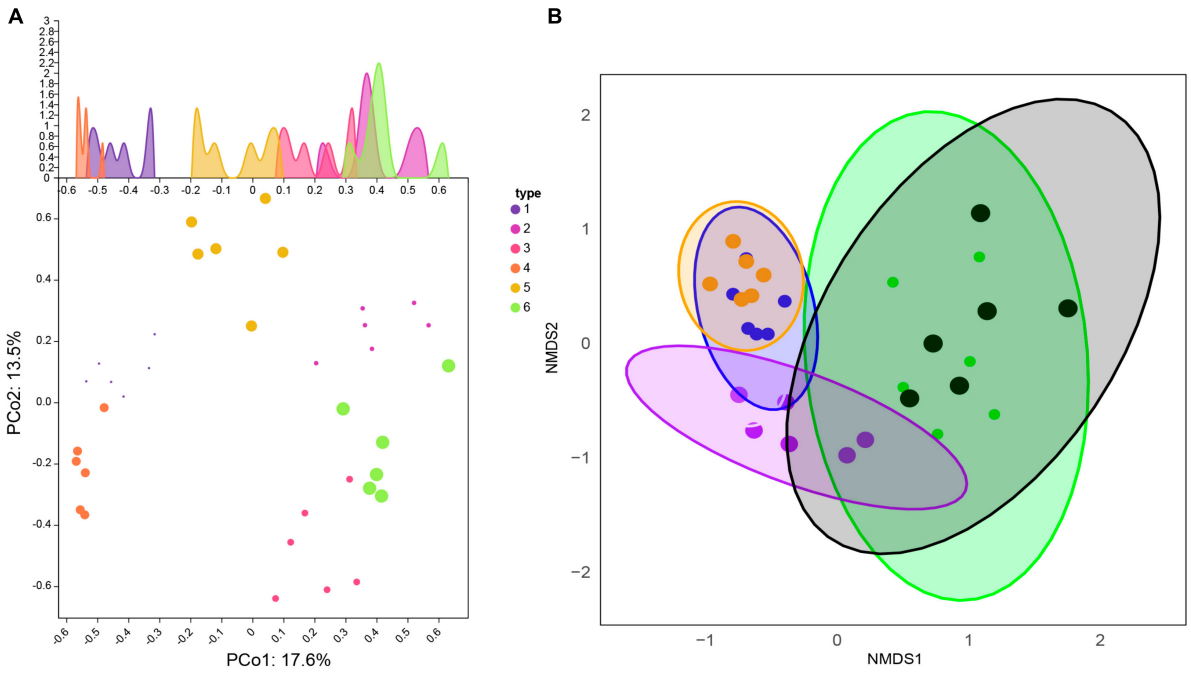


FIGURE 3
Comparison of β-diversity differences within and between Xinjiang and Fujian soil samples. **(A)** Principal Coordinate Analysis (PCoA). The horizontal and vertical coordinates represent two different principal components. **(B)** Similarity of fungal communities among different types of soils based on Non-metric Multidimensional Scaling (NMDS) analysis.

Based on differences in soil physicochemical properties and fungal composition at the genus level, non-biological environmental driving factors influencing fungal community composition were identified using RDA (**Figures 5C, D**). The explained variation in fungal diversity by environmental variables was 24.59% for Fujian soil and 24.36% for Xinjiang soil. The results revealed that in Fujian soil, *Hygrocybe* and *Archaeorhizomyces* were mainly driven by TP, TN, and $Na^+$, while *Phlebia* was primarily driven by pH. In Xinjiang soil, TP, TN, ASL, and OC primarily drove the dominant genera *Fusarium*, *Penicillium*, and *Preussia* in Xinjiang deciduous broadleaf forest. *Chaetomium* and *Archaeorhizomyces* were the dominant genera in Xinjiang grassland, primarily driven by the non-biological factor TK, consistent with the results presented in **Figure 5B**.

## 3.6 Functional analysis of soil fungal communities

Based on predictions using FUNGuild, the ecological functions of fungi were inferred (**Figure 6**). These analyses indicated that the fungal community in Xinjiang soil was primarily involved in wood saprotrophy, soil saprotrophy, plant nutrient provision, and facilitation of other material exchanges in the soil. In contrast, the predicted dominant ecological functions of fungal microorganisms in Fujian soil were focused on wood saprotrophy and ectomycorrhizal associations. Moreover, a significant portion (20–50%, $P < 0.01$) of the fungal community in Xinjiang soil samples was categorized as saprotrophs with undefined ecological functions.

## 3.7 Neutral community model analysis

Assembly mechanisms of the fungal communities in different regions and three different vegetation types using the neutral community model (NCM). Based on the OTUs dataset, the NCM explained a relatively low proportion of microbial assembly (The $R^2$ values for Xinjiang and Fujian were 0.260 and 0.354, respectively, and the migration rates were 0.004 and 0.006, respectively) (**Figure 7**). These results suggest that the assembly of fungal communities in the two regions is primarily influenced by deterministic processes.

# 4 Discussion

## 4.1 Effects of environmental factors on microbial community structure

Soil ecosystems mediate material (e.g., carbon, nitrogen, and phosphorus) exchange, energy transfer, and act as the conduit for information communication among various animals, plants, and microorganisms (Massalha et al., 2017; Kumawat et al., 2022). As crucial members of the carbon and nitrogen cycles, microorganisms can respond rapidly to environmental changes, help remediate inorganic and organic matter, and form intimate associations in networks with other microbes, plants, and animals (Falkowski et al., 2008; Ma et al., 2023). Previous studies have indicated that the structure and diversity of soil microbial communities are influenced by environmental factors including pH, nitrogen, phosphorus, potassium, temperature, humidity, and other climatic

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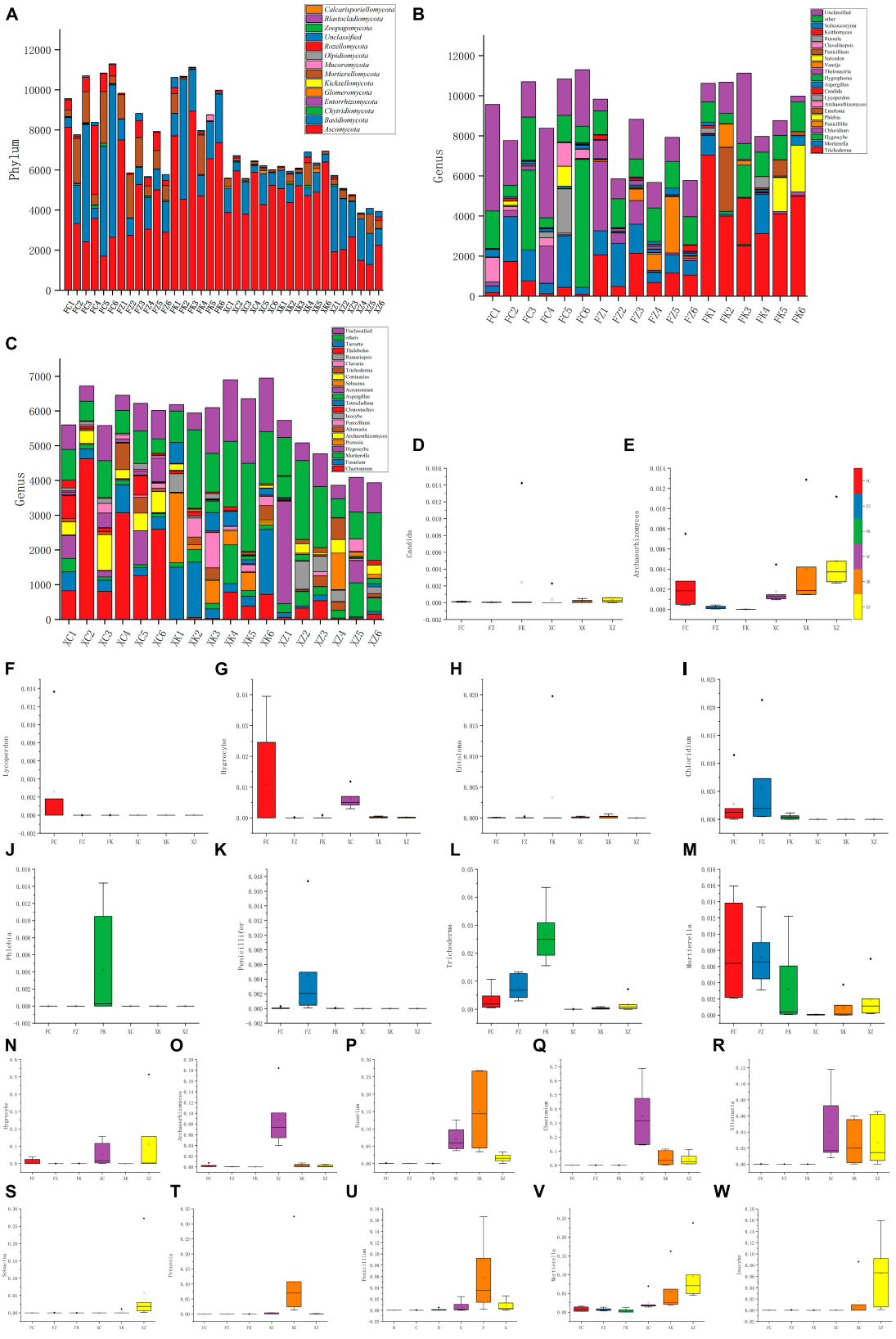


FIGURE 4

Composition of fungal communities at different taxonomic levels in all samples. **(A)** Relative abundance distribution of dominant fungal phyla. *X*-axis = sample ID, *Y*-axis = relative abundance of dominant fungal phyla. **(B,C)** The relative abundance of the same phylum at a deeper taxonomic level (genus). **(D–M)** Distribution of the top 10 fungal genera with the highest abundance in Fujian and Xinjiang soils. **(N–W)** Distribution of the top 10 fungal genera with the highest abundance in Xinjiang and Fujian soil. *X*-axis = the six samples, *Y*-axis = relative abundance of the genera in the corresponding samples.

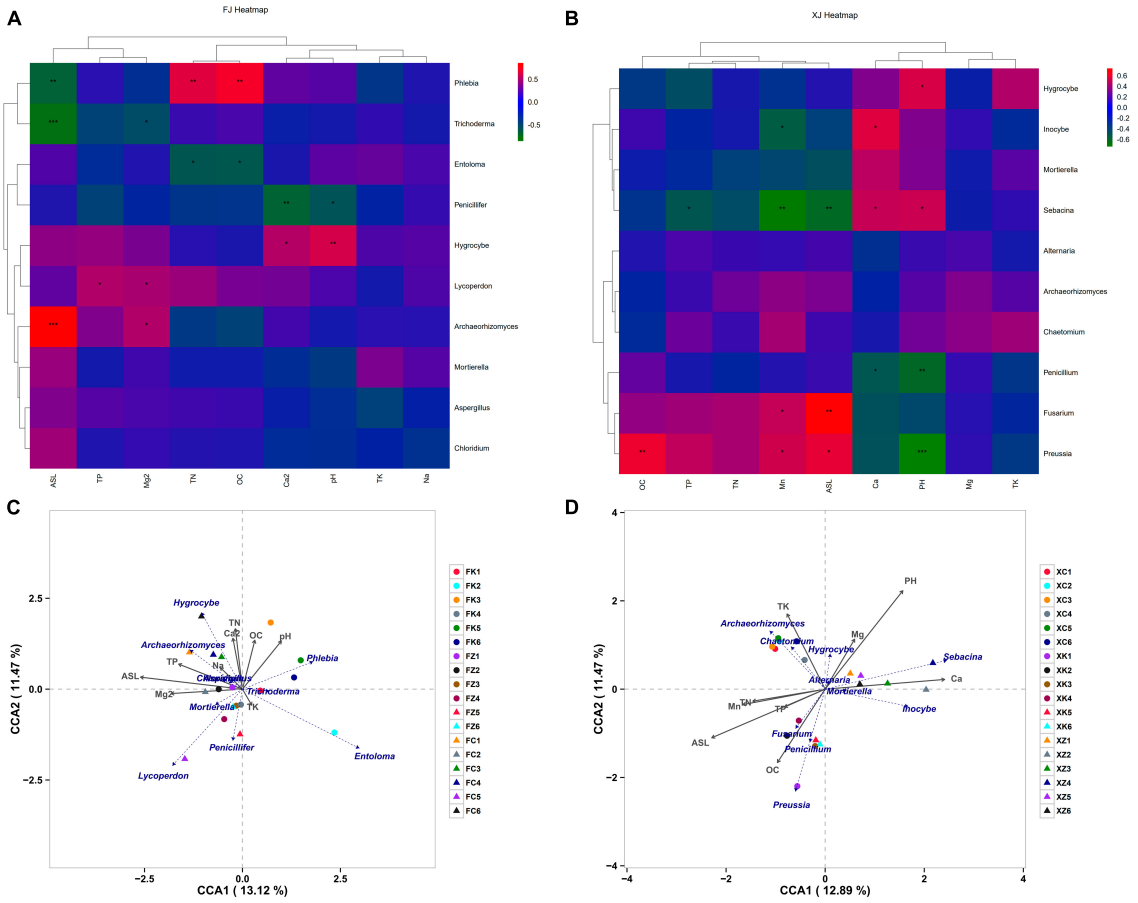


FIGURE 5
Correlation analysis of environmental factors and dominant species in soil samples, **(A,B)** represent the correlation analysis of soil microbial community composition and soil physicochemical factors based on Spearman correlation in Fujian and Xinjiang, respectively. **(C,D)** Represent Canonical Correspondence Analysis (CCA) of fungal communities based on soil physicochemical properties in Fujian Province and Xinjiang Province, respectively. Note: The horizontal axis represents soil physicochemical factors, the vertical axis represents dominant fungal at the genus level in each sample. Colors represent Spearman correlation, and asterisks indicate significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

conditions (Xu et al., 2014; Aßhauer et al., 2015) as well as related to geographic parameters (Hanson et al., 2012), however most such studies have focused on bacterial populations and not fungal. As expected, our data indicate significant differences in soil pH value, nitrogen, phosphorus, potassium, and organic matter content between the coastal regions of Fujian and the remote inland areas of Xinjiang. Fujian soils were acidic, with pH values ranging from 3.97 to 5.5, whereas the soil locations sampled in Xinjiang were predominantly alkaline, with only the Xinjiang broadleaf forest exhibiting acidic pH value (5.61), with the rest ranging above that to a pH of 8.98. A negative correlation was seen between soil pH and soil fungal abundance and diversity, which differed from a previous study (Rousk et al., 2009), but may be more broadly consistent with most fungi preferring acidic conditions. In addition, many fungi acidify their environment thus contribution to acidification of soils. Thus, it is possible that the alkaline soil in Xinjiang is less hospitable to fungi and/or since there are less fungi in the Xinjiang soils, less (fungal) mediated acidification occurs, and hence the soil is more alkaline.

Phosphorus content in the root zone soils of the three vegetation types sampled in Xinjiang was significantly higher than in the (three different vegetation types) soils examined from Fujian. Nitrogen and potassium levels did not show the same trend. Organic matter content in broadleaf forests from both regions were similar (and highest of the soils tested). However, despite the higher organic matter content in the soils of broadleaf forests (of both Xinjiang and Fujian), this did not correlate with fungal diversity. This suggests that the fungi in the root zone soils of broadleaf forests may remain limited, consistent with what has been reported by others (Averill et al., 2014).

## 4.2 Alpha and beta diversity analysis of microbial community

To better examine total species abundance and microbial diversity, we selected a sample size validated by the species accumulation curve trend. Using α-diversity indices (Shannon, Simpson, Chao1, ACE) and examining the distribution of microbial communities at various taxonomic levels, we assessed fungal diversity and abundance. The Chao1 index showed that species richness and the number of unassigned species were significantly higher in the Fujian samples as compared to the Xinjiang soil samples. Overall, the Fujian soil samples exhibited higher

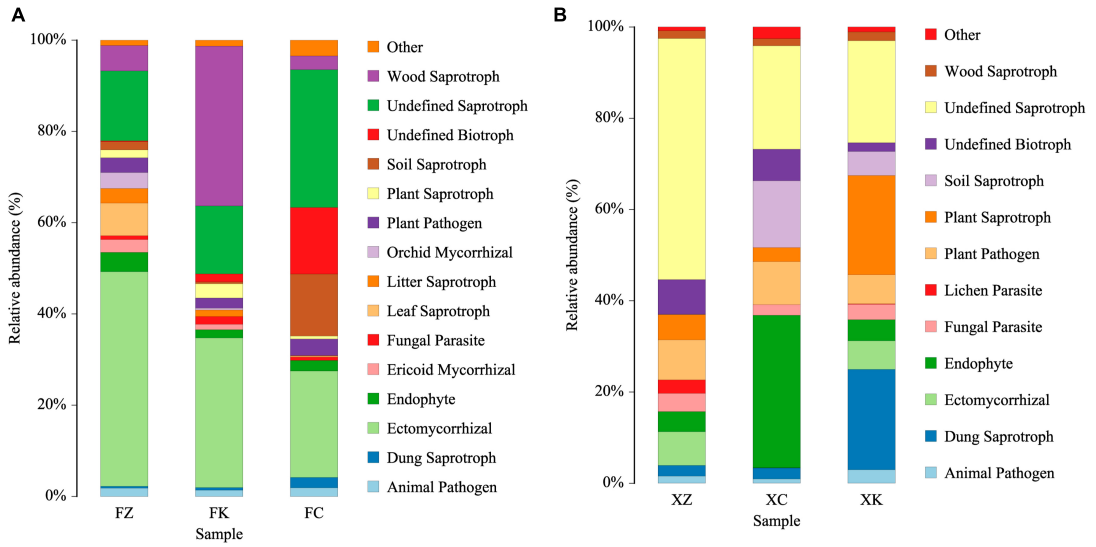


FIGURE 6
FUNGuild analyses predicting ecological functions of identified fungi. **(A)** Relative abundance of microbial community functions in Fujian soil samples. **(B)** Relative abundance of microbial community functions in Xinjiang soil samples.

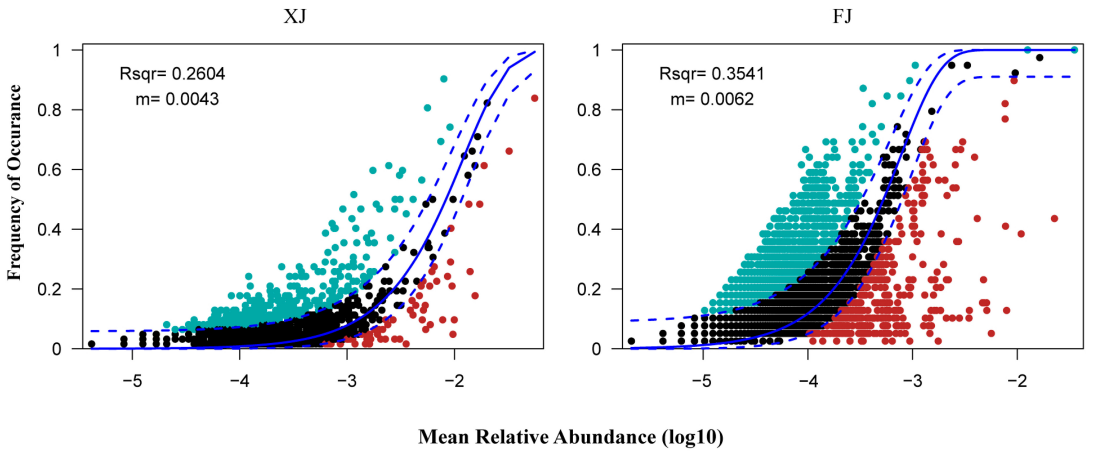


FIGURE 7
Fit of the neutral community model (NCM) in two regions. The solid blue lines indicate the best fit to the NCM as in Sloan et al. (2006), and the dashed blue lines represent the 95% confidence intervals around the model prediction. The OTUs that occur more or less frequently than predicted by the NCM are shown in aquamarine or red colors, respectively. $R^2$ indicates the fit to the NCM. The m represents the migration rate at the community level.

fungal richness and diversity at the phyla and unassigned levels, while the Xinjiang soil samples were more diverse at the identifiable genus level.

Principal Component Analysis (PCA) and Non-metric Multidimensional Scaling (NMDS) were used to analyze the structural composition and variation of fungal soil community (Quast et al., 2013; Zhu et al., 2022). Although the distribution of the 36 samples (in the two-dimensional analytic space) was relatively dispersed, a clear clustering of samples from the same region (and even more so from the same location within the region) was seen. It is challenging to determine whether the observed differences in the fungal soil microbial community structure result from distinct geographical locations and varying vegetation root and cover communities, or if different fungal communities play a role in shaping the vegetation. The most likely scenario is that they mutually influence each other. However, there was also a certain degree of random variation among soil samples within the same region, which indicates considerable environmental plasticity of the soil fungi within the same "habitat."

## 4.3 Analysis of dominant flora and functional flora

Our data reveal the presence of fungi from at least 31 distinct phyla in the soils of the Xinjiang and Fujian ecosystems. At the phylum level, the fungal soil root-associated microbiota in Fujian and Xinjiang province included 31 and 30 different

class, respectively, of which 24 were shared (found in both). Five classes, the Archaeosporomycetes, Blastocladiomycetes, Calcarisporiellomycetes, Cystobasidiomycetes, and Paraglomeromycetes, were found to be unique to Xinjiang, with five other classes, the Entorrhizomycetes, Rhizophydiomycetes, Xylonomycetes, Zoopagomycetes, and Endogonomycetes, unique to Fujian. Eight classes, namely the Sordariomycetes, Agaricomycetes, Mortierellomycetes, Saccharomycetes, Eurotiomycetes, Tremellomycetes, Pezizomycetes, and Dothideomycetes, were highly abundant and shared between the two regions. Previous studies have reported the wide distribution of the Agaricomycetes in soil environments, where they play an important role in decomposition and transformation of soil organic matter, facilitating the breakdown and conversion of organic compounds (Hibbett and Donoghue, 2001; Moore et al., 2015; Yi et al., 2019).

Furthermore, Xinjiang soils had a relatively high abundance of the class Archaeosporomycetes, second only to the Sordariomycetes and Agaricomycetes in grassland soils. Archaeosporomycete spores and mycelia can interact with soil microorganisms, plant roots, and other fungi, establishing a mycorrhizal symbiosis with plant roots and providing water and nutrients to plants (Fan et al., 2022). This might be related to the arid and low-rainfall climate in Xinjiang. Sordariomycetes and Agaricomycetes, as functional fungal groups involved in soil organic matter, were found to be widespread across six habitats, consistent with previous research results. Due to their broad organic matter degradation capabilities, the Sordariomycetes and Agaricomycetes become the dominant fungi in plant root soils.

The fungal microbial community structure in Fujian soil was found to be dominated by the following top 10 genera—*Archaeorhizomyces*, *Candida*, *Chloridium*, and *Entoloma*. In Xinjiang coniferous forest soil, the dominant fungal genera included *Chaetomium*, *Fusarium*, *Mortierella*, *Inocybe*, and *Hygrocybe*. In addition, *Trichoderma*, known for various plant beneficial effects, including protection (of plants) from pathogenic microorganisms (Sharma et al., 2017) and disease (Woo et al., 2023), was found to be a dominant fungal genus in Fujian. On the other hand, certain types of *Fusaria*, a dominant fungal genus identified in the Xinjiang soil samples, are plant pathogens (Berasategui et al., 2023). However, some *Fusaria* may have beneficial functions, such as organic matter decomposition and nutrient cycling (Mukjang et al., 2022). Furthermore, one of the dominant genera (*Sebacina*) found in Xinjiang is known to form symbiotic relationships with plant roots, promoting plant growth by providing nutrients and enhancing plant tolerance to environmental stress (Weiss et al., 2016; Lupini et al., 2023). These fungal functional groups can help plants enhance their tolerances to environmental stress and play crucial roles in plant growth under long-term environmental stress conditions (Weiss et al., 2011; Perez-Lamarque et al., 2022). While not all dominant fungal species can be attributed to directly aiding plants in stress management, their distinct microbial characteristics, in some instances, are likely linked to alterations in the soil environment. Significant variations in genera distribution were observed across different samples, suggesting that differences in geological habitats, soil properties, and climatic conditions either contribute to these variations and/or could be, even if to a small degree, determined by their respective fungal communities. However, our findings suggest that fungal community structures are influenced by spatial distance, potentially due to limitations in dispersal and interactions with environmental heterogeneity (Fierer et al., 2005; Ferrari et al., 2016). Thus, environmental conditions and geographic variations play a crucial role in shaping these fungal microbial communities.

Our canonical correspondence analysis (CCA) results showed a significant positive correlation between the fungal genera *Hygrocybe* and *Archaeorhizomyces* in Fujian soil and nitrogen and phosphorus content, consistent with these fungal groups being involved in soil material cycling and energy flow processes. In Xinjiang, the abundance of *Fusarium* and *Penicillium* may indicate their participation in nitrogen and phosphorus energy cycling that could significantly alter the nutrient characteristics of the soil. Soil pH was positively correlated with many of the identified dominant fungal populations, confirming the predictive role of pH as a major abiotic driver in shaping soil fungal communities (Tan et al., 2020; Yang et al., 2020).

Herein, the ecological functions of soil fungi have been a focal point of interest. Comparative analysis, using tools such as FUNGuild (Nguyen et al., 2016), revealed that the dominant fungal microbial communities in Fujian soil mainly consisted of ectomycorrhizal, soil saprotrophs, and wood saprotrophs. In contrast, the dominant fungal microbial communities in Xinjiang soil were primarily composed of endophytes, plant saprotrophs, dung saprotrophs, and intriguingly a significant number of undefined saprotrophs. It is well-known that mycorrhizal fungi play a crucial role in enhancing plant nutrient uptake and supply, improving stress resistance, and influencing the structure and diversity of plant communities (Harrier and Watson, 2004). These fungi form mutualistic associations with specific plant species, leading to the dominance of certain plant species in soils with ectomycorrhizal symbiosis. The results of the community functional analysis performed as part of this work showed that mycorrhizal fungi in Xinjiang constituted only a small fraction of the total, while the majority was unidentified saprophytic fungus. However, mycorrhizal fungi, as beneficial symbionts of plant roots, form symbiotic relationships with over 70% of plant species worldwide (Gao et al., 2019). Therefore, we speculate that some of the saprophytic fungi present in the root soil microbiomes of the three vegetation types in Xinjiang may possess partial functions similar to mycorrhizal fungi. These data also highlight a significant reservoir of uncharacterized fungi, and further research is needed to explore the microbial communities in these soils in greater detail.

The NCM results indicated that the assembly processes of fungal communities in Fujian and Xinjiang are primarily influenced by deterministic processes. Deterministic processes may include environmental filtering and interspecies competition, which play important roles in both regions (Stegen et al., 2012). However, compared to Fujian, Xinjiang exhibits a greater influence of deterministic processes on fungal community assembly, potentially due to its unique environmental conditions and selective pressures. Furthermore, the higher migration rate observed in Xinjiang suggests that the fungal communities in this region have higher dispersal abilities and may have stronger connectivity with other fungal communities. This could be one of the reasons for the observed differences in community assembly processes between the two regions.

## 5 Conclusion

Climate variations and spatial distances collectively contribute to the evolution of soil heterogeneity, thereby influencing the structure and diversity of soil fungal communities. In this study, by comparing two distinct regions, the coastal Fujian and the far inland Xinjiang areas, with three distinct regions within each of these areas, we identified the main fungal taxa in these ecosystems. Fungi involved in energy cycling and organic matter degradation were identified, including *Sordariomycetes*, *Agaricomycetes*, *Mortierellomycetes*, *Saccharomycetes*, and others. In addition, ecological functional differences were observed among the dominant fungal microbial communities between soils with different vegetation root systems. For instance, most of the fungal communities in Xinjiang grassland soil were endophytic fungi, likely contributing to enhancing plant resistance to environmental stress, and hence selected for by the plants as well exerting selective pressure on specific plants. In contrast, Fujian grassland soil exhibited a significant presence of fungal plant pathogens and parasites that impact plant growth, potentially indicating that pathogenic processes are more active in these ecosystems. Our results provide important insights into the interactions among soil microorganisms in different vegetation types and the primary functional roles of fungal microbial communities under the constraint of spatial distances.

## Data availability statement

The original contributions presented in this study are included in this article/supplementary material, further inquiries can be directed to the corresponding authors.

## Author contributions

SL: Conceptualization, Methodology, Writing – original draft. CX: Data curation, Writing – original draft. LL: Data curation, Writing – original draft. NK: Writing – review and editing. MZ: Data curation, Writing – original draft. ZZ: Data curation, Writing – original draft. WZ: Resources, Writing – original draft. CY: Resources, Writing – original draft. HS: Writing – original draft. PL: Writing – review and editing. XG: Writing – review and editing. JQ: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Arbuscular mycorrhizal fungal communities in soils where astragalus had grown for 2 years were similar to those in the abandoned farmland

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**Purpose:** Astragalus-cultivated soils are enriched in arbuscular mycorrhizal fungi (AMF); however, the community changes of AMF between years in stragalus-cultivated soils are still unclear.

**Methods:** To illustrate this, using high-throughput amplicon sequencing and quantitative real-time PCR, we analyzed the AMF communities of the abandoned farmlands and interannual astragalus-cultivated soils for 1-, 2-, 3-, and 4-years, including community composition, dominant, core, specific and significantly fluctuating AMF, co-occurrence network, alpha diversity, and beta diversity.

**Results:** A total of 74 OTUs were classified into one phylum, Glomeromycota; one class, Glomeromycetes; four orders; four families; and six genera. The 2-year soil had the highest number of reads among the interannual soils. Only one OTU was shared among all interannual soils. The treatments significantly affected the Ace, Shannoneven, and Shannon estimators of the communities. The 2-year soil had the highest richness, evenness, and diversity among all interannual soils and was the closest to the abandoned farmland in terms of alpha diversity. *Glomus* of the family Glomeraceae was the dominant genus present in all treatments, and the composition of the dominant genus in interannual soils was different. Both *Glomus* and *Diversispora* were the core AMF in interannual soils, and specific AMF existed in different interannual soils. *Glomus* is a genus that exhibits significant interannual variation. The interannual time significantly affected the network connectivity. The results of the principal coordinate analysis showed that the community composition of the interannual soils was close to each other and separated from the abandoned farmland, and that the interannual time significantly affected the community composition.

**Conclusion:** Among the interannual soils, the 2-year soil may be more suitable for *A. sinensis* seedling rotation.

KEYWORDS

**arbuscular mycorrhizal fungi, astragalus, interannual soil, network, alpha diversity, beta diversity**

# Introduction

Astragalus (Huangqi in Chinese, also known as Astragali Radix) has a long history of medicinal and edible value in China and is the dried root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao and *Astragalus membranaceus* (Fiscn.) Bqe (Chen et al., 2020). Astragalus is widely utilized in Chinese medicine and the food industry because it functions to tonify Qi, strengthens the body's immunity, and regulates blood sugar (Sun et al., 2017; Xu et al., 2022).

Dingxi in Gansu Province is an original production region for astragalus (Durazzo et al., 2021; Sheng et al., 2021). Astragalus is a perennial medicinal plant, sown in the first year and harvested in the second, third and/or fourth year (Sun et al., 2020; Huang et al., 2022). Astragalus belongs to the legume family and can form a symbiotic system with arbuscular mycorrhizal fungi (AMF), which are abundant in the soil where the host plant grows (Lin et al., 2019; Primieri et al., 2022). Dingxi is also the main cultivation area for *Angelica sinensis*. The seedlings usually cultivated in abandoned farmlands. However this approach cultivating the seedlings is not sustainable (Wang et al., 2019; Xu et al., 2020). Hence, in past studies, four types of crop-cultivated soil were tested to cultivate *A. sinensis* seedlings, and the results indicated that the *A. sinensis* seedlings cultivated in the astragalus-cultivated soil were of good quality, and we found that the astragalus-cultivated soil was a suitable soil for fostering *A. sinensis* seedlings (Jin et al., 2018; An et al., 2023). Bai et al. (2019) cultivated *A. sinensis* seedlings on a crop-cultivated field followed by pea-astragalus rotation, and the results showed that high-quality seedlings could be harvested by pea-astragalus rotation farmland. We conducted experiments on the cultivation of *A. sinensis* seedlings in both alpine meadow soils and crop-cultivated soils, with the aim of to investigating the characteristics of the fungal community composition and function within the rhizosphere of *A. sinensis* seedlings (An et al., 2020). We found that the relative abundance of AMF in astragalus-cultivated soil was higher than that in other crop-cultivated soils and alpine meadow soil, indicating that astragalus-cultivated soil was enriched with more AMF (An et al., 2023). This was one of the reasons for fostering high-quality seedlings of *A. sinensis*.

AMF are widespread root endosymbionts of terrestrial plants that play a beneficial role in sustainable agriculture. They aid in the absorption of phosphorous and nitrogen by the host, resulting in enhanced host productivity, while in turn they receive carbon sources from their hosts in exchange (Lee et al., 2013; Wang et al., 2017). The available evidence suggests that crop rotation changes the diversity and composition of AMF in the soil, and some crops increase the abundance of AMF, while others produce the opposite effect (Sosa-Hernández et al., 2019; Wang et al., 2023).

It is not clear how AMF community composition changes among the interannual soils from the cultivating seedling stage (the first year) to the forming herb stage (the second, third, and fourth years) in astragalus-cultivated soils. Therefore, we hypothesize that the AMF community composition varied significantly among the interannual soils, and that the AMF community composition of the 2-year soil was similar to that of the abandoned farmland. To test this hypothesis, we investigated the AMF community composition in interannual astragalus soils. From the perspective of AMF communities, the results will help to provide a theoretical basis for the selection of astragalus interannual soil for the cultivation of *A. sinensis* seedlings.

# Materials and methods

## Study site and experimental design

The test site was located at Dingxi Institute of Agricultural Science (N 35°58′, E 104°62′), with an altitude of 1,915 m, average annual temperature of 7.2°C, annual sunshine hours of 2,500 h, average annual frost-free period of 140 d, and average annual rainfall of over 400 mm. Field experiment design was a random complete block design. The treatments were prepared by culturing astragalus for 1, 2, 3, and 4 years, as well as the control treatment with abandoned farmland (Supplementary Figure S1). In this study, the abandoned farmland is defined as land that is not cultivated and on which a variety of plants grow freely. Each plot of astragalus cultivation was three meters by six meters. Before the test was implemented, broad bean (*Vicia faba* L.) was planted in the field and completed one growing season. We maintained consistent plot management. Each plot was cultivated by *A. membranaceus*, and the plants were harvested on schedule.

## Sample preparation

After harvesting the plants in the first, second, third, and/or fourth years, both interannual soils and abandoned 4-year farmland soils were sampled to a depth of about 20 cm using the randomized five-point sampling method. Each plot sample was mixed and packed in sterile bags, recorded, and stored. Leaves, roots, stones, and other debris were removed from the samples, and the samples were air-dried. After the four replication samples were collected, they were mixed together and transferred to sterile tubes.

## DNA extraction and amplification

DNA was extracted from the samples using E.Z.N.A.® soil DNA kit (Omega). The quality of genomic DNA was examined by 1% agarose gel electrophoresis, and DNA concentration and purity were determined using a NanoDrop2000 (Thermo Scientific). Genomic DNA was used as a template for amplification using ABI GeneAmp®9700 PCR (ABI). Specific primer pairs (Zhu et al., 2016) with barcodes are shown in Table 1. PCR reaction system consisted of 20 μL, including 5 × FastPfu Buffer 4 μL, 2.5 mM dNTPs 2 μL, 5 μM forward primer 0.8 μL, 5 μM reverse primer 0.8 μL, FastPfu DNA polymerase (TransGen Biotech) 0.4 μL, bovine serum albumin 0.2 μL, template DNA 10 ng, and dd $H_2O$ to reach a final volume of 20 μL. PCR amplification conditions were as follows: initial 95°C for 3 min, denaturation 95°C for 30 s, annealing 55°C for 30 s, extension 72°C for 45 s, first amplification 32 cycles/s amplification 25 cycles, and

TABLE 1 Primer design for nested PCR.

| Sequencing region | Base sequence |
|---|---|
| 338F/806R | 338F (ACTCCTACGGGAGGCAGCAG) |
| | 806R (GGACTACHVGGGTWTCTAAT) |
| AMV4.5NF/AMDGR | AMV4.5NF (AAGCTCGTAGTTGAATTTCG) |
| | AMDGR (CCCAACTATCCCTATTAATCAT) |

extension 72°C for 10 min. The PCR products were detected using 2% agarose gel electrophoresis.

## Library construction and sequencing

PCR products from the same sample were mixed and recovered on a 2% agarose gel. The recovered products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), detected by 2% agarose gel electrophoresis, and quantified using a Quantus™ Fluorometer (Promega).

The purified PCR products were used to construct the library using NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific): (i) splice linkage; (ii) removal of splice self-linked fragments using magnetic bead screening; (iii) enrichment of library template using PCR amplification; and (iv) recovery of PCR products using magnetic beads to obtain the final library. Sequencing was performed using the Illumina MiSeq PE300 platform (Shanghai Meiji Biomedical Technology Co., Ltd.). The raw data were uploaded to the NCBI SRA database (Accession: PRJNA905095).

## OTU clustering and species annotation

Raw FASTQ files were de-multiplexed using an in-house Perl script, and then quality-filtered by FASTP (Chen et al., 2018) (V 0.19.6) and merged by FLASH (Magoc and Salzberg, 2011) (V 1.2.11) as per the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and truncated reads shorter than 50 bp, and reads containing ambiguous characters were discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapping sequence. The maximum mismatch ratio in the overlap region was 0.2. Reads that could not be assembled were discarded; (iii) samples were distinguished according to the barcode and primers, and the sequence direction was adjusted; barcodes were matched exactly, and the mismatch in primer matching was two nucleotides.

The optimized sequences were clustered into operational taxonomic units (OTUs) using UPARSE (Stackebrandt and Goebel, 1994; Edgar, 2013) (V 7.1) with a 97% sequence similarity level. Annotation for each sequence was implemented in the UNITE (V 8.0) database. The most abundant sequence for each OTU was selected as a representative sequence. The samples were normalized according to the minimum number of sample sequences. The taxonomy of each OTU representative sequence was analyzed using the RDP Classifier (Wang et al., 2007) (V 2.13) against the UNITE (Release 8.0) database with a confidence threshold of 0.7, and the community composition of each sample was counted at different taxonomic levels.

## Co-occurrence network

The OTUs correlation matrix of interannual soils was studied by selecting the top 50 species in terms of total abundance at the genus level and calculating Spearman's correlation coefficients ($-0.5 \leq r \leq 0.5$, $p < 0.05$) between species. NetworkX was used to construct and analyze the co-occurrence network.

## Quantitative real-time PCR and absolute abundance

Primer pairs AMV4.5NF/AMDGR were used to determine AMF abundance by ABI 7300 ABI7300 Fluorescent Quantitative PCR System (Applied Biosystems, United States). The standard curve was constructed with the plasmid vector (pMD18-T, 2692 bp). Each PCR reaction was carried out in a 20 μL qPCR reaction mixture containing 10 μL ChamQ SYBR Color qPCR Mix (2×) (Vazyme Biotech, China), 0.8 μL PCR forward and reverse primers (both 5 μM), 0.4 μL X ROX Reference Dye1, 2 μL DNA template, and 6 μL double distilled water (dd $H_2O$). Quantitative real-time PCR reactions were set to 95°C for 3 min, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 1 min. Analysis of amplification and melting curves for AMF gene quantification showed excellent specificity of the qPCR with an efficiency of 96.44%. AMF gene standard curve $y = -3.4104\,x + 40.509\left(R^2 = 0.9988\right)$.

To allow for a quantitative comparison, the relative abundance values of specific taxa were converted into absolute abundance values by multiplying by the corresponding quantitative fluorescence values (copies × $10^6$/g).

## Statistical analyses

ACE and Shannon indices were calculated using MOTHUR (Schloss et al., 2009) (http://www.mothur.org/wiki/Calculators). Data were analyzed with principal coordinate analysis (PCoA) based on Bray–Curtis, Anosim test (permutations 999), Kruskal–Wallis rank sum test (multiple test corrected fdr and *post-hoc* test Scheffe), and One-ANOVA analysis with Tukey multiple comparisons. Statistical analyses were performed using the Meguiar's BioCloud platform (https://cloud.majorbio.com) and Origin (2022).

# Results

## AMF community

The dilution curve results showed that the number of OTUs in all samples flattened out with increasing sequencing numbers; therefore, the amount of sequencing data was large enough to reflect the AMF diversity information in the samples (Supplementary Figure S2). Pan-OTU is the total number of species found in all samples, and core-OTU is the number of species shared by all samples. As the sample size increased, the pan-OTUs showed an increasing upward trend (Supplementary Figure S3A), whereas the core-OTUs showed a decreasing trend (Supplementary Figure S3B).

After splicing, filtering, and removing chimeras from the raw reads of 20 samples, 513,233 reads were obtained with 89,479 reads for 1-year soil, 115,574 reads for 2-year soil, 99,396 reads for 3-year soil, and 99,293 reads for 4-year soil. The 2-year soil had the highest number of reads. The fluorescence quantification results showed that the interannual soil treatment significantly affected the absolute abundance of AMF, with 2-year soil being significantly higher than the 1- and 4-year soils (Figure 1).

To minimize the effects of sequencing depth on alpha and beta diversity measures, the number of sequences from each sample was

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rarefied to 19,814 according to the minimum number of sample sequences. A total of 74 OTUs were obtained by species annotation and were classified into the phylum Glomeromycota, four orders, four families, six genera, and 27 species (Table 2). At the order level, the treatments affected the distribution of species, with only the order Glomerales present in all treatments and the variability in the distribution of the other three orders (Table 3).

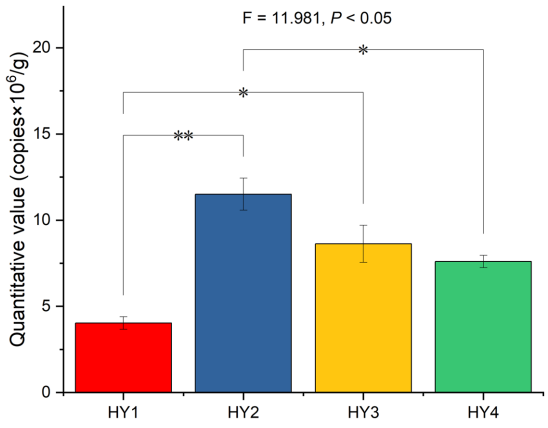


FIGURE 1
Effect of interannual soil treatment on absolute quantitative values based on One-ANOVA with Tukey tests, * $p \leq 0.05$ and ** $p \leq 0.01$. HY1, 1-year soil; HY2, 2-year soil; HY3, 3-year soil; HY4, 4-year soil.

## Community composition and alpha diversity

Only one OTU was shared among the interannual soil samples (Figure 2). The 2-year soil had the highest number of OTUs (43 OTUs), followed by the 3-, 4-, and 1-year soils. The 2-year soil had the highest number of unique OTUs (19 OTUs), the 3-year soil had unique 17 OTUs, and the 1- and 4-year soils had two unique OTUs. Thus, the 2-year soil exhibited a rich diversity of AMF species.

Alpha diversity was used to describe the abundance and diversity of a community, including the Ace estimator for community richness, Shannoneven estimator for community evenness, and Shannon estimator for community diversity. One-ANOVA analysis with Tukey tests was used to investigate the Ace, Shannoneven, and Shannon of the communities. The treatments significantly affected the Ace estimator (Figure 3A). The Ace of 1-year soil was significantly lower than that of the abandoned farmland, and the Ace of 2-year soil was of no difference to that of the abandoned farmland. The Ace of the 1-, 3-, and 4-year soils were significantly lower than that of the 2-year soil, which was with the highest Ace. The treatments significantly affected the Shannoneven estimator (Figure 3B). The Shannoneven of the 3-year soil was significantly lower than that of 2-year soil and

TABLE 2 Taxonomic of the species annotation to OTUs.

| Order | Family | Genus | Species |
|---|---|---|---|
| Diversisporales | Diversisporaceae | *Diversispora* | *Glomus* acnaGlo2 VTX00155 |
| Glomerales | Diversisporaceae | *Glomus*_f_Diversisporaceae | *Glomus* Glo32 VTX00124 |
| Paraglomerales | Paraglomeraceae | *Glomus*_f_Glomeraceae | *Glomus* Glo3 VTX00074 |
| unclassified_c_Glomeromycetes | unclassified_c_Glomeromycetes | *Paraglomus* | *Glomus* group B *Glomus* Glo G8 VTX00340 |
| | | unclassified_c_Glomeromycetes | *Glomus* group B *Glomus* lamellosu VTX00193 |
| | | unclassified_f_Diversisporaceae | *Glomus intraradices* VTX00105 |
| | | | *Glomus* MO G18 VTX00064 |
| | | | *Glomus* MO G20 VTX00143 |
| | | | *Glomus* MO G22 VTX00125 |
| | | | *Glomus* MO G23 VTX00222 |
| | | | *Glomus* MO G4 VTX00166 |
| | | | *Glomus* MO G7 VTX00199 |
| | | | *Glomus mosseae* VTX00067 |
| | | | *Glomus* sp. VTX00234 |
| | | | *Glomus* sp. VTX00301 |
| | | | *Glomus* sp. VTX00304 |
| | | | *Glomus* VeGlo18 VTX00342 |
| | | | *Glomus versiforme* VTX00061 |
| | | | *Glomus viscosum* VTX00063 |
| | | | *Glomus* Whitfield type 17 VTX00195 |
| | | | *Glomus* Wirsel OTU16 VTX00156 |
| | | | *Paraglomus* Glom 1B.13 VTX00308 |
| | | | unclassified_c_Glomeromycetes |
| | | | unclassified_f_Diversisporaceae |
| | | | unclassified_g_*Diversispora* |
| | | | unclassified_g_*Glomus*_f_Glomeraceae |
| | | | unclassified_g_*Paraglomus* |

TABLE 3 Distribution of AMF in the interannual soils at Order level.

| Order | CK | HY1 | HY2 | HY3 | HY4 |
|---|---|---|---|---|---|
| Diversisporales | × | ✓ | ✓ | ✓ | ✓ |
| Glomerales | ✓ | ✓ | ✓ | ✓ | ✓ |
| Paraglomerales | × | × | × | ✓ | × |
| unclassified_c_Glomeromycetes | × | ✓ | ✓ | × | × |

✓ means present, and × means not present.

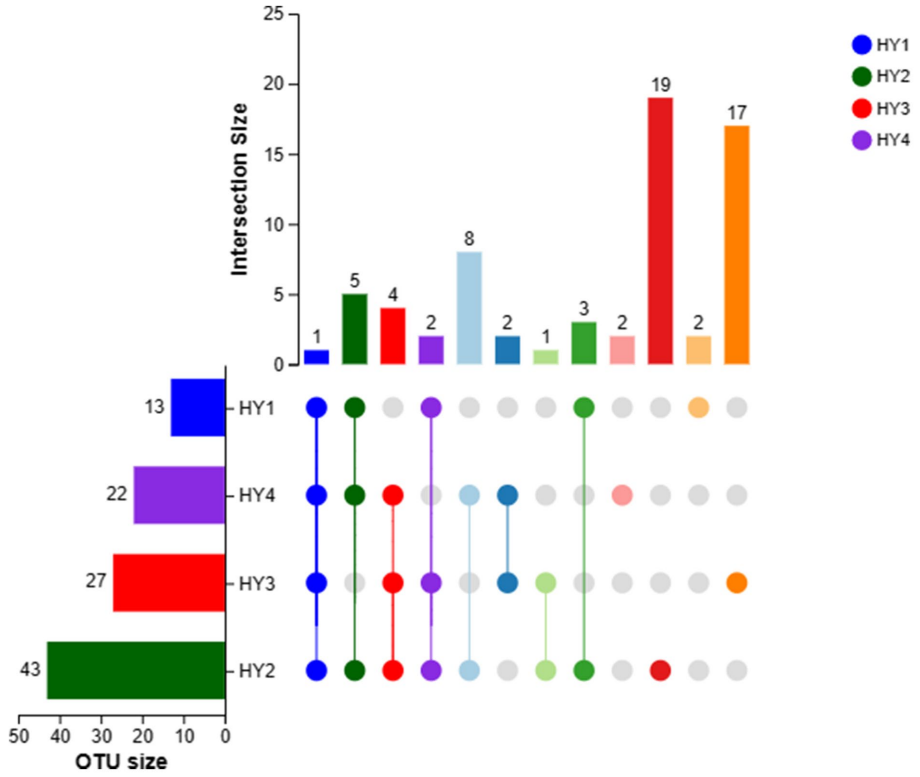


FIGURE 2
UpSet of OTUs in interannual soils. The meanings of HY1, HY2, HY3, and HY4 were illustrated in Figure 1.

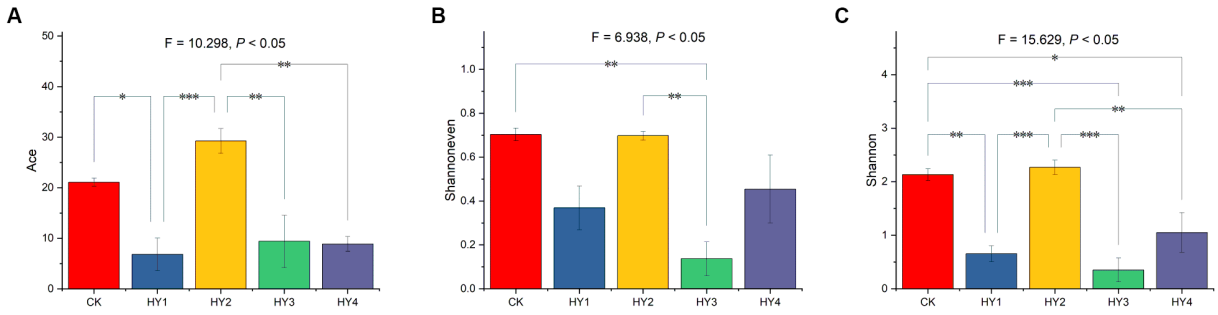


FIGURE 3
Comparisons of Ace **(A)**, Shannoneven **(B)**, and Shannon **(C)** were based on One-ANOVA with Tukey tests, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. The content of each estimator is expressed as mean ± SE ($n = 4$). CK, abandoned farmland; the meanings of HY1, HY2, HY3 and HY4 were illustrated in Figure 1.

that of the abandoned farmland. There was no significant difference for Shannoneven between abandoned farmland and 2-year soil. The Shannoneven of the 3-year soil was significantly lower than that of the 2-year soil. The 2-year soil was with the highest Shannoneven in the interannual soils. Also, the treatments significantly affected Shannon estimator (Figure 3C). The Shannon of the 1-, 3-, and 4-year

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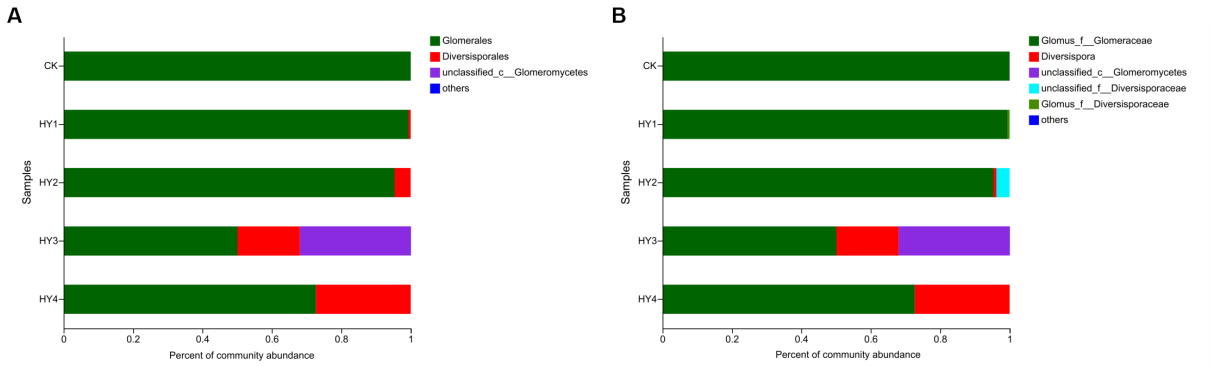



FIGURE 4
Community barplots of the relative abundances at order level **(A)** and genus level **(B)**. The meanings of CK, HY1, HY2, HY3, and HY4 were illustrated in Figure 3. f stands for family, and c for class.

TABLE 4 **Compositions of the dominant genus among interannual soils.**

| Treatments | Dominant genus |
|---|---|
| CK | *Glomus* of family Glomeraceae (99.97%) |
| HY1 | *Glomus* of family Glomeraceae (99.36%) |
| HY2 | *Glomus* of family Glomeraceae (95.31%), unclassified of family Diversisporaceae (3.80%) |
| HY3 | *Glomus* of family Glomeraceae (50.09%), unclassified of class Glomeromycetes (31.95%), *Diversispora* (17.95%) |
| HY4 | *Glomus* of family Glomeraceae (72.67%), *Diversispora* (27.32%) |

soils were significantly lower than that of the abandoned farmland, and the Shannon of 2-year soil was of no difference to that of the abandoned farmland. The Shannon of the 1-, 3-, and 4-year soils were significantly lower than that of the 2-year soil, which was with the highest Shannon. In conclusion, the 2-year soil had the highest Ace richness, Shannoneven evenness, and Shannon diversity in the interannual soils, and was the closest to the abandoned farmland in terms of alpha diversity.

## Dominant, core, specific, and significantly fluctuating AMF

The order Diversisporales was more frequently present to all interannual soils compared to the abandoned farmland (Figure 4A). At the genus level (Figure 4B), *Diversispora* was present in all interannual soils, and its abundance increased with interannual time. In this study, the genus with relative abundance (RA) of ≥1% was considered the dominant genus. The composition of the dominant genera differed among interannual soils (Table 4). The genus *Glomus* of the family Glomeraceae was present in all treatments with a relative abundance of more than 50%, while its relative abundance was more than 90% in the one- and 2-year soils. *Diversispora* was the dominant genus in the 3- and 4-year soils.

The core AMF in interannual soils were identified by Venn analysis (Figure 5), and they were the genera *Glomus* and *Diversispora*. In this study, specific AMF are those that are unique in one treatment and do not appear in others. There was one specific genus *Glomus* (RA < 1%) in the 1-year soil. The specific genus for the 2-year soil was

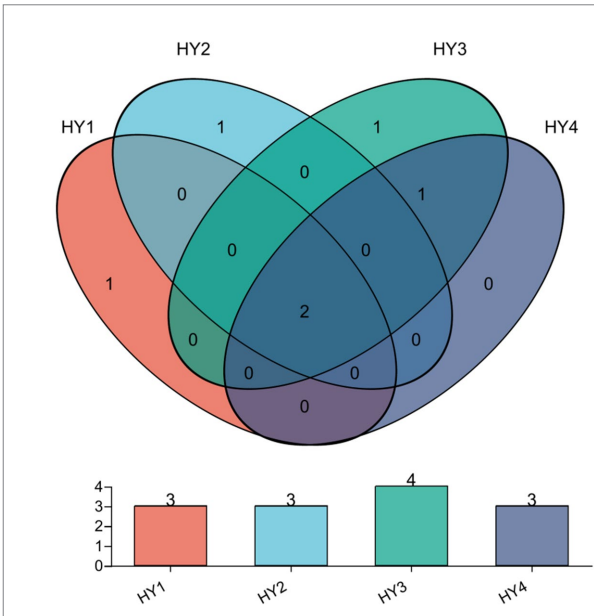



FIGURE 5
Venn diagrams at Genus level on the interannual soils. The meanings of HY1, HY2, HY3, and HY4 were illustrated in Figure 1.

an unclassified genus of the family Diversisporaceae, and it was the dominant genus in the 2-year soil. The specific genus for the 3-year soil was *Paraglomus* (RA < 0.1%), whereas no specific genus was present in the 4-year soil.

The Kruskal–Wallis test was used to analyze the species that showed significant differences among the interannual soils. Six species

showed significant differences in relative abundance among the interannual times, and these results were consistent with those of absolute abundance (Figure 6). Thus, genus *Glomus* varied significantly among the interannual soils. Except for the species (unclassified_class_Glomeromycetes), species with significant variations in relative abundance were mainly found in the order Glomerales.

## Co-occurrence network and beta diversity

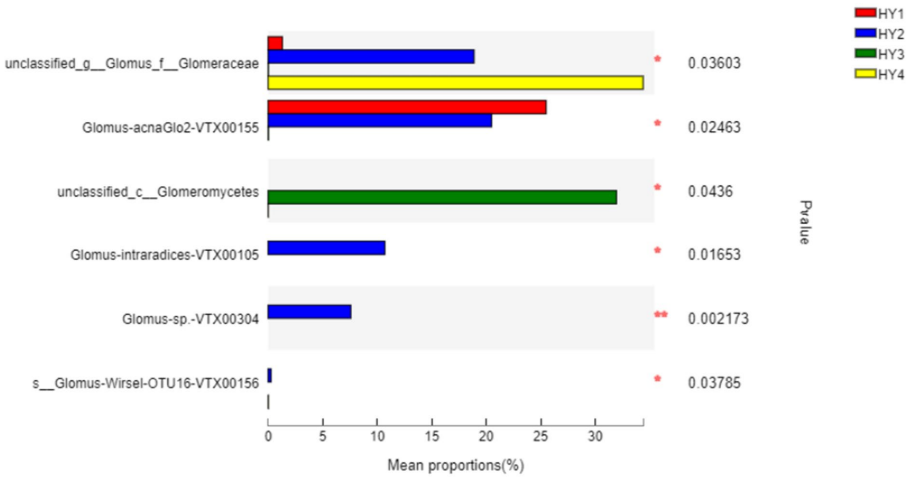
The community co-occurrence networks of interannual soils reflected relationships between species under specific environmental conditions (Supplementary Figure S4). One-ANOVA analysis of the network degree showed that the interannual time significantly ($p < 0.01$) affected the connectivity of networks ($HY3^{a} > HY2^{b} > HY4^{bc} > HY1^{c}$; lowercase letters in the right superscript indicate the results of Tukey multiple comparisons, $p < 0.05$). The network connectivity of the 3-year soil was significantly higher than that of other soils; and its clustering coefficient was the largest, indicating that the interrelationship among species in the 3-year soil was the strongest.

Beta diversity was used to analyze the diversity of the community along the environmental gradient. PCoA analysis was used to investigate the effect of treatments and interannual times on the community composition. The results showed that the community compositions of the interannual soils were close to each other and separated from the abandoned farmland, indicating that the community compositions of the interannual soils were different from those of the abandoned farmland (Figure 7A). Anosim test based on relative adundance ($R = 0.5150$, $p = 0.0010$) showed that the treatments significantly affected the community composition, and the consistent result was supported by Anosim test based on absolute abundance ($R = 0.4938$, $p = 0.0010$). The community compositions of the 1-year soil, 2-year soil, 3-year soil, and 4-year soil were significantly different from those of the abandoned farmland, and these results were supported by the statistical validation based on the relative and absolute abundance (Figure 7A).

The interannual times significantly affected the community composition, which was supported by Anosim test based on the relative abundance ($R = 0.3785$, $p = 0.0030$) and absolute abundance ($R = 0.3550$, $p = 0.0030$) (Figure 7B). The Anosim test showed no significant difference between the community composition of the 1-year soil and the 2-year soil, and between the 3-year soil and the 4-year soil, while the community composition of the 2-year soil was significantly different from that of the 3-year soil and the 4-year soil. These results based on the relative and absolute abundance were consistent (Figure 7B). Thus, significant changes in the community composition among interannual soils occurred in the 3-year soil.

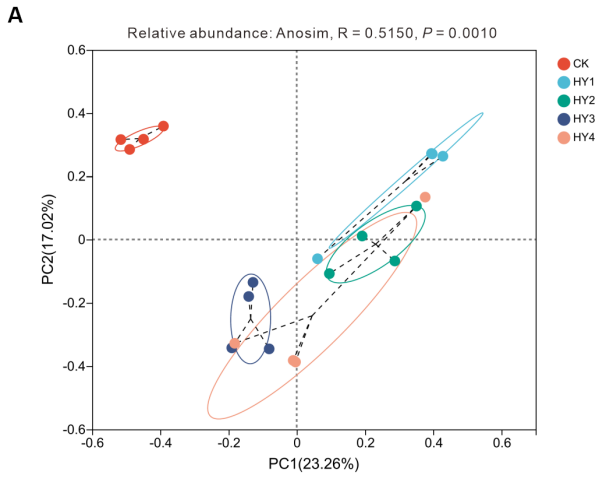
# Discussion

In this study, we investigated the effect of interannual time on the AMF community in astragalus soils using the Illumina sequencing technology and quantitative real-time PCR. The amount of sequencing data reflected the AMF diversity in the samples. A total of 74 OTUs were identified by species annotation. The 2-year

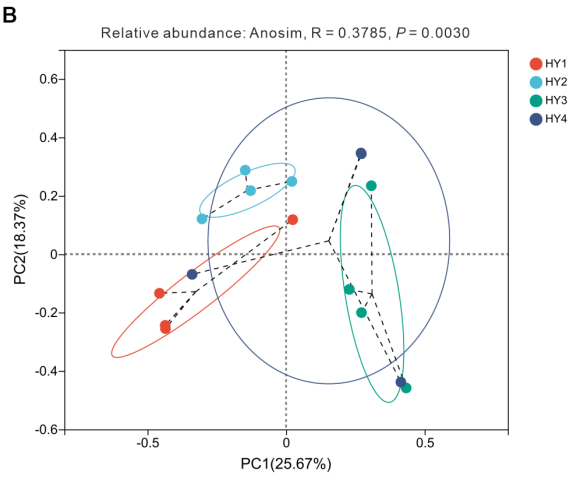



| Kruskal–Wallis test | Absolute abundance |
|---|---|
| unclassified_g__Glomus_f__Glomeraceae | H = 8.587, $P < 0.05$ |
| Glomus-acnaGlo2-VTX00155 | H = 10.539, $P < 0.05$ |
| s__unclassified_c__Glomeromycetes | H = 8.120, $P < 0.05$ |
| Glomus-intraradices-VTX00105 | H = 10.253, $P < 0.05$ |
| Glomus-sp.-VTX00304 | H = 14.619, $P < 0.01$ |
| Glomus-Wirsel-OTU16-VTX00156 | H = 8.454, $P < 0.05$ |

FIGURE 6
Kruskal-Wallis H test bar plot of species that differed significantly among interannual soils. Y-axis indicated species name at species level, X-axis indicated the mean relative abundance of species, and colored bars indicated different groups. *$p \leq 0.05$ and **$p \leq 0.01$. The meanings of HY1, HY2, HY3, and HY4 were illustrated in Figure 1. Kruskal-Wallis statistics based on the absolute abundance were shown in the table. g stands for genus, f for family, and c for class.




| Anosim test | Relative abundance | Absolute abundance |
|---|---|---|
| CK vs HY1 | R = 0.7865, *P* = 0.0340 | R = 0.7969, *P* = 0.0340 |
| CK vs HY2 | R = 1.0000, *P* = 0.0340 | R = 1.0000, *P* = 0.0340 |
| CK vs HY3 | R = 0.5573, *P* = 0.0340 | R = 0.5521, *P* = 0.0340 |
| CK vs HY4 | R = 0.7500, *P* = 0.0340 | R = 0.7500, *P* = 0.0340 |




| Anosim test | Relative abundance | Absolute abundance |
|---|---|---|
| HY1 vs HY2 | R = 0.4479, *P* = 0.0600 | R = 0.3542, *P* = 0.1200 |
| HY3 vs HY4 | R = 0.2812, *P* = 0.0680 | R = 0.2865, *P* = 0.0680 |
| HY2 vs HY3 | R = 0.4948, *P* = 0.0340 | R = 0.5000, *P* = 0.0340 |
| HY2 vs HY4 | R = 0.4531, *P* = 0.0340 | R = 0.4219, *P* = 0.0340 |

FIGURE 7
Principal coordinate analyses of the community compositions to treatments **(A)** and interannual soils **(B)** based on OUT level. Anosim statistics based on the relative and absolute abundance were shown in the table. R, degree of explanation of the difference between samples; *P*, significant test value. The meanings of CK, HY1, HY2, HY3, and HY4 were illustrated in Figure 3.

soil had the highest number of reads among the interannual soils, which was consistent with the results of AMF absolute abundance based on fluorescence quantification. This evidence strongly suggests that 2-year soil was highly enriched with AMF. All AMF belonged to the class Glomeromycetes in the phylum Glomeromycota; however, the distribution of AMF at the order level showed variability, with the order Glomerales occurring in all interannual soils.

The distribution characteristics of OTUs indicated that the 2-year soil had rich species diversity, and the Shannon analysis concluded that the diversity of the 2-year soil was significantly higher than that of the 1-, 3-, and 4-year soils. The results of both the analyses were consistent. In a 3-year continuous crop study, AMF diversity was found to be significantly higher in a 3-year continuous soil than in a 2-year soil, concluding that the continuous crop was beneficial to the accumulation of AMF diversity (Cui et al., 2018). This study showed that the interannual soils significantly affected the ACE of the community; similar results were found in a study of soybean continuous crop years, where the relative abundance of AMF communities varied significantly among soybean continuous crop soils (Cui et al., 2018). However, in all interannual soils, the maximum richness, evenness, and diversity of the community occurred in the 2-year soil, indicating that the 2-year continuous astragalus crop contributed to the increase in richness, evenness, and diversity of the AMF community.

Ace, Shannoneven, and Shannon estimators decreased significantly from 2-year soil to 3-year soil; however, the results of the beta diversity showed that the 3-year soil was the year in which significant changes in community composition occurred, thus indicating that the AMF community composition changed significantly during the third year of continuous astragalus cropping. In conclusion, the community composition of the 2-year soil had highest richness, evenness, and diversity among interannual soils, and all of them were close to the richness, evenness, and diversity of the abandoned farmland. In a previous study, we found that *A. sinensis* seedlings grown in Astragalus-cultivated soils were of good quality and the AMF abundance was higher than other soils (An et al., 2023). Therefore, from the perspective of AMF community, soil with 2 years of continuous astragalus may be more suitable for *A. sinensis* seedlings rotation. Many studies have shown that implementing a rational crop rotation strategy can increase the abundance, diversity, and composition of AMF in the soil. For example, the abundance of AMF was increased through wheat (Higo et al., 2013) and maize (Moitinho et al., 2020) rotation.

The genus *Glomus* had a high relative abundance in all treatments and was the dominant genus, whereas the genus *Diversispora* dominated in the 3- and 4-year soils. The genus *Glomus* has been found to be dominant in AMF communities within soils and rhizosphere in many studies (Xiao et al., 2022), including those conducted on soybean continuous cropping soils (Cui et al., 2018), rhizosphere of *Atractylodes lancea* in the Chongqing region (Cao et al., 2020), and rhizosphere of *Pinellia ternate* in Hangzhou and Guiyang (Shi et al., 2017). The dominant AMF genera in the potato rhizosphere in central Inner Mongolia included *Glomus* and *Diversispora* depending on the sampling site (Zhang et al., 2020). However, Lin et al. (2019) studied AMF diversity in the soils of karst habitats, and the results showed that *Rhizophagus* was the dominant genus. Chen et al., 2022 studied AMF diversity in cotton growing regions in Xinjiang, and successfully iedentied AMF spores based on morphological and molecular examinations. They found that *Paraglomus* was the dominant genus. In addition, the genera *Glomus* and *Diversispora* were the core AMF in all interannual soils. 1-, 2-, and 3-year soils possessed their own unique genera, and this may be due to the distinct ecological niches caused by specific habitats, or may be attributed to species drift (Dini-Andreote and Raaijmakers, 2018).

*Glomus intraradices* has been extensively studied, and now this species belongs to the genus *Rhizophagus*, known as *Rhizophagus intraradices* (Reference URL: https://invam.ku.edu/). Inoculation with *G. intraradices* improved phosphorus uptake in the phosphorus-deprived state of olive trees (Jiménez-Moreno et al., 2018), and another study showed that inoculation with *G. intraradices* increased root colonization of *Panax ginseng*, increased the content of monomeric and total ginsenosides, and improved root activity as well as polyphenol oxidase and peroxidase activities (Tian et al., 2019). In this study, the higher abundance of *G. intraradices* in the 2-year soil may have favored the growth of rotational crops.

Alpha and beta diversities were used to analyze the characteristics of AMF communities on a macro level, and co-occurrence networks can reflect the relationship between community individuals at the micro level. Although the 3-year soil was the year in which the richness, evenness, and diversity of the AMF community decreased and the composition changed significantly, the network analysis of the 3-year soil showed that the relationship between the community individuals became closer. This suggests that there is an inevitable relationship between changes in species interrelationships and changes in community alpha and beta diversities.

## Conclusion

This study reported the variation in AMF communities among interannual astragalus soils. The *Glomus* was the dominant genus present in all treatments, and the composition of the dominant genus in interannual soils was different. AMF community composition varied significantly among the interannual soils. The community composition of the 2-year soil was significantly different from that of the three and 4-year soils. Significant changes in the community composition among interannual soils occurred in the 3-year soil. Although a significant difference in beta diversity between the 2-year soil and abandoned farmland was noted, the Ace, Shannoneven, and Shannon estimators in the 2-year soil were the closest to the abandoned farmland. Therefore, among the interannual soils, the 2-year soil may be more suitable for *A. sinensis* seedlings rotation.

After thoroughly analyzing the results, we identified three questions that warrant further investigation. For example, (1) why did the AMF community within the 2-year soil exhibit the highest richness among all samples? (2) why was the abundance of wasteland soils higher than expected? (3) why did a significant shift in the microbial community composition occur within the 3-year soil?

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI - PRJNA905095 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA905095).

## Author contributions

Z-GA: Formal analysis, Software, Writing – original draft, Writing – review & editing. H-SS: Data curation, Funding acquisition, Writing – review & editing. Z-JC: Data curation, Methodology, Writing – original draft. Y-FH: Methodology, Writing – original draft. RW: Investigation, Writing – original draft. R-HL: Resources, Writing – original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1293496/full#supplementary-material

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# Long-term phosphorus fertilization reveals the phosphorus limitation shaping the soil micro-food web stability in the Loess Plateau

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The intricate decomposition pathways within soil micro-food webs are vital for cycling soil organic carbon and nutrients, influencing the quality, productivity, and sustainability of soil systems. However, the impact of diverse phosphorus addition on these organic decomposition pathways still needs to be explored. In an 8-year experiment, phosphorus (P) fertilizer was added at varying levels (0 kg ha$^{-1}$, CK; 60 kg ha$^{-1}$, P60; 120 kg ha$^{-1}$, P120; and 180 kg ha$^{-1}$, P180), to investigate the response of the soil micro-food web. The results revealed a significant effect of phosphorus addition on soil microorganisms and nematodes, with P60 exerting a greater influence than other treatments. At P60, the Shannon index of nematodes and fungi surpassed other treatments, indicating higher diversity, while the Shannon index of bacteria was lower. The Chao1 index of bacteria and fungi at P60 was higher, contrasting with the lower index for nematodes. Metabolic footprints of bacterivores and omnivores–predators (BFMF and OPMF) were higher at P60, while metabolic footprints of fungivores and plant parasites (FFMF and PPMF) were lower, signifying altered energy flow. Functional metabolic footprints and energy flow analysis unveiled a stable soil micro-food web structure at P60, with enhanced energy conversion efficiency. Network analysis illustrated positive correlations between fungi, fungivorous nematodes (FF), and omnivorous-predatory nematodes (OP) at P60, while P120 and P180 showed positive correlations among bacteria, bacterivorous nematodes (BF), and OP. Path analysis underscored the higher contribution rate of BF-C, FF-C, and OP-C to soil organic carbon at P60 compared with P120 and P180. These findings suggest that nutrient interactions between fungi and nematodes regulate soil micro-food web decomposition under low phosphorus concentrations. In contrast, interactions between bacteria and nematodes dominate at high phosphorus concentrations. The study indicates that adding phosphorus has nuanced bottom-up effects, intricately shaping the structure and activity of the pathways and underscoring the need for a comprehensive understanding of nutrient dynamics in soil ecosystems.

KEYWORDS

**Loess Plateau, soil micro-food web, nematode metabolic footprint, decomposition pathway, phosphorus**

# 1 Introduction

Soil is the largest organic carbon pool in terrestrial ecosystems, the carbon storage of which exceeds the sum of vegetation and atmospheric carbon pools (Lehmann and Kleber, 2015). Indeed, soil organic carbon (SOC) is crucial in providing ecosystem services and regulating the global carbon cycle. However, small changes in the global SOC pool will significantly impact atmospheric $CO_2$ concentration (Kirschbaum, 2000). This change in SOC depends on the balance between plant inputs and microbial decomposition outputs, which can be further affected by soil phosphorus (P) fertilizers (Jackson et al., 2017). Mismanagement or misunderstanding of phosphorus fertilizers can have disastrous effects on soil quality and productivity (Poirier et al., 2014). An important agricultural management practice also provides carbon and nutrient sources to soil micro-food web through decomposition pathways (Das et al., 2012). The soil micro-food web represents a consumer–resource interaction network composed of "microbes–protozoa–nematodes" or "microbes–nematodes" and other soil microorganism groups with different functions (Lavelle, 1997). Modifications in the structure of these webs not only signify alterations in the energy pathways of organic matter but also exert a profound influence on aboveground plant growth through intricate feedback mechanisms. Among soil nematodes, categories encompass bacterivores, fungivores, plant-parasitic, and omnivore–predator nematodes, each contributing to distinct carbon flow pathways, such as the bacterial channel, the fungal channel, and the root channel. Specifically, within these pathways, the bacterial channel involves the collaboration of bacteria and bacterivorous nematodes and the fungal channel involves fungi and fungivorous nematodes, while the root channels are inhabited by plant-parasitic nematodes (Thakur and Geisen, 2019). Increasing evidence shows that the interconnections between these channels play a critical role in regulating soil food webs (Ferris, 2010; Kou et al., 2020; Wu et al., 2021). The interaction between soil microorganisms and nematodes directly affects the connectivity of the soil micro-food web (Figure 1). This interaction holds significant implications for organic matter decomposition, carbon storage, nutrient recycling and redistribution, soil respiration, and aggregate formation (Ferris et al., 2001). Utilizing microorganisms and nematodes as a starting point for examining soil micro-food webs offers valuable insights into the nutrient cycling mechanisms in the ecosystem. It is of great scientific significance for the in-depth understanding of the underground ecological processes in which soil organisms participate under the addition of phosphorus and for revealing soil health and quality (Jiang et al., 2018).

Soil microorganisms and nematodes are pivotal contributors and indicators of subterranean ecosystems properly functioning. Microbial communities in the soil significantly influence carbon sequestration, nutrient cycling, decomposition of plant residue, and productivity supply (Fang et al., 2016). Various ecological indicators related to soil nematodes illuminate the response of the soil micro-food web to resources and their contribution to ecosystem functions (Ferris et al., 2001; Briar et al., 2012). Furthermore, the interactions between soil microorganisms and nematodes act as indicators of ecosystem health. Soil nematodes play a crucial role in shaping the composition of

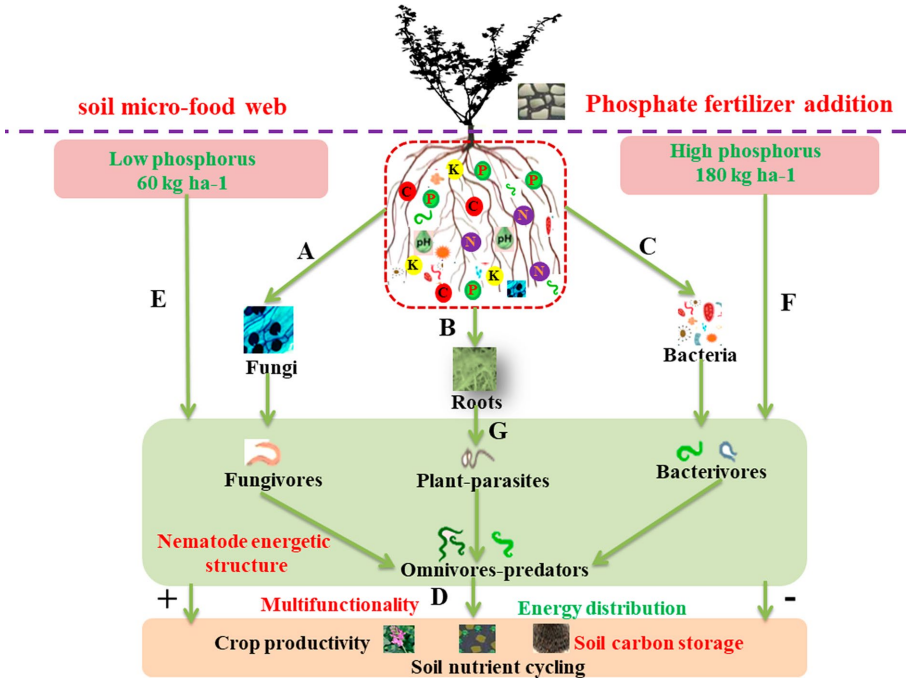


FIGURE 1
A conceptual framework showing how soil multifunctionality in sustainable agriculture emerges as the result of the underlying food web structure, which is fueled by energy and nutrients coming from phosphorus (P) fertilizer applications to the agroecosystem. Phosphorus fertilizers increase the energy flux through microbivores and herbivores by promoting soil microbial biomass **(A,C)** and plant growth **(B)**, respectively. These changes would increase the energy flux through omnivores-carnivores and alter nematode energetic structure, enhancing soil multifunctionality **(D)**. When nutrients were limited, plant root growth and rhizodeposition would be stimulated, and more energy flux through herbivores would disrupt the energy flux balance and change soil multifunctionality **(G)**. Conversely, when nutrients were sufficient, more energy flux by microbivores would favor soil multifunctionality **(E,F)**.

microbial communities. Alterations in the structure of the nematode community trigger dynamic shifts in the microbial community, impacting dominant species. This influence is evident in the selective predation of soil nematodes on specific microorganisms. In meeting their growth needs, soil nematodes may preferentially consume more rapidly growing microorganisms, leading to changes in the structure and diversity of microbial communities (Marschner and Kalbitz, 2003; Jiang et al., 2018; Wang et al., 2018). The bottom-up relationship between soil nematodes and microbial communities plays a pivotal role in regulating the structure of the soil ecosystem. This relationship is directly related to the soil biogeochemical processes, thereby promoting soil ecosystem function and altering the soil ecological environment (Sohlenius et al., 1988; Francini et al., 2018). In general, disturbances in the soil environment trigger the trophic chain through a bottom-up mechanism. This bottom-up regulation results in alterations within bacterial and fungal communities due to primary production or resource input, which subsequently extend to higher trophic levels (Kou et al., 2020; Nguyen et al., 2020). Environmental disturbance can lead to bottom-up regulation within the trophic levels of the soil micro-food web, potentially impacting critical ecosystem functions, including nutrient cycling and soil production (Thakur and Geisen, 2019). In a context where human activities exert multifaceted impacts on soil systems, the analysis of key factors that drive cascading effects within trophic interactions is vital for the effective management of soil health and plant productivity (Barnes et al., 2017).

To more accurately represent the pathways of the soil micro-food web and carbon utilization in nematode production and respiration, Ferris (2010) introduced the concept of the soil nematode metabolic footprint (NMF) as a measure of carbon fluxes within the soil nematode food web. Metabolic footprints can be divided into enrichment footprints and structural footprints. Enrichment footprints are the metabolic footprints of those nematodes that respond most quickly to resource enrichment. Structural footprints are metabolic footprints of nematodes at higher trophic levels that may have regulatory functions in the food web (Ferris, 2010). Scholars are increasingly recognizing the importance of soil nematodes as biological indicators of the impact of human and soil disturbance on soil food webs (Hu et al., 2016). Previous studies have shown that phosphorus addition mainly affects the quantity and quality of organic resources, thereby affecting the composition and activity of soil biological communities (Aerts et al., 2003). It has been suggested that low concentrations of phosphorus addition can improve soil nematode and bacterial community diversity, while too high phosphorus concentrations can improve soil fungal community diversity (Bissett et al., 2011; Hu J. et al., 2017). Consequently, we speculate that the outcomes of phosphorus addition will differ depending on the quantity applied. However, only some studies have attempted to explore the mechanisms by which soil food webs respond to such differences in resource management, and our study helps fill this knowledge gap.

The Loess Plateau in northwestern China has a long farming history. Due to the special ecological environment and soil texture, the Loess Plateau suffers from severe soil erosion, making it one of the most vulnerable regions in the world (Li et al., 2008; Fu et al., 2010). Alfalfa, an excellent legume forage for popularization and planting, has the characteristics of high yield, rich nutrition, and strong ecological adaptability. Its root system has strong nodule nitrogen (N) fixation, increasing soil organic matter, improving the regional ecological environment, and promoting the development of animal husbandry (Abbas et al., 2022). However, continuous alfalfa planting has seriously consumed soil moisture and phosphorus availability, resulting in a short, vigorous growth period and low alfalfa yield, and also caused the degradation of alfalfa artificial grassland (Gu et al., 2018; Wang et al., 2021). Nevertheless, the phosphorus addition exhibited a noticeable influence on the nitrogen fixation within alfalfa root nodules (Jia et al., 2006). Therefore, choosing the appropriate amount of phosphorus benefits the growth and soil fertility improvement of alfalfa. In summary, this study used an 8-year fertilization experiment to investigate the effects of different phosphorus addition on alfalfa soil and explore their effect on the decomposition pathway of soil micro-food web. We hypothesized that the amount of phosphorus application significantly influences the community structure, diversity, and metabolic footprint of microorganisms and nematodes, thereby impacting the decomposition pathways within the micro-food web. At low phosphorus concentrations, fungi, fungivorous nematodes, and predatory/omnivorous nematodes are primarily engaged in fungal decomposition pathways, enhancing carbon storage. Conversely, at high phosphorus concentrations, bacteria, bacterivorous nematodes, and predatory/omnivorous nematodes primarily participate in bacterial decomposition pathways, potentially reducing carbon storage.

# 2 Materials and methods

## 2.1 Site description

This study carefully selected an experimental site which is situated within the Gansu Agricultural University Comprehensive Experimental Station for dry farming on the Loess Plateau in the northwestern region of China. The experimental site belongs to Dingxi City in Gansu Province (35°28′N, 104°44′E with an average altitude of 1,970 m. In this area, the mean annual temperature is 6.4°C, the mean annual precipitation is 390 mm, and the mean annual evaporation is 1,531 mm. The experimental site is located in a semi-arid middle temperate zone area, a typical rainfed farming area with one crop annually. The basic properties of soil were characterized by organic matter content of 8.04 g $kg^{-1}$, total nitrogen content of 0.82 g $kg^{-1}$, and total phosphorus content of 1.07 g $kg^{-1}$.

## 2.2 Experimental design and soil sampling

In this experiment, alfalfa was planted in April 2014 using a drill method with a seeding rate of 22.5 kg $ha^{-1}$, 10 rows per plot, and a row spacing of 30 cm. It had four treatments, including no phosphorus (CK), low phosphorus with 60 kg $ha^{-1}$ (P60), moderate phosphorus with 120 kg $ha^{-1}$ (P120), and high phosphorus with 180 kg $ha^{-1}$ (P180). Phosphorus fertilizer was applied every 3 years (2014, 2017, and 2020), and nitrogen fertilizer was applied yearly (N 50 kg $ha^{-1}$). Each treatment with three replicates was applied to a plot with a dimension of 4 m × 3 m. The nitrogen fertilizer used in the experiment was urea (46% pure nitrogen), and the phosphorus fertilizer was superphosphate (12% pure $P_2O_5$). In addition to nitrogen and phosphorus fertilizers, no other organic and inorganic fertilizers were

added in this experiment. The experiment was carried out under natural conditions without irrigation.

Soil samples were collected in June 2022 at the full flowering stage of alfalfa head stubble. There were 12 samples (4 treatments × 3 replicates). Soil samples were collected from the tillage layer of 0–20 cm depth. In each plot, five soil cores were randomly collected with a 2.5 cm diameter auger, and then, the samples were hand-mixed and passed through a 2-mm screen. Each sample was subdivided into two parts to determine general soil properties and stored at −80°C for the high-throughput sequencing of microorganisms.

## 2.3 Analysis of soil physicochemical properties

In this study, selected soil properties were measured, including soil moisture, pH, organic carbon (OC), total nitrogen (TN), total phosphorus (TP), and available phosphorus (AP). Soil water content (SW) was measured by drying fresh soil samples at 105°C to constant weight. Soil pH was measured in a soil: water (1:2.5) extract with a pH meter (Mettler Toledo, Switzerland). SOC was determined using the dichromate oxidation method (Sanmanee and Suwannaoin, 2009). This involved weighing 0.50 g of an air-dried soil sample, adding a potassium dichromate-sulfuric acid solution, and thoroughly mixing the sample and solution with a mixer, followed by sequential digestion and titration. Total nitrogen was analyzed by the Kjeldahl method (Brookes et al., 1985). First, 1.00 g of an air-dried soil sample should be weighed, fully enveloped in nitrogen-free weighing paper, and carefully positioned at the base of the digestion tube. Subsequently, 2 g of a copper sulfate-potassium sulfate accelerator and 5 mL of concentrated sulfuric acid are added to the tube, which was then covered with a curved neck funnel. The prepared sample was then placed in the digestion furnace and heated until the solution in the tube turns a light blue-green or off-white color. Upon completion of the digestion process, the tube was removed and allowed to cool to room temperature. Finally, the cooled sample was analyzed using a Kjeldahl Azotometer to determine its TN content. Total phosphorus was determined by the molybdenum blue method (Levine et al., 1955). Initially, 0.5 g of an air-dried soil sample was weighed in a digestion tube, which was moistened with a small amount of water. Next, 8 mL of concentrated sulfuric acid and 10 drops of perchloric acid were added. The tube was then placed in the digestion furnace for processing. Once digestion was complete, the tube was removed and allowed to cool to room temperature. The digestion liquid was then transferred from the tube to a 100 mL volumetric flask, diluted to the mark with water, and set aside for measurement. For the test solution, 10 mL was transferred to a 50 mL stoppered colorimetric tube, diluted with water to 40 mL, and supplemented with a drop of 2,4-dinitrophenol indicator. The pH of the solution was adjusted to a slight yellow hue using 0.5 mol/L sulfuric acid solution and 2 mol/L sodium hydroxide solution. Subsequently, 5 mL of molybdenum antimony antireagent was added, and the volume was brought up to 50 mL with water, followed by thorough mixing. After a 30-min interval, colorimetry was performed at a wavelength of 880 nm.

Soil AP was determined by the Olsen method (Do et al., 2007). Generally, 2.5 g of the air-dried soil sample was weighed and then transferred to a 250 mL conical flask. Then, 100 mL of sodium bicarbonate solution was added as the extraction agent, and the flask was securely sealed. Subsequently, the mixture was agitated on a constant-temperature reciprocating shaker for 30 min and then promptly filtered into a 150 mL dry Erlenmeyer flask using phosphorus-free filter paper, which was designated for analysis. Subsequently, 10 mL of the filtrate was transferred into a 50 mL volumetric flask, to which 5 mL of molybdenum antimony anti-chromogenic agent was added, and the volume was adjusted to the mark. After 30 min of resting period, colorimetric analysis was performed at a wavelength of 880 nm.

## 2.4 DNA extraction and high-throughput sequencing

The E.Z.N.A Soil kit (Omega Bio-tek, Norcross, GA, United States) isolated total genomic DNA from a 0.5 g soil sample, and the DNA concentration and purity were detected with a NanoDrop 2000 UV–VIS spectrophotometer (Thermo Scientific, Wilmington, United States). The quality of its extraction was detected by 1% agarose gel electrophoresis (Porazinska et al., 2009). The V4 region of the 18S rRNA gene of nematodes was amplified with the primers NF1(5′-GGTGGTGCATGGCCGTTCTTAGTT-3′) and 18S r2bR (5′-TACAAAGGGCAGGGACGTAAT-3′) (Beauregard et al., 2010). The PCR amplification was performed for each soil DNA extract in triplicate and combined into a single composite sample. The thermal cycling conditions were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, followed by 72°C for 10 min for primers NF1/18S r2bR. For bacteria, the V3-V4 region of the 16S rRNA gene was amplified using the primer pair 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Wang and Wang, 1996). The thermal cycling conditions were as follows: pre-denaturation at 98°C for 2 min, denaturation at 98°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min, 30 cycles. For fungi, the ITS1 region was amplified using the primer pair ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′) (Yang et al., 2017). The thermal cycling conditions were as follows: 95°C for 5 min; 15 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, followed by 72°C for 7 min for primers ITS1F/ITS1R. PCR products were gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, United States) (Mueller et al., 2014; Sinclair et al., 2015). The resultant PCR products were combined at equimolar concentrations before being sequenced on an Illumina MiSeq sequencer at the Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Paired-end (PE) reads obtained from Illumina sequencing were initially spliced based on overlap relationships, simultaneously undergoing quality control and filtration. After distinguishing the samples, both OTU clustering and species taxonomy analyses were performed. Based on the OTU cluster analysis results, diversity index analysis was performed on OTUs. Using the taxonomic information, community structure statistical analysis was performed at the fungal and bacterial phylum levels and the nematode genus level. Based on the above analysis, in-depth statistical and visual analyses were conducted on community composition and phylogenetic information of each treatment. The microbial and nematode DNA sequences of the 12 soil samples were deposited in the SRA of the NCBI database under Accession nos. NCBI: PRJNA988166 and SRA: SUB13581533.

## 2.5 Data analysis

### 2.5.1 Calculation of soil microbial and nematode diversity index

Calculate a diversity index of bacteria, fungi, and nematodes. The Chaol index reflected the richness of microbial and nematode communities (Chao, 1984), and the Shannon index indicated the diversity of microbial and nematode communities (Shannon, 1997).

$$S_{chao1} = S_{obs} + \frac{n_1(n_1-1)}{2(n_2+1)}$$

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} \ln \frac{n_i}{N}$$

where $S_{obs}$ is the number of OTUs observed; $n_1$ is the data with one sequence (e.g., singular); $n_2$ is the data with two sequences (e.g., even); $n_i$ is the number of OTUs containing sequence $i$; and $N$ is the total number of sequences. The number of OTUs in the sample was evaluated using the chao1 index. The larger the Chao1 index, the larger the number of OTUs, indicating more species in the sample. The larger the Shannon value, the higher the community diversity.

Indices such as the nematode Basal Index (BI), Structural Index (SI), Enrichment Index (EI), Trophic Diversity Index (TD), Free-living Nematode Maturity Index (MI), Plant-parasitic Nematode Maturity Index (PPI), and Channel Index (CI) were served to reflect the food web structure, nutrient enrichment status, and decomposition pathways of soil ecosystems (Bongers, 1990; Ferris and Bongers, 2006).

### 2.5.2 Metabolic footprints of nematode communities

The nematode metabolic footprints were calculated using the fresh weight of nematodes listed in the "Nematode-Plant Expert Information System."[1]

$$NMF = \Sigma\left(N_t \times \left(0.1 \times \left(W_t \div m_t\right) + 0.273\left(W_t^{0.75}\right)\right)\right)$$

In the formula: $N_t$ is the abundance of $t$-type nematode group; $m_t$ is the c-$p$ value of $t$-type nematode group; and $W_t$ is the biomass of $t$-type nematode group (Ferris, 2010).

Nematode flora analysis assessed enrichment footprints (Fe) and structural footprints (Fs). Taking the coordinate point of (SI, EI) as the center position, sequentially connecting (SI-0.5Fs/k, EI), (SI + 0.5Fs/k, EI), (SI, EI-0.5Fe/k), and (SI, EI + 0.5Fe/k), the formed diamond area was the functional metabolic footprint of the nematode community (Zhang et al., 2015).

### 2.5.3 Energy flow analysis of soil food web

The energy flow analysis of the soil food web referred to the method by Ferris and Bongers (2006). The coordinate point of the vertex was (50, 86.6), the coordinate point of the lower left corner of the triangle was (0, 0), and the coordinate point of the lower right corner was (100, 0). Geometry knowledge calculated the coordinate points that determine the center point of a triangle and the midpoints of three sides. Seven coordinate points of each treatment were calculated from the relative metabolic footprints of bacterivorous, fungivorous, and plant-parasitic nematodes. After setting the coordinate points of each process, we selected all of them to draw a scatter diagram, in which the drawing of the three sides of the triangle and the midline of each side can be presented by adding a solid line or a dashed line, finally deleting the coordinate axis.

### 2.5.4 Correlation ecological network and structural equation model

This study assessed the relationship between microorganisms and nematodes using correlation network analysis. To simplify the correlations between microbes and nematodes, we focused on the relative abundance at the genus level, thereby excluding interactions between genera within the same species. We screened for all possible Spearman correlations among genera appearing in at least three samples. If the Spearman correlation coefficient ($r$) > 0.6 and $p$ < 0.05, there was a valid co-occurrence between genera (Deng et al., 2012). The network visualization was performed using Gephi v0.9.2.

Path analyses were used to reveal nematode metabolic footprints and microbial community relationships. The arrows and their path coefficients indicated the direction and strength of the relationships between these potential variables. Model fit to the data was evaluated using the $X^2$ value and associated $p$ value. Three tests, namely, Comparative Fit Index (CFI), Goodness of Fit (GFI), and Root Mean Square Error of Approximation (RMSEM), were used to assess model fit. The optimal model was identified by progressively eliminating non-significant paths. Path analyses were conducted using Amos 24.0 software (Arbuckle, 2006).

### 2.5.5 Statistical analysis

One-way ANOVA was performed to investigate whether different phosphorus treatments significantly affected soil physicochemical properties, microorganisms, and nematode communities. Differences between all soil parameter means were compared using the least significant difference (LSD) ($p$ < 0.05). Charts and figures of soil properties, microorganisms, and nematode communities were generated by SPSS Statistics 22 (SPSS Inc., Chicago, IL, United States) and Origin 2021, respectively. Canoco 5.0 software was used to determine the interactions between soil physicochemical properties, microorganisms, and nematode communities. The correlation network was carried out using R software packages "igraph."

# 3 Results

## 3.1 Environmental factors

Soil physicochemical properties are shown in Table 1. There was no significant difference between SW and TN. SOC, TP, and AP increased significantly with increasing phosphorus addition ($p$ < 0.05), while soil pH decreased with increasing phosphorus addition, and the P180 treatment was significantly higher than that of CK ($p$ < 0.05).

1 http://plpnemweb.ucdavis.edu/nemaplex

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TABLE 1 Soil physical and chemical properties under different phosphorus addition treatments.

| Factors | CK | P60 | P120 | P180 |
|---|---|---|---|---|
| Soil water (%) | 7.75 ± 0.15a | 8.04 ± 0.12a | 7.69 ± 0.43a | 7.89 ± 0.12a |
| Soil organic carbon (g·kg$^{-1}$) | 11.01 ± 0.12c | 11.30 ± 0.07b | 11.60 ± 0.06a | 11.67 ± 0.03a |
| Total nitrogen (g·kg$^{-1}$) | 0.78 ± 0.01a | 0.80 ± 0.02a | 0.83 ± 0.01a | 0.79 ± 0.01a |
| Total phosphorus (g·kg$^{-1}$) | 0.82 ± 0.01c | 0.90 ± 0.01b | 0.94 ± 0.02a | 0.98 ± 0.01a |
| Available phosphorus (mg·kg$^{-1}$) | 4.07 ± 0.27c | 5.57 ± 0.09b | 6.05 ± 0.02a | 6.21 ± 0.06a |
| pH | 8.31 ± 0.02a | 8.29 ± 0.01a | 8.27 ± 0.02ab | 8.24 ± 0.01b |
| C/N | 14.13 ± 0.16a | 14.18 ± 0.36a | 13.98 ± 0.11a | 14.69 ± 0.21a |

Values are mean ± SE in triplicate replicates. Different lowercase letters indicate significant differences with a value of $p < 0.05$ based on the ANOVA. C/N, Ratio of carbon to nitrogen.

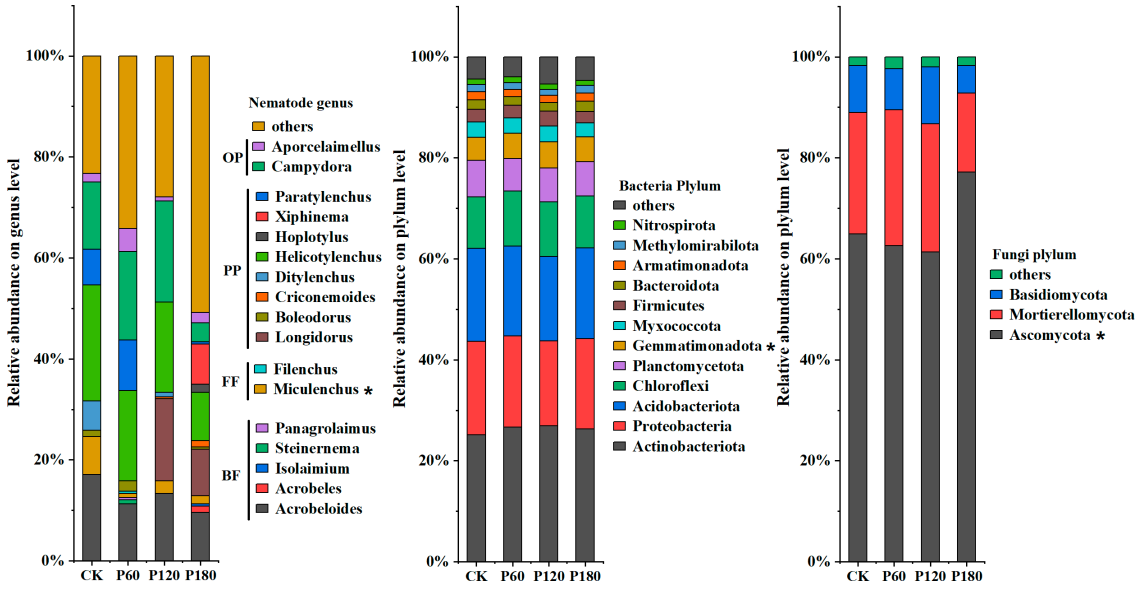


FIGURE 2
Relative abundance of soil microorganism and nematode communities (greater than 0.1%) in different phosphorus treatments: CK, P60, P120, and P180. Dominant genera: relative abundance of soil nematodes is greater than 10%; common genera: relative abundance of soil nematodes is 1–10%; rare genera: relative abundance of soil nematodes is less than 1%. BF, Bacterivorous nematodes; FF, Fungivorous nematodes; PP, Plant parasitic nematodes; and OP, Omnivorous predatory nematodes. The asterisk (*) indicated a significant difference between treatments ($p < 0.05$).

## 3.2 Community structure characteristics of soil microorganisms and nematodes

In this study, high-throughput sequencing was used to identify soil microorganisms and nematode communities under different phosphorus treatments (Figure 2). The results showed significant differences in the response of soil microbial and nematode communities to different phosphorus addition treatments ($p < 0.05$). At the soil nematode genus level, *Miculenchus* had significant differences between different phosphorus addition treatments ($p < 0.05$); at the bacterial phylum level, *Gemmatimonadota* showed a significant difference ($p < 0.05$), and at the fungal phylum level, *Ascomycota* displayed a significant difference ($p < 0.05$). In addition, different phosphorus addition treatments influenced the relative abundance of nematode trophic groups. Specifically, the relative abundance of plant-parasitic nematodes in CK and P120 treatments was significantly higher compared with P60 treatment and P180 treatment. The CK treatment demonstrated a significantly higher relative abundance of bacterivorous and fungivorous nematodes in comparison to other treatments. Conversely, the P180 treatment exhibited a noticeable decrease in the abundance of omnivorous-predatory nematodes compared with the other treatments.

## 3.3 Alpha diversity of soil microorganism and nematode communities

Shannon and Chao1 indexes of soil microorganisms and nematode communities (Figure 3) were measured to evaluate the α diversity of soil microorganisms and nematode communities under different phosphorus additions. Regarding the Shannon index, soil nematodes and fungi in the P60 treatment displayed a higher value than other treatments. Similarly, for the Chao1 index, soil bacteria and fungi under the P60 treatment surpassed other treatments.

## 3.4 Analysis of soil nematode metabolic footprint and food web energy flow

The bacterivore's metabolic footprint (BFMF) and omnivores-predators' metabolic footprint (OPMF) in the P60 treatment were higher than those of other treatments. In contrast, the fungivore's metabolic footprint (FFMF) and plant parasites' metabolic footprint (PPMF) were lower than those of other treatments. The opposite was true for the P180 treatment (Figure 4A). Flora analysis showed

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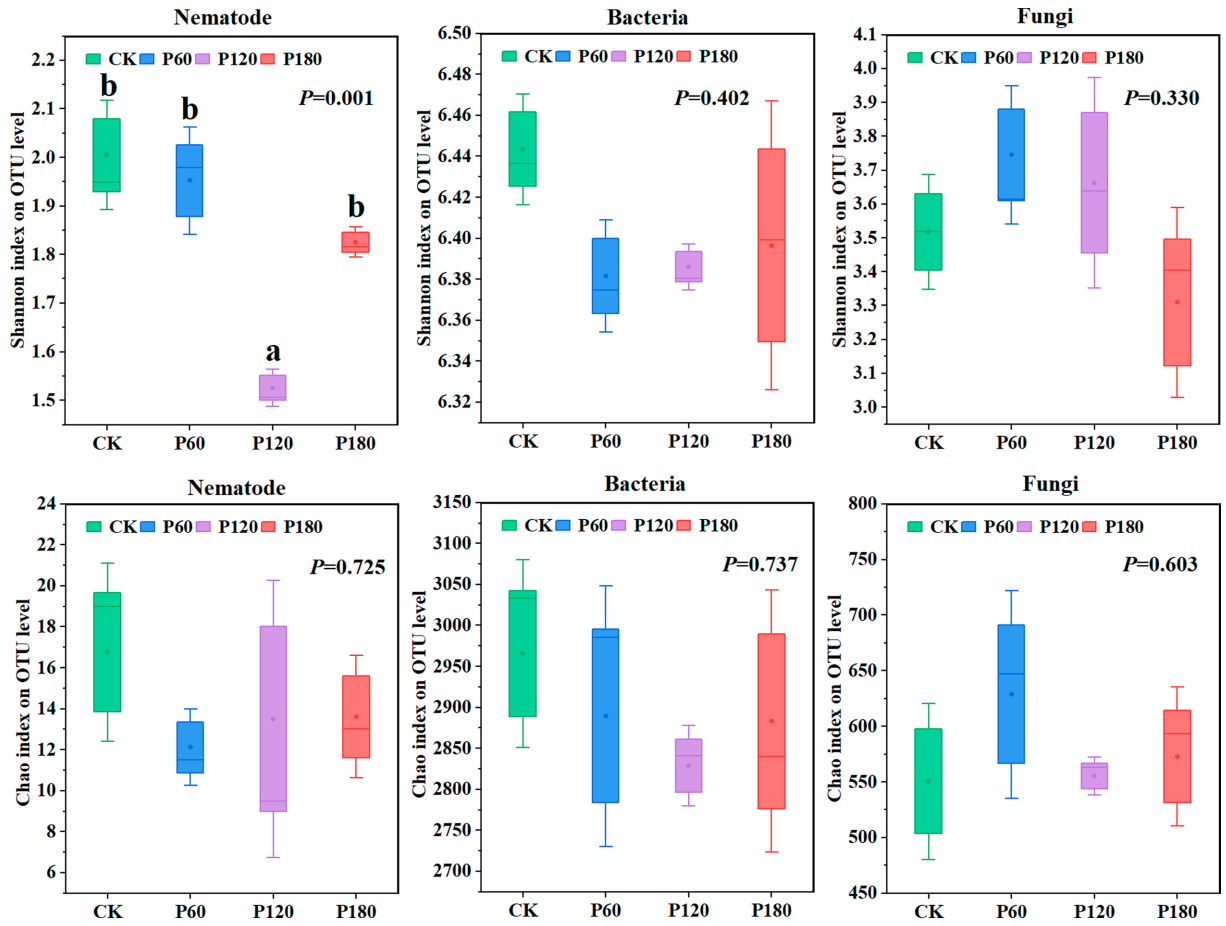


FIGURE 3
Diversity of soil microorganism and nematode communities under different phosphorus treatments. Different lowercase letters indicate significant differences with a value of $p < 0.05$ based on the ANOVA.

(Figure 4B) that CK, P60, P120, and P180 treatments were all in the C quadrant. The functional footprint of soil nematodes (diamond area) shows that the P60 treatment is higher than other treatments. The soil nematode enrichment index (center position of the diamond) increased with the increased amount of phosphorus added. The soil nematode structure index (center position of the diamond) decreased with the increased amount of phosphorus added. Food web energy flow analysis (Figure 4C) showed that the P60 treatment had the largest proportion of bacterial and fungal energy flow channels. In contrast, the proportion of plant energy flow channels was the smallest, and the P120 treatment was on the contrary. P180 treatment was between P60 and P120 treatments, indicating that energy conversion and utilization efficiency in soil food webs at P60 treatment was relatively high.

## 3.5 Analysis of functional indices of soil nematode communities

The functional index of the soil nematode community, as shown in Figure 5, reveals several findings. The Free-living Nematode Maturity Index (MI) for the P60 treatment surpasses other treatments. Conversely, both the plant parasitic nematode maturity index (PPI) and the ratio of PPI to MI (PPI/MI) were lower in the P60 treatment. These indexes suggested that the P60 treatment experiences fewer external disturbances, indicating a more stable ecological environment. As phosphorus application increases, indices such as the nematode basal index (BI), channel index (CI), and trophic diversity index (TD) demonstrate a declining trend. Significant differences ($p < 0.05$) were observed in BI and CI across treatments. This pattern indicated that lower phosphorus concentrations would reduce environmental disruptions, thereby enhancing the resistance of the soil food web. Furthermore, fungi predominantly dominated the degradation pathway in the soil food web at low phosphorus concentrations, while bacteria prevail at high phosphorus concentrations.

## 3.6 Soil micro-food web relationships

The interaction between soil microorganisms and nematodes became less frequent and weaker as the level of phosphorus addition increased (Figure 6; Table 2). Compared with the CK treatment, the phosphorus addition treatment increased the total number of links in the network. Within the phosphorus addition treatment, the P60 treatment exhibited more links than other treatments, with increased negative connections and decreased positive connections.

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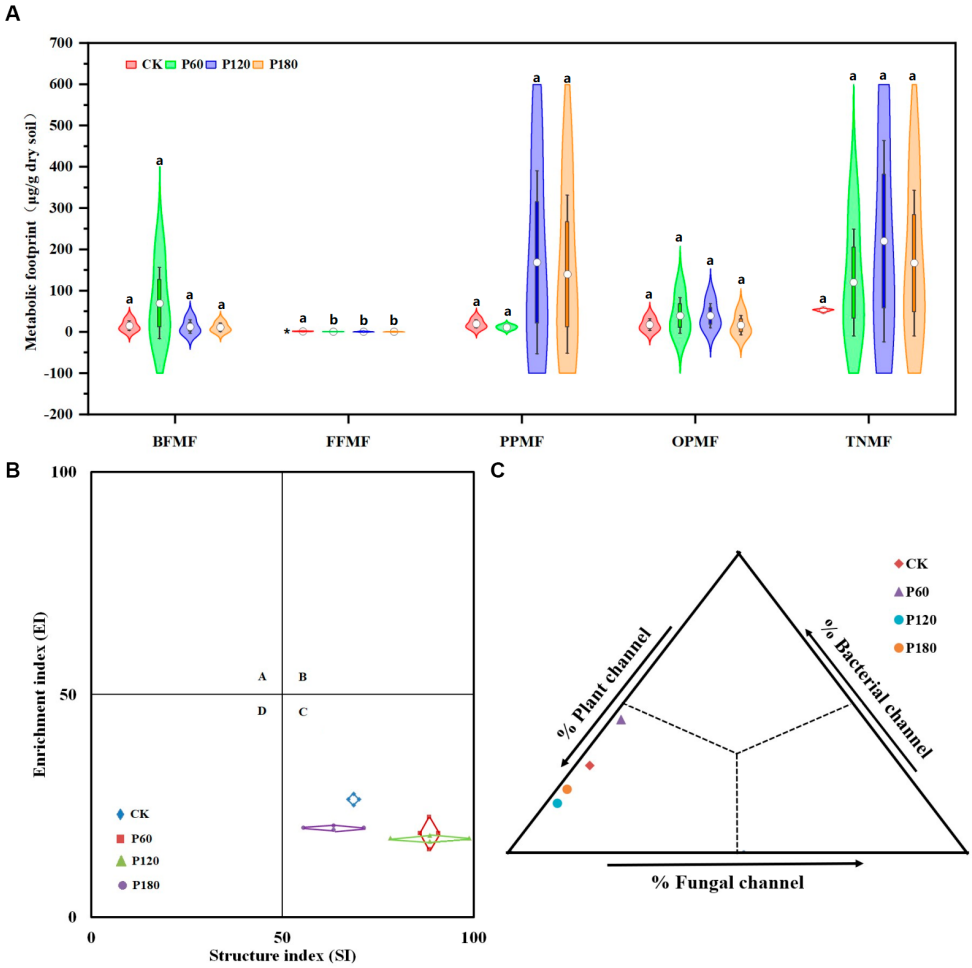


FIGURE 4
Metabolic footprint, floristic analysis, and food web energy flow analysis of soil nematodes. **(A)** Metabolic footprint; **(B)** Flora analysis; **(C)** Food web energy flow analysis; the piano box size depends on the interquartile range of the data. A large box means that the data distribution is discrete, and the data fluctuate greatly. Small means of the dataset are concentrated. The upper side of the box is the 75% quantile, the lower side is the 25% quantile, and the white dot on the violin plot represents the average value. BFMF, Bacterivores metabolic footprint; FFMF, Fungivores metabolic footprint; PPMF, Plant parasites metabolic footprint; OPMF, Omnivores-predators metabolic footprint; and TNMF, Total nematode metabolic footprint. The asterisk (*) indicated a significant difference between treatments ($p < 0.05$). Different lowercase letters indicate significant differences with a value of $p < 0.05$ based on the ANOVA.

In contrast, the P180 treatment displayed an inverse trend. In the CK and P60 treatments, there was a positive correlation among fungi, FF, and OP. In the P120 treatment, a positive correlation existed between bacteria, BF, and OP. Meanwhile, the P180 treatment showed a negative correlation between OP and both BF and bacteria but a positive correlation between BF and bacteria. Consequently, the low phosphorus treatment revealed a positive correlation among fungi, FF, and OP, suggesting that fungi predominantly drive the soil food web degradation channel. In contrast, the high phosphorus treatment demonstrated a positive correlation among bacteria, BF, and OP, indicating bacteria as the primary degradation channel. Path analysis was utilized to evaluate carbon flow within the soil food web, taking into account diverse components of soil microorganisms and nematodes across varying phosphorus additions. The contribution rates of BF-C, FF-C, and OP-C to SOC were notably higher in the low-concentration phosphorus treatment compared with the high-concentration phosphorus treatment (Figure 7).

## 3.7 Correlation analysis between soil microorganism and nematode communities and soil physicochemical properties

The analysis employed soil microorganism and nematode community at the genus levels as response variables, with SW, pH, SOC, TN, AP, and TP serving as explanatory variables in the redundancy analysis (Figures 8A–C). Redundancy analysis of environmental factors and soil nematode communities showed that *Acrobeloides*, *Isolaimium*, *Filenchus*, *Hoplotylus*, and *Longidorus* were positively correlated with SOC, TP, AP, and TN; *Helicotylenchus*, *Ditylenchus*, and *Miculenchus* were positively correlated with SW and PH (Figure 8A). Redundancy analysis of environmental factors and soil bacteria communities showed that *Nocardioides*, *Streptomyces*, and *Arthrobacter* were positively correlated with SW, SOC, TN, AP, and TP; *Microvirga*, *Nitrospira*, *Solirubrobacter*, and *UTCFX1* were positively correlated with pH (Figure 8B). The effects of environmental

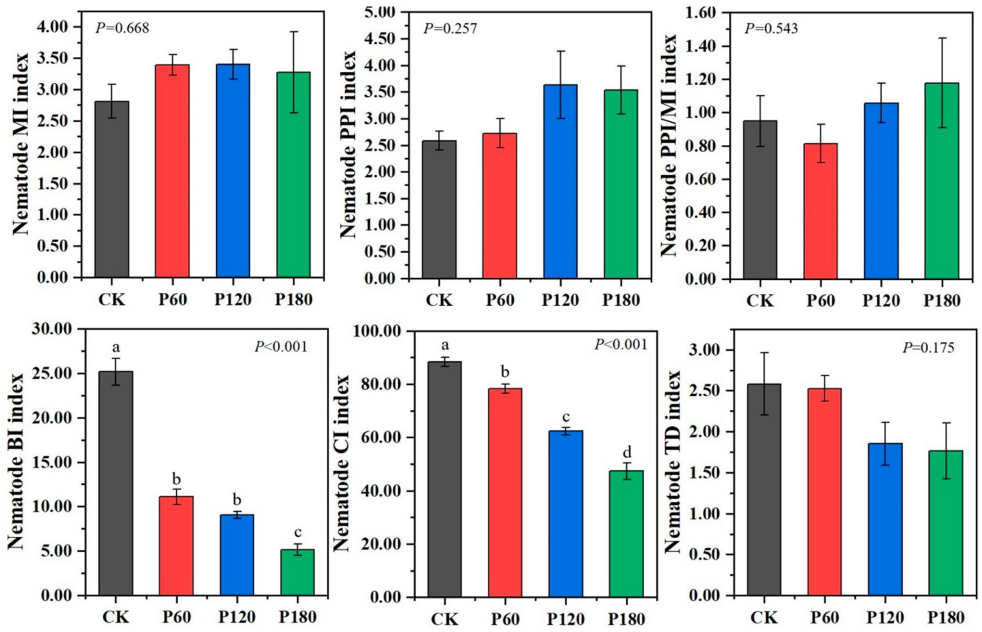


FIGURE 5
Functional structure index of soil microorganism and nematode communities under different phosphorus treatments. Different lowercase letters indicate significant differences with a value of $p < 0.05$ based on the ANOVA.

factors on soil fungal communities showed that *Titaea*, *Neonectria*, *Phaeomycocentrospora*, and *Fusariella* were positively correlated with SOC, TN, AP, and TP; *Paracylindrocarpon*, *Coniosporium*, *Beauveria*, and *Chaetomium* were positively correlated with SW and PH (Figure 8C). The path analysis further indicated (Figure 8D) that SOC was the predominant environmental factor influencing the soil bacterial community ($p < 0.05$). In contrast, the soil nematode community was primarily affected by SW ($p < 0.05$). No single environmental factor significantly impacts soil fungal communities in isolation. Instead, soil fungal communities are influenced by a multitude of environmental factors. Both soil TP and AP exert indirect effects on microorganisms.

# 4 Discussion

## 4.1 Responses of microbial and nematode communities to physicochemical factors

P addition increased soil phosphorus availability, thereby affecting plant productivity and altering soil carbon inputs (Feng and Zhu, 2019). Moreover, phosphorus addition can change soil physical and chemical properties, thereby impacting the growth, activity, and community structure of the soil micro-food web components, ultimately affecting soil ecological functions. In this study, SOC emerged as the pivotal factor, impacting the bacterial community. As the main carbon source, SOC provided the energy and C-based nutrients, which are essential for soil bacteria. Bacteria can obtain more energy and nutrients when SOC levels are higher, which encourages bacterial growth and increases bacterial abundance. Simultaneously, changes in SOC concentration alter the interactions between soil microorganisms, including symbiosis, competition, and predation, all of which can have an impact on bacterial communities. Previous studies have shown that soil bacterial richness and abundance were significantly related to SOC content (Xia et al., 2011; Yuan et al., 2012), which was consistent with our research results. In terrestrial ecosystems, micro-food webs within soil ecosystems are instrumental in the carbon cycle, particularly in mediating litter decomposition and soil carbon sequestration. The process of soil carbon sequestration is influenced by an array of factors, including soil microbial biomass, community structure, secondary metabolites, and soil physical and chemical characteristics (Frey et al., 2006). Furthermore, the origin of stable soil carbon is predominantly driven by microbial activities, underscoring the pivotal role of microorganisms in stable carbon synthesis. Notably, the contribution to soil carbon sequestration varies between bacteria and fungi. Studies indicated that communities dominated by fungi have a superior capacity for carbon sequestration compared with those dominated by bacteria (Frey et al., 2006; Grandy and Neff, 2008). Soil nematodes predominantly inhabited the water film surrounding soil particles. Their vital activities, including movement, predation, and reproduction, are intricately linked to soil moisture levels (Yeates, 1979). Enhanced soil moisture facilitates these activities, resulting in a rise in both the abundance and diversity of nematodes. Additionally, increased moisture content improves resource availability, which indirectly influences nematode community dynamics. This suggests that soil nematode communities are influenced not only by direct environmental factors but also by indirect bottom-up effects stemming from substrate availability (Hu Z. et al., 2017). Redundancy analysis revealed a close relationship between soil pH and the communities of soil bacteria, fungi, and nematodes. Numerous studies have identified soil pH as the primary determinant in shaping microbial

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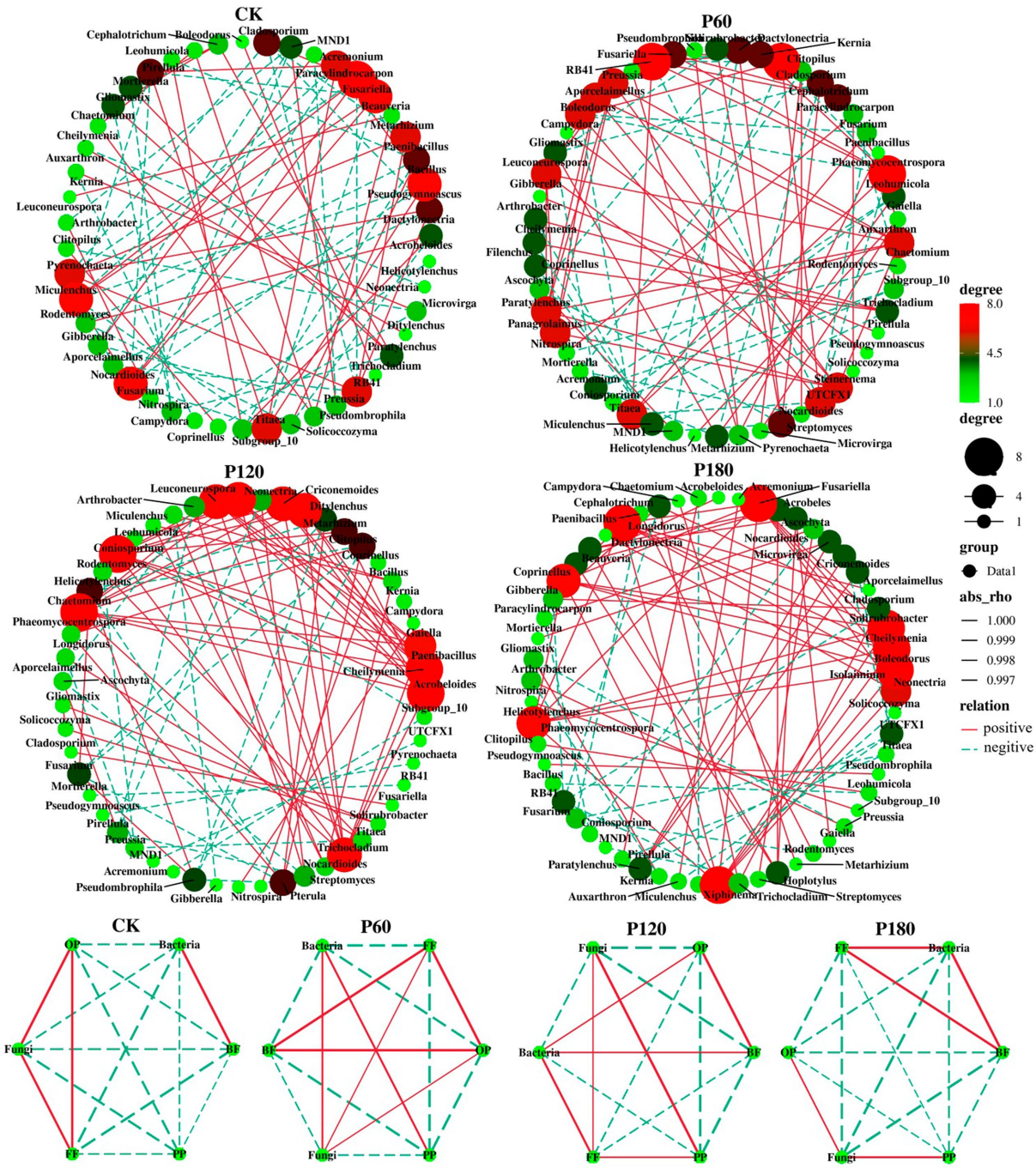


FIGURE 6
Network visualization of soil micro-food web interaction strength under different phosphorus treatments. The size of each node is proportional to the centrality score, with genera with larger centrality representing the key species of each network. Lines between each pair of nodes indicate a strong positive (red) or negative (dashed blue) interaction, and the thickness of the line indicates the strength of the correlation.

TABLE 2 Correlation network parameters under different phosphorus addition treatments.

| Index | CK | P60 | P120 | P180 |
|---|---|---|---|---|
| Number of nodes | 49 | 52 | 51 | 57 |
| Total links | 84 | 101 | 103 | 92 |
| Positive links | 41 | 65 | 77 | 70 |
| Negative links | 43 | 36 | 25 | 22 |
| Average degree | 3.53 | 3.85 | 4 | 3.19 |
| Average node size | 8.62 | 9.07 | 8.33 | 8.13 |

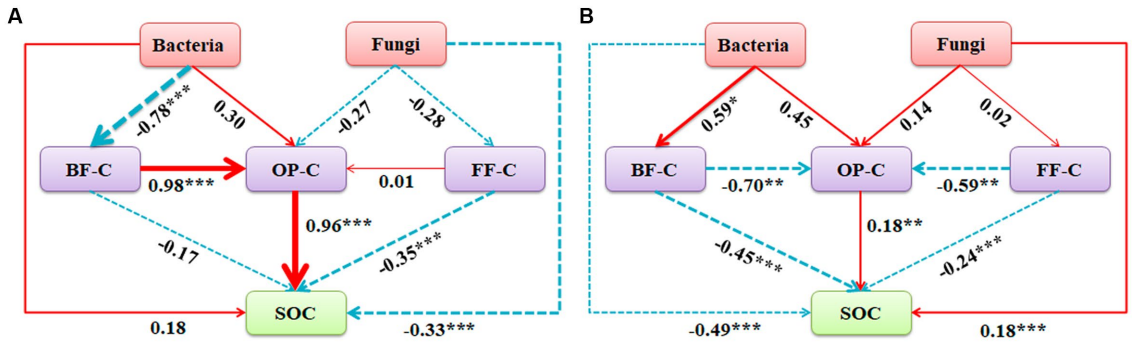


FIGURE 7
Path analysis model of decomposition pathway of soil micro-food webs with **(A)** low phosphorus concentration and **(B)** high phosphorus concentration. (**A**, $X^2 = 6.049$, df = 3, $p = 0.791$, CFI = 0.865, GFI = 0.895, RMSEA = 0.078, NFI = 0.904, NNFI = 0.892; **B**, $X^2 = 3.445$, df = 3, $p = 0.328$, CFI = 0.980, GFI = 0.906, RMSEA = 0.052, NFI = 0.924, NNFI = 0.903). The width of the arrow is proportional to the strength of the path coefficient, and the continuous red and broken blue arrows represent positive and negative relationships. Path analysis show direct and indirect effects of soil microorganism and nematode communities on carbon sequestration and bacterial and fungal community composition: first principal coordinate PCA1; BF-C, Carbon metabolic footprint of bacterivores; FF-C, Carbon metabolic footprint of fungivores; OP-C, Carbon metabolic footprint of omnivores-predators; SOC, Soil organic carbon.

communities (Fierer and Jackson, 2006; Lauber et al., 2009), which is consistent with the results of this study. Microorganisms exhibit varying pH preferences and tolerances. Long-term phosphorus addition has been shown to lower soil pH, subsequently altering microbial community composition and diversity. This shift in pH is beneficial to certain microbes in new environmental conditions while suppressing or diminishing others. Additionally, soil pH influences nutrient forms and availability, subsequently impacting microbial growth and activity. Alterations in microbial communities consequently affect the composition and diversity of microbivorous nematodes, as these nematodes depend on microorganisms for sustenance. Changes in microbial populations directly influence their food sources and habitats. Thus, soil pH exerts a bottom-up effect on the entire microbial food web, initiating microbial community alterations and extending to higher trophic levels.

## 4.2 Effects of phosphorus addition on structure and function of soil micro-food web

Rhizosphere nutrients constitute the fundamental energy source of the soil micro-food web. Plant roots and their secretions significantly influence the energy flow within the soil micro-food web, stimulating the proliferation of plant-parasitic, bacterivorous, and fungivorous nematodes. This proliferation, in turn, impacts the micro-food web through the predation of these nematode types by higher trophic-level nematodes (Yeates and Bongers, 1999). Our study indicated that high phosphorus concentrations were advantageous for fungivorous and plant-parasitic nematodes (Porazinska et al., 2009). This may be because high concentrations of phosphorus led to an increase in soil fungal populations, the primary food source for fungivorous nematodes, resulting in a higher proportion of fungivorous nematodes (Beauregard et al., 2010). Moreover, phosphorus addition increased soil nutrients, stimulated alfalfa root growth, and promoted the reproduction of herbivorous nematodes, thereby increasing the proportion of plant-parasitic nematodes (Beauregard et al., 2010; Xiaofang and Li, 2020). This finding confirmed the strong interplay between plant roots and plant-parasitic nematodes and supported the previous research to a certain extent. Microbivorous nematodes not only expedite micro-food web turnover by feeding on microorganisms but their metabolic processes also provide feedback effects on plant growth. The interaction between soil nematodes and microorganisms transcends a simple predator–prey dynamic. Different nutritional types of nematodes exert varied impacts on microbial communities. Research has shown that soil nematode activity significantly inhibited bacterial and fungal proliferation. Furthermore, the existence of microbivorous nematodes increased the complexity and variability of the soil ecosystem and micro-food web structure (Sauvadet et al., 2016). The results of this study showed that fungi in low-concentration phosphorus soils have a negative correlation with plant-parasitic nematodes and a positive correlation with fungivorous nematodes and omnivorous-predatory nematodes. In high-P soils, bacteria exhibit a negative correlation with plant-parasitic nematodes and a positive correlation with bacterivorous and omnivorous-predatory nematodes. Analysis based on the structural characteristics and energy flow direction of the soil micro-food web suggested that a short-term increase in plant-parasitic nematodes elevated the consumption of plant root nutrients. Because nematodes were larger and had longer survival times, their community dynamics lag behind those of microorganisms. Simultaneously, in environments with different phosphorus concentrations, bacteria and fungi are more susceptible to external soil stress, affecting community growth, thereby regulating soil nematode communities through a bottom-up approach.

Energy flow pathways in the soil food web, classified based on energy sources, comprise bacterial, fungal, and plant-based channels (Moore and William Hunt, 1988). The varied feeding habits of nematode trophic groups enable them to represent these energy flows at a higher trophic level (Deng et al., 2012). Analysis of these flows revealed that in P60 treatment soil, bacterial and fungal pathways predominate, while the plant energy flow channels had the smallest proportion. This dominance suggests a relatively high efficiency in

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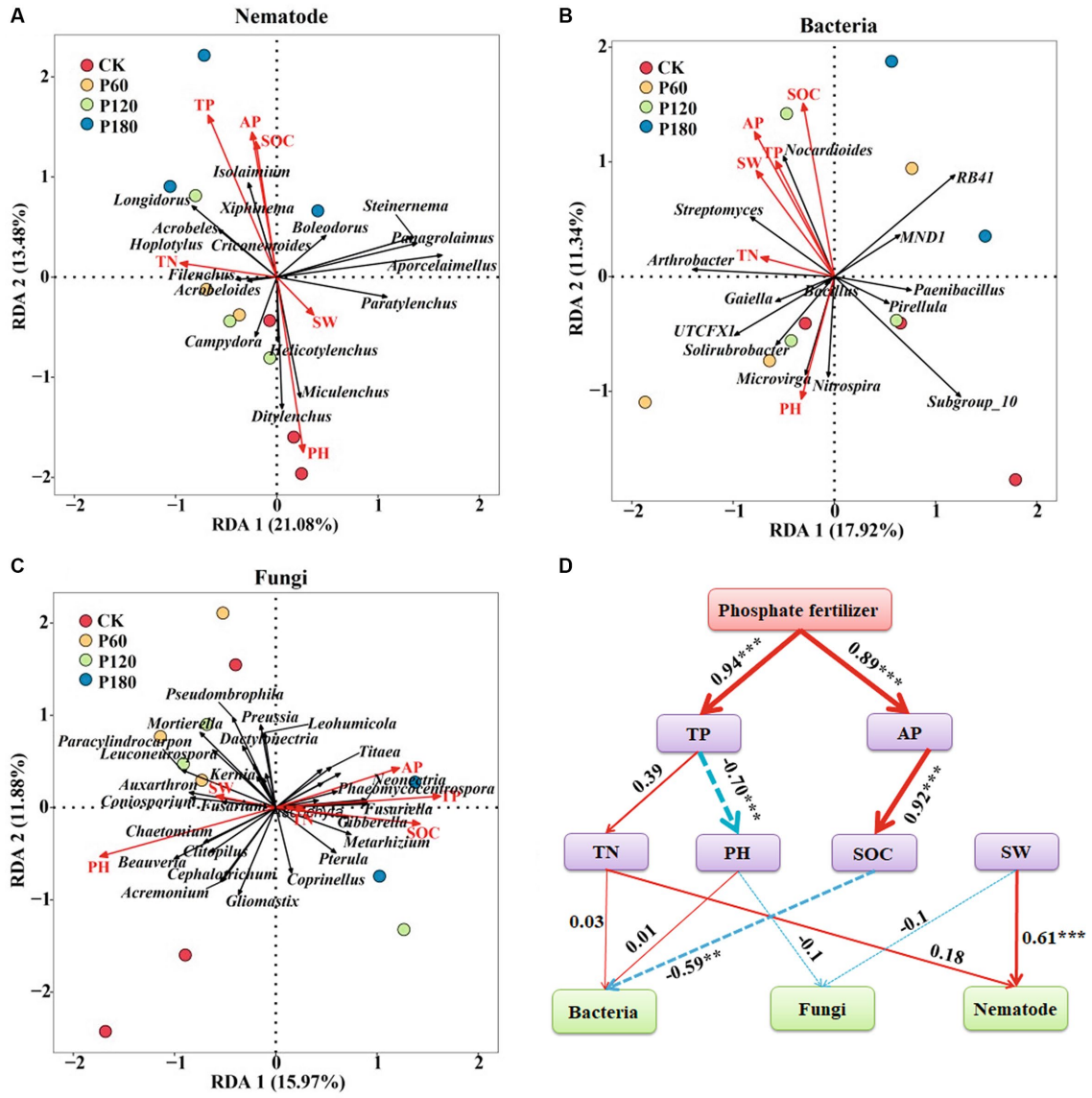


FIGURE 8
Correlation analysis between soil microorganism and nematode communities and soil physicochemical factors. **(A)** Redundancy analysis of soil nematode community and environmental factors. **(B)** Redundancy analysis of soil bacterial community and environmental factors. **(C)** Redundancy analysis of soil fungal community and environmental factors. **(D)** Path analysis model of soil microorganism and nematode communities and environmental factors ($X^2 = 72.43$, df = 32, $p = 0.791$, CFI = 0.956, GFI = 0.921, RMSEA = 0.052, NFI = 0.944, NFI = 0.923). The width of the arrows is proportional to the strength of the path coefficient. The continuous red and broken blue arrows represent positive and negative relationships. Path analysis model show direct and indirect effects of environmental factors on microorganisms and nematode communities. SW, Soil water content; SOC, Soil organic carbon; TN, Total nitrogen; TP, Total phosphor; and AP, Available phosphorus.

energy conversion and utilization within the P60 treatment soil food web. Specifically, the P60 treatment soil nematode community exhibited an elevated carbon utilization rate and an enhanced capacity to regulate the food web, maintaining a balanced predator–prey dynamic (Deng et al., 2012). Our research indicated that both the biomass of fungivorous nematodes and the total nematode biomass are greater in low-P soils compared with high-P soils. This disparity suggests that low-P soils, being less disturbed, offer more stable conditions for nematodes, particularly for large-sized k-strategy species treatment (Ferris, 2010). Fauna analysis demonstrated that the functional footprint of soil nematodes in the P60 treatment exceeded as compared with other treatments, implying that the P60 treatment enhanced soil nutrient levels and improved the soil environment. Consequently, this results in reduced soil disturbance and a more mature, stable food web structure. Furthermore, nematode functional metabolic footprints were larger at lower phosphorus concentrations, indicating a greater carbon flux through the decomposition pathway of the soil food web. This increase results in a greater allocation of carbon to higher trophic levels, where it was fixed by organisms at these levels (Ferris and Bongers, 2006).

The free-living nematode maturity index (MI) and the plant-parasitic nematode maturity index (PPI) were served as key indicators of high-intensity interference in a short period (Lenz and Eisenbeis, 2000). A higher MI value suggests a less disturbed environment and a

more stable nematode community, while the opposite was true for a more intense disturbance (Neher, 2010). The plant PPI represents the proportion of plant parasitic nematodes selected by r2 and k2, which can reflect the ability of such nematodes to resist habitat disturbance and their reproductive strength (Ferris et al., 2001). Compared with MI and PPI, the ratio of maturity index of plant parasitic nematodes to free-living nematodes (PPI/MI) played an important role in responding to the soil ecological environment and coping with external environmental interference. It was more representative in terms of the subsequent recovery status. The higher PPI/MI value indicated a greater degree of disturbance in the habitat where the nematode community resided (Bongers, 1990). In this study, applying low-concentration phosphorus fertilizer improved the MI and reduced PPI and PPI/MI. These results suggested that low phosphorus levels enhanced soil environmental stability. Such an outcome arises because prolonged phosphorus addition compels local organisms to develop survival mechanisms that counter the effects of low-concentration phosphorus fertilizers. Consequently, the P60 treatment displayed the highest MI value and the lowest PPI and PPI/MI values, indicating minimal external disturbances and a more stable ecological environment. In contrast, the MI values diminished in the P120 and P180 treatments while the PPI and PPI/MI values increased. This trend may be attributed to the enhanced soil nutrients from phosphorus addition, promoting robust growth in above-ground plants and fostering healthy root systems. Such conditions deter plant-parasitic nematodes, demonstrating that high phosphorus concentrations reduce plant-parasitic nematode viability and hindering their survival and reproduction. Overall, this research supported the use of phosphorus fertilizers in restoring degraded grasslands.

## 4.3 The effect of phosphorus addition on the decomposition pathway of soil micro-food web

The ecological network indicated a positive correlation among fungi, FF, and OP at low phosphorus levels but no positive correlation between bacteria and BF. This implies that, under low phosphorus addition, the decomposition pathway of fungi exhibited greater strength compared with high phosphorus levels. Bacteria and fungi employ distinct metabolic abilities for the breakdown of substrates with varying qualities. The alteration in decomposition pathways within the soil food web played a crucial role in influencing the rate of soil carbon loss. Notably, the bacterial decomposition pathway, conducive to rapid nutrient turnover, surpassed the fungal decomposition pathway in this aspect (de Vries et al., 2011). The quality of readily available refractory substrates affects the transition of bacterial and fungal decomposition pathways in soil food webs, thereby largely influencing carbon turnover (Fabian et al., 2017). In this study, elevated phosphorus addition levels resulted in the predominance of bacterial decomposition pathways within the soil food web. However, as phosphorus concentration diminishes, the substrate exhibits increased resistance to degradation, thus favoring fungal decomposition. Therefore, increasing phosphorus may drive the successional trend of the community from fungi to bacteria. In addition, it was also observed that organic carbon increased at low phosphorus supply and relatively strong fungal decomposition pathways, suggesting that fungi had larger protective carbon pools and larger carbon retention ratios than bacteria. This effect improved the physical environment for carbon stabilization and promoted the accumulation of microbial-derived organic matter (Frey et al., 2006; Danger et al., 2016). Variations in both bacterivores and fungivore nematodes were driven by phosphorus supply and soil microbial communities (Ferris and Matute, 2003). Under different phosphorus levels, this study found that fungi, fungivores, and omnivores-predators were positively correlated at low phosphorus supply. However, bacteria, bacterivores, and omnivores-predators were positively correlated at high phosphorus supply, positively supporting the view that low-P and high-P supply enhance fungal and bacterial decomposition pathways, respectively (Rooney et al., 2006). Moreover, the decomposition pathways of bacteria and fungi in the soil food web need to be separated, as the two pathways work simultaneously, and different carbon transfer and utilization processes occur between them (Prescott and Vesterdal, 2021). Different phosphorus supplies regulated the community composition and decomposition pathways of microorganisms and nematodes differently.

Basis index (BI), channel index (CI), enrichment index (EI), and structure index (SI) can provide a large amount of information for food web dynamic processes in environments of stress, enrichment, stable structure, and rapid decomposition (Ferris and Tuomisto, 2015). Compared with other treatments, the enrichment index (EI) under the P60 treatment decreased, indicating that the number of Ba1 and Fu2 nematodes, such as *Acrobeles*, *Acrobloides*, and *Miculenchus*, was significantly reduced. A lower enrichment index (EI) value indicated a decrease in food resources at this level of phosphorus addition (Bongers et al., 1997), leading to fewer nutrient inputs from external sources into the soil. The elevated structural index (SI) under P60 treatment was attributed to the elevated number of cp3-5 nematode groups, especially the omnivore-predator cp3-5 nematodes, which may signify a higher degree of connectivity in the soil food web, with longer food chains, greater soil resilience (Ferris et al., 2001), and a more stable soil food web. The CI values were greater than 60 under the CK, P60, and P120 treatments and less than 60 under the P180 treatment. This distinction confirmed that soil food webs with lower phosphorus concentrations predominantly decompose via fungal channels, while those with higher phosphorus concentrations used bacterial channels. The decomposition of organic matter in the soil food web was carried out through different channels. Fungi break down substances that are rich in cellulose, lignin, and high carbon-to-nitrogen ratios, whereas bacteria process nitrogen-rich substances (Yeates et al., 1993; Wardle et al., 2003). Excessively higher nitrogen content also raised carbon content. However, a high soil organic matter C/N ratio can be crucial for long-term sustainable plant production in perennial or natural systems. In this state, the degradation pathway in the soil micro-food web is dominated by fungi (Ferris et al., 2001). As the phosphorus fertilizer concentration increases, the BI index gradually decreases. This decline suggested that greater phosphorus addition might amplify environmental disturbances, thus undermining the resilience of the soil food web. This may be related to the potential toxic impact of phosphorus addition on soil nematodes (Ferris and Bongers, 2006). Indeed, phosphorus supply serves many purposes and benefits. In addition to carbon fixation, phosphorus is a component of many important organic compounds that constitute crops. Phosphorus is present in nucleic acids, nucleoproteins, phospholipids, phytochemicals, adenosine phosphate, and many enzymes in crops. It plays an

important role in energy conversion and metabolism of crops, affecting the synthesis and operation of carbohydrates and nitrogen and fat metabolism. It also enhanced the resistance of plants against stresses, such as drought, cold, and salinity.

## 5 Conclusion

This study has enhanced our comprehension of the mechanisms, governing alterations in soil micro-food web decomposition pathways under varying phosphorus supply conditions. Within the decomposition pathway of the soil micro-food web, phosphorus supply facilitated a noticeable shift between bacteria and fungi, thereby promoting the transition between bacterivores and fungivores. Notably, our experimental findings elucidate that low phosphorus supply reinforces the fungal decomposition pathway, whereas high phosphorus supply strengthens the bacterial decomposition pathway. The intricate nutrient interactions between soil microorganisms and nematode communities emerge as primary drivers of carbon flow in the decomposition pathways. Consequently, low phosphorus supply exerts a more pronounced regulatory effect on the decomposition pathway of the soil food web compared with high phosphorus supply, impacting the metabolic processes of carbon within the soil food web.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

## Author contributions

LiaL: Data curation, Investigation, Methodology, Software, Writing – original draft. ZL: Conceptualization, Funding acquisition, Methodology, Writing – review & editing. LinL: Conceptualization, Data curation, Methodology, Writing – review & editing. YN: Conceptualization, Investigation, Methodology, Writing – review & editing. YZ: Investigation, Methodology, Software, Data curation, Writing – review & editing. RH: Investigation, Project administration, Software, Supervision, Writing – review & editing. JL: Investigation, Methodology, Software, Writing – review & editing. LN: Data curation, Formal analysis, Investigation, Software, Supervision, Writing – original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Cultural techniques capture diverse phosphate-solubilizing bacteria in rock phosphate-enriched habitats

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Phosphorus (P) deficiency is a common problem in croplands where phosphate-based fertilizers are regularly used to maintain bioavailable P for plants. However, due to their limited mobility in the soil, there has been an increased interest in microorganisms that can convert insoluble P into a bioavailable form, and their use to develop phosphate-solubilizing bioinoculants as an alternative to the conventional use of P fertilizers. In this study, we proposed two independent experiments and explored two entirely different habitats to trap phosphate-solubilizing bacteria (PSBs). In the first experiment, PSBs were isolated from the rhizoplane of native plant species grown in a rock-phosphate (RP) mining area. A subset of 24 bacterial isolates from 210 rhizoplane morphotypes was selected for the inorganic phosphate solubilizing activities using tricalcium phosphate (TCP) as the sole P source. In the second experiment, we proposed an innovative experimental setup to select mycohyphospheric bacteria associated to arbuscular mycorrhizal fungal hyphae, indigenous of soils where agronomic plant have been grown and trapped in membrane bag filled with RP. A subset of 25 bacterial isolates from 44 mycohyphospheric morphotypes was tested for P solubilizing activities. These two bacterial subsets were then screened for additional plant growth-promoting (PGP) traits, and 16S rDNA sequencing was performed for their identification. Overall, the two isolation experiments resulted in diverse phylogenetic affiliations of the PSB collection, showing only 4 genera (24%) and 5 species (17%) shared between the two communities, thus underlining the value of the two protocols, including the innovative mycohyphospheric isolate selection method, for selecting a greater biodiversity of cultivable PSB. All the rhizoplane and mycohyphospheric PSB were positive for ammonia production. Indol-3-acetic acid (IAA) production was observed for 13 and 20 isolates, respectively among rhizoplane and mycohyphospheric PSB, ranging, respectively, from 32.52 to 330.27 $\mu g\ mL^{-1}$ and from 41.4 to 963.9 $\mu g\ mL^{-1}$. Only five rhizoplane and 12 mycohyphospheric isolates were positively screened for $N_2$ fixation. Four rhizoplane PSB were identified as siderophore producers, while none of the mycohyphospheric isolates were. The phenotype of one PSB rhizoplane isolate, assigned to *Pseudomonas*, showed four additive PGP activities. Some bacterial strains belonging to the dominant genera *Bacillus* and *Pseudomonas* could be considered potential candidates for further formulation of biofertilizer in order to develop bioinoculant consortia that promote plant P nutrition and growth in RP-enriched soils.

KEYWORDS

rock phosphate, phosphate-solubilizing bacteria, bioinoculants, rhizoplane, hyphosphere, mining area

## Introduction

Agricultural production in conventional cultural systems relies on continual inputs of chemical P-fertilizers. However, their industrial production and processing is costly and can lead to environmental concerns if applied in excess (Hudson-Edwards, 2016). Therefore, to reduce the environmental footprint of agricultural practices, sustainable alternatives that reduce over-reliance on chemical fertilizer applications while maintaining crop production, have been tested (Shen et al., 2011). Studies have examined the various functions of microbial sources found in the root environment to produce microbial-based agricultural inputs which are effective in improving plant growth with reduced mineral intake (Compant et al., 2019; Elhaissoufi et al., 2022). Therefore, Plant Growth-Promoting Rhizobacteria (PGPR), bacteria that promote plant growth, have become the focus of attention of researchers (Richardson et al., 2009; Vacheron et al., 2013). Particularly, phosphate-solubilizing bacteria (PSB) have been proposed as a feasible solution to ameliorate the bioavailable-phosphate deficiency in cropping systems. These bacteria convert insoluble and organic P compounds into soluble and bioavailable forms ($H_2PO_4^-$ and $HPO_4^{2-}$) with the aid of organic acids and enzymes such as phosphatases and phytases (Goldstein, 1995; Ahmed et al., 2021). This, in turn, allows them to benefit from P while also enabling other organisms, such as plants, to access it as a nutrient source (Raymond et al., 2021). As such, these bacteria have been highlighted as key players in the biogeochemical P cycle (Tian et al., 2021; Ducousso-Detrez et al., 2022a,b). Thus, the development of phosphate-solubilizing bio-inoculants has been suggested as an alternative to the regular use of P fertilizers. Moreover, some particular PGPR/PSB strains have been shown to possess several growth-promoting properties, such as the production of hormones, antibiotics, and enzymes to enhance plant growth and protection against pathogen attacks (Kour et al., 2021; Alotaibi et al., 2022). An example of this was demonstrated by *Aneurinibacillus aneurinilyticus* CKMV1, which was shown to possess several plant growth-promoting activities, including N-fixation, indole-3-acetic acid production, siderophore synthesis, hydrogen cyanide production, and antifungal activity (Chauhan et al., 2017). Siderophore production is a crucial factor for promoting plant growth. Indeed, siderophores are high-affinity iron-chelating compounds that can acquire ferric $Fe^{3+}$ from mineral phases, scavenging it to make it available as the preferred form of $Fe^{2+}$ for uptake by plant roots (Vessey, 2003).

Furthermore, the use of microbial and mineral P resources together has been gaining more attention as a potential solution. When used in combination, they may act synergistically to increase the agronomic efficiency of mineral fertilizers and supply the essential nutrients and functions needed for plant growth and development. Notably, the combined use of PSB-based inoculants and rock phosphate (RP) has led to a successful "microbial-P mineral alliance" (Bargaz et al., 2018; Tahir et al., 2018). Indeed, RP is a geological P-rich rock used alone in some agricultural systems to efficiently improve crop production with lower costs and environmental damage than water-soluble P chemical fertilizers (Sharma et al., 2013; Ahemad and Kibret, 2014). However, due to its low solubility in water (largely linked to its chemical, crystallographic, and mineralogical composition of its apatites), RP reactivity (i.e., the rate of orthophosphate ion release when applied directly to soils under favorable soil conditions) and its agronomic effectiveness are generally lower than those of commercial fertilizers (Verma et al., 2012). Nevertheless, a number of studies have evidenced improved P availability in soils when RP is combined with PSB (Bargaz et al., 2018; Elhaissoufi et al., 2022), with significant increases in P uptake, shoot/root biomass, and yield performances compared to inoculation treatments used singly (Kaur and Reddy, 2015; Manzoor et al., 2016; Adnan et al., 2017; Ditta et al., 2018; Elhaissoufi et al., 2020).

The identification of PSB has thus become a relevant research field for the development of a more sustainable farming system that benefits the environment and society, and increases farmers' income (Verma et al., 2015; Lobo et al., 2019). Here, the literature clearly shows that numerous commercial PSB strains and bioinoculants including PSB strains are already available on the global market (Owen et al., 2015; Koskey et al., 2021). However, the success of microbial inoculation is also open to debate (Basiru and Hijri, 2022). Indeed, as its effectiveness is highly dependent on environmental conditions (i.e., strongly constrained by soil properties, and indigenous microbial populations in particular). To face such a challenge, a larger diversity in the set of cultivable PSB isolates could provide a response to the soil diversity in which inoculants will be used. It could also open new ways for better mechanistic understanding of the PSB phenotype.

Based on this hypothesis, new PSB identification therefore required new trapping strategies, based for instance on new media for *in vitro* selection and culture of isolates. It is also crucial in promoting a diversification of sampling environments and habitats, each inhabited by its own microbiome, leading potentially to isolates not yet identified.

Screenings for PSB are typically performed from rhizospheric soils or roots, where it is widely accepted that PSB are abundant (Ashok et al., 2012; Baliah et al., 2016; Alotaibi et al., 2022). PSB have also been screened from the soil environment along the extraradical hyphae of arbuscular mycorrhizal fungi (AMF): the mycohyphosphere (Johansson et al., 2004; Toljander et al., 2006; Lecomte et al., 2011). In this microhabitat, it is known that some bacteria can adhere to the surface of AMF hyphae (Ordonez et al., 2016; Taktek et al., 2017). Nonetheless, the identity and potential roles of microbes associated with AMF hyphae, along with the mechanisms enabling the recruitment and cooperation of beneficial microbes, remain inadequately understood. However, there is evidence indicating that bacteria associated with AMF can exhibit diverse functional traits, such as nitrogen fixation, phosphate mobilization, growth hormone production, biofilm production, and biocontrol against pathogens (Barea et al., 2002; Frey-Klett et al., 2007; Ujvári et al., 2021). These traits may play a significant role in

maintaining the health of AMF symbiosis and fostering plant growth [reviewed in Basiru et al. (2023) and Wang L. et al. (2023)]. Thus, Cruz and Ishii (2011) successfully isolated probable endobacteria such as *Bacillus* and *Paenibacillus* isolates from *Gigaspora margarita* spores that exhibited multiple PGP traits, including P solubilization. Similarly, Battini et al. (2017) showed positive effects of AMF and their associative endobacteria isolated from AMF spores, regarding facilitation of P uptake under P-limiting conditions. Benefits also occur for AMF and hyphae-associated PSB communities, as interactions provide key resources to each other (Zhang et al., 2016). In particular, AMF hyphae exudates (sugars, amino acids, carboxylates) may provide key nutrients for bacterial growth, while attachment of bacteria with P solubilizing capacity to the extraradical AMF hyphae can allow the AMF to get additional soluble orthophosphate ions (Jansa et al., 2013; Zhang et al., 2018; Jiang et al., 2021). Additionally, PSB can access nutrients with limited diffusion in soils, inside the mycorrhizosphere (Ordonez et al., 2016). As a result, there is a growing interest in exploring the diversity and functions of bacteria associated with AMF hyphae (Toljander et al., 2007; Faghihinia et al., 2022; Basiru et al., 2023). Speculations regarding potential positive interactions between PSB and AMF in nutrient acquisition have increased, making the isolation of mycohyphospheric PSB a promising avenue for developing formulations of biofertilizer inoculants (Jansa et al., 2013; Zhang et al., 2016).

Consequently, in this study, we proposed two independent experiments and explore two entirely different habitats to trap PSB isolates in RP-rich soils. In the first experiment the PSBs were isolated from the rhizoplane of native plant species growing in a rock-phosphate mining area. In the second experiment, we proposed an innovative experimental setup to trap mycohyphospheric bacteria associated to arbuscular mycorrhizal fungal hyphae, indigenous of soils where agronomic plant have been grown. The selected PSB were further screened for additional conventional plant growth-promoting traits, and the isolates were identified by 16S rRNA sequencing and phylogenetic analysis. The taxonomic and functional diversity of the selected isolates were compared and discussed as a potential component in further inoculant formulations for increasing crop production, especially in RP-enriched soils.

# Materials and methods

## Experimental setup for isolation of rhizoplane bacteria

### Sampling sites

In order to isolate PSB from the rhizoplane, samplings were performed in a former RP mining area located in the National Nature Reserve of Geological Interest of the Lot department in France (44° 22′ 22″ N, 1° 41′ 16″ E). Three locations (L1: 44°21′03,70″N; 1°41′26,66″E – L2: 44°21′44,65”N; 1°41′16,02″E – and L3: 44°29′15,85”N; 1°48′13,19″E) were identified, each providing two sites characterized by contrasting P concentrations due to mining activities in the past (high P and low P). Indeed, during mining operations, the most RP-enriched soil fractions were exported and used to manufacture P fertilizers, while the finer mine tailings were left in place. This resulted in surface soils that were locally enriched in phosphate (hereinafter referred to as P soils) side by side with soils not enriched in phosphate (nP soils).

In each site, the soil physicochemical properties, including pH, soil texture, and chemical composition, were assessed by the CIRAD-US Analyse laboratory in Montpellier, France, utilizing inductively coupled plasma spectrometry, atomic emission spectrometry, and X-ray fluorometry (Ducousso-Detrez et al., 2022b). The six soils exhibited near-neutral pH levels, ranging between 6.9 and 7.3. Regarding granulometry, all topsoil (0–30 cm) exhibited elevated levels of clay, with additional higher percentages of coarse sands observed in P soils.

In nP soils, total P concentrations ranged from 1,057.1 to 1,496.3 mg/kg, while in P soils, they varied from 2,880.0 to 13,927.9 mg/kg. Similarly, available Olsen P concentrations in P soils ranged from 46.1 mg/kg to 339.5 mg/kg, while nP soils were characterized by values ranging from 5.04 to 12.82 mg/kg. Through the comparison of matched soils from the same location, P and Olsen P ratios, ranging from 2.07 to 13.05 and from 9.14 to 41.96 Pi, respectively, were derived. These ratios allowed for the classification of P sites as high-P sites and nP sites as low-P sites.

### Plant sampling

In January 2019, samples of native plant species (*Bromus sterilis, Dactylis glomerata, Taraxacum officinalis,* and *Ranunculus bulbosus*) were collected from various mining sites. For each of the six sites surveyed, three individual plants per plant species were collected, resulting in the harvest of a total of 72 plants. These plants were carefully placed in sterile plastic Whirl-Pack bags and transported to the laboratory with the aid of an ice pack. Subsequently, the roots were separated from the aerial parts, and the soil adhering to roots was eliminated by vigorously shaking the roots. Fresh root segments were washed by shaking in 90 mL of sterile saline solution (NaCl, 0.85% W/V) before to be plated on Tryptic Soy Agar (TSA) agar medium Petri dishes. Colonies were picked up after one week of culture and spread over TSA nutrient plates. Serial subcultures were then performed on the same medium and, if required, isolate purification was conducted (Figure 1A).

## Experimental setup for isolation of mycohyphospheric bacteria

Mycohyphospheric bacterial isolates were selected through an experimental process consisting of three steps. The first was conducted with the aim of trapping mycorrhizal structures (spores and hyphae) of native AMF. It was conducted at the Montreal Botanical Garden (Montreal, QC, Canada) in June 2018, in a plot with a Black Chernozem soil used exclusively for organic farming practices. Small bags (5 cm width x 10 cm length) made of a nylon membrane (pore size of 200 µm) containing igneous RP (with 54% of $P_2O_5$, Quebec, Canada) or sedimentary RP (with 39% of $P_2O_5$, Morocco) were placed near the roots of potato, tomato, leek, and maize plants. After three months, the RP bags were removed from the soil. A supplementary step was conducted to extend the time required for spore development and increase the number of spores inside the RP bags. It was carried out in a greenhouse (22°C with a photoperiod of 8-16 h) with RP bags placed near the roots of plants grown in pots. The soil substrate was watered to field capacity with tap water as needed and fertilized weekly with a Hoagland solution without P. After six months, bags were

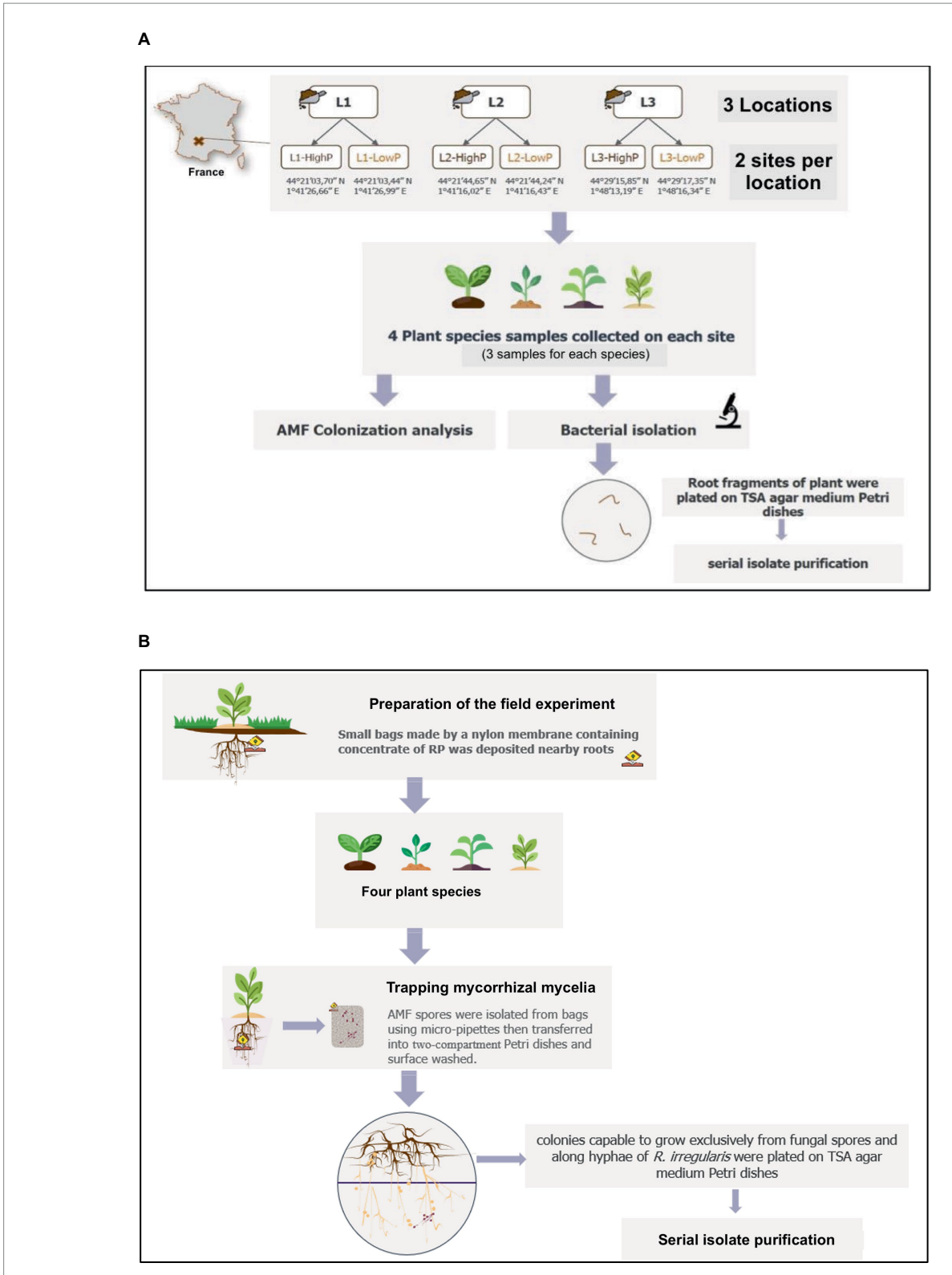


FIGURE 1
Experimental setup for root **(A)** and mycohyphosphere **(B)** bacteria isolation.

collected from each pot. The contents of each bag were placed on a Petri dish, covered with sterile distilled water. Subsequently, utilizing gentle and repetitive circular motions, hyphae with attached spores were gathered around a glass Pasteur pipette and transferred into another Petri dish. The spores/hyphae mixture underwent successive washes with sterile water containing EDTA (0.5%). Finaly, under a stereomicroscope, spores were individually collected using a 20 µm micropipette and transferred into a microtube containing sterile distilled water. In these conditions, 30–40 spores per 10 g of soil were successfully obtained.

The third step of the experimental setup aimed to isolate bacterial strains from AMF spores previously trapped and harvested from the RP bags. It was performed *in vitro*, using a two-compartment Petri dish design, as previously described by St-Arnaud et al. (1995) then modified by Taktek et al. (2017). This design was used to isolate mycohyphospheric bacteria able to grow on the surface of AMF hyphae, utilizing the extraradical mycelium exudates as a sole source of energy. Thus, the AMF *Rhizophagus irregularis* DAOM 197198 (originally isolated from Pont-Rouge, QC, Canada) was grown on *Agrobacterium rhizogenes*-transformed carrot (*Daucus carota* L.) roots (IRBV, QC, Canada), in the first Petri dish compartment filled with 20 mL of M medium (Becard and Fortin, 1988). The second compartment received 20 mL of M medium without any carbon source or vitamins and was kept root-free by trimming the roots to exclusively facilitate the growth of AMF extraradical hyphae. Both media were solidified with 0.4% (W/V) of Phytagel (Sigma-Aldrich, Oakville, ON, Canada). The plates were incubated for approximately 5 weeks at 25°C in the dark until the hyphae colonized the second compartment. The spores, which had been previously confined within RP bags and manually retrieved through microscopic observations, were subsequently placed onto the *R. irregularis* hyphae growing in the distal compartment. The Petri dishes were then incubated for 5 days at 25°C and observed daily to check the viability of hyphae. The bacterial colonies capable of growing along the *R. irregularis* hyphae, without any visible damage, were isolated and reinoculated repeatedly until single morphotypes were obtained on 10% TSA (QueLab, QC, Canada). Hereafter, they were referred to as mycohyphospheric bacteria (Figure 1B).

## *In vitro* screening for inorganic phosphate solubilizing bacteria

After serial purification, the rhizoplane and mycohyphospheric bacterial isolates were spread on modified National Botanical Research Institute's Phosphate (NBRIP) agar medium, containing tricalcium phosphate (TCP) (Nautiyal, 1999). NBRIP plates were incubated at 28°C for 14 days and colonies forming a clear solubilization halo, which results from their TCP solubilizing activity, were taken as PSB.

The phosphomolybdenum blue method was performed to quantitatively assess the P-solubilization capabilities of isolates in a solution. In this method, P ions react with acid ammonium molybdate to generate phosphomolybdenum complexes. These complexes are then reduced by ascorbic acid, leading to the formation of a highly pigmented phosphomolybdenum blue species. The degree of blue coloration, measured spectrophotometrically at 820 nm, correlates proportionally with the phosphate concentration (Howe and Mellon, 1940; Aliyat et al., 2020). Thus, NBRIP medium in a 250 mL flask was inoculated with an aliquot of an overnight bacterial culture (OD 600 nm = 0.7) and incubated under shaking conditions for 7 days at 28°C. After centrifugation (for 10 min, 12,000 g), 0.5 mL of supernatant was initially added to trichloroacetic acid (10% W/V), followed by a mixture containing ammonium molybdate and ascorbic acid in a sulfuric acid solution. The flask was then placed in the dark for the color reaction. The quantitative estimation of P solubilization in the bacterial culture supernatant was determined against a $KH_2PO_4$ standard curve.

## Culture preservation and maintenance

The selected PSB isolates were stored in 25% glycerol at −80°C. After preservation, all subcultures were performed in 50 mL of 10% TSA medium (Difco Laboratories Inc. Detroit, MI, USA) at room temperature 25°C, with continuous agitation at 150 rpm on a gyratory shaker, for 48 h.

## Identification of PSB and phylogenetic analysis

### DNA extraction

Bacterial cells were grown overnight at 28°C, in 100 mL Erlenmeyer flaks containing TSA medium (150 rpm). Cells were then harvested by centrifugation (10,000 *g*, 10 min at 4°C) and washed twice by centrifugation in saline solution. Washed cells were frozen in liquid nitrogen, lyophilized for 24 h, and resuspended in 1 mL of CTAB (2% hexadecyltrimethylammonium bromide, 1.4-M NaCl, 0.2% 2-mercaptoethanol, 20-mM EDTA, 100 mM Tris–HCl pH 8.0 (Sigma-Aldrich, Oakville, ON, Canada). Total genomic DNA was extracted using the FastDNA® SPIN Kit and FastPrep® Instruments (MP Biomedicals, Montreal, QC, Canada). Following extraction, the DNA was re-suspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.4), quantified using the spectrophotometer NanoDrop, and diluted in TE to give a concentration of 50 ng $\mu L^{-1}$.

### PCR amplifications

The gene encoding the 16S rRNA was amplified by the polymerase chain reaction (PCR) using the combination of universal primers pA (AGAGTTTGATCCTGGCTCAG) and pH (AAGGAGGTG ATCCAGCCGCA) (Edwards et al., 1989). The PCR mixture consisted of deoxynucleotides at 200 µM each, 0.25 µM of each primer, 2.5 µM $MgCl_2$, with 5 µL PCR buffer and 0.25 U of *Taq* DNA polymerase (5 Prime GmbH, Hamburg, Germany) (from (Taktek et al., 2015), with minor modifications). The following PCR conditions were used: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 7 min.

### Purification and sequencing of PCR products

The sizes of the PCR products were determined in 1% (W/V) Agarose TBE (Tris-Borate-EDTA, pH 8.3). The PCR products harboring an expected size of around 1,500 bp were used for further process.

The PCR purification and sequencing was performed by the Centre d'Expertise et de Service, Génome Québec (Montreal, QC, Canada). Sequences were identified using BLAST Nucleotide searches at NCBI website. All sequences were deposited in GenBank and their accession numbers are shown in Tables 1, 2.

## Further *in vitro* screening of PSB for additional plant growth promoting traits

All bacterial isolates that were identified as positive for phosphate solubilizing activity through at least, one subculture on NBRIP were referred to as PSB, as they possess the metabolic capability to solubilize phosphate. To further investigate them for different conventional plant

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TABLE 1 Taxonomic identification of rhizoplane isolates.

| Root PSB isolates | Sample site | Seq. length (bp) | Identity (%) | Coverage (%) | Family | Species | Accession number |
|---|---|---|---|---|---|---|---|
| 7* | L1-HighP | 1,111 | 94 | 100 | Pseudomonadaceae | *Pseudomonas mohnii* | OQ876719 |
| 8* | L1-HighP | 991 | 91 | 100 | Sphingomonadaceae | *Novosphingobium resinovorum* | OQ876703 |
| 19 | L1-HighP | 1,135 | 99 | 100 | Pseudomonadaceae | *Pseudomonas* sp. | OQ876718 |
| 23 | L1-HighP | 989 | 99 | 100 | Moraxellaceae | *Acinetobacter rhizosphaerae* | OQ876702 |
| 69 | L1-LowP | 714 | 99 | 100 | Alcaligenaceae | *Achromobacter xylosoxidans* | OQ876701 |
| 82 | L2-HighP | 1,073 | 98 | 100 | Pseudomonadaceae | *Pseudomonas azotoformans* | OQ876700 |
| 90 | L2-HighP | 942 | 99 | 100 | Paenibacillaceae | *Paenibacillus xylanexedens* | OQ876716 |
| 92 | L2-HighP | 979 | 99 | 99 | Pseudomonadaceae | *Pseudomonas* sp. | OQ876699 |
| 93 | L2-HighP | 988 | 98 | 99 | Pseudomonadaceae | *Pseudomonas* sp. | OQ876714 |
| 96 | L2-HighP | 723 | 98 | 100 | Paenibacillaceae | *Brevibacillus* sp. | OQ876713 |
| 99 | L2-HighP | 1,156 | 99 | 96 | Paenibacillaceae | *Paenibacillus purispatii* | OQ876723 |
| 132 | L2-LowP | 1,097 | 99 | 100 | Pseudomonadaceae | *Pseudomonas* sp. | OQ876722 |
| 133* | L2-LowP | 857 | 89 | 99 | Paenibacillaceae | *Paenibacillus amylolyticus* | OQ876721 |
| 136* | L2-LowP | 1,193 | 95 | 99 | Xanthomonadaceae | *Stenotrophomonas maltophilia* | OQ876736 |
| 146 | L3-HighP | 1,146 | 99 | 100 | Bacillaceae | *Bacillus thuringiensis* | OQ876735 |
| 153* | L3-HighP | 790 | 96 | 100 | Pseudomonadaceae | *Pseudomonas* sp. | OQ876729 |
| 149 | L3-HighP | 1,060 | 99 | 100 | Bacillaceae | *Bacillus nitratireducens* | OQ876734 |
| 164 | L3-HighP | 1,068 | 99 | 100 | Pseudomonadaceae | *Pseudomonas fluorescens* | OQ876727 |
| 174 | L3-HighP | 983 | 99 | 100 | Pseudomonadaceae | *Pseudomonas fluorescens* | OQ876720 |
| 185 | L3-LowP | 1,092 | 98 | 99 | Pseudomonadaceae | *Pseudomonas* sp. | OQ876712 |
| 187* | L3-LowP | 1,053 | 97 | 98 | Paenibacillaceae | *Paenibacillus polymyxa* | OQ876711 |
| 196* | L3-LowP | 826 | 96 | 100 | Brevibacteriaceae | *Brevibacterium* sp. | OQ876710 |
| 202* | L3-LowP | 577 | 96 | 100 | Paenibacillaceae | *Brevibacillus* sp. | OQ876709 |
| 203 | L3-LowP | 1,025 | 99 | 100 | Bacillaceae | *Bacillus* sp. | OQ876708 |

*Indicates isolates with a percentage of identity below 98%, which is the generally accepted threshold for sequence similarity at the species level for bacteria (Yarza et al., 2014). These strains require thorough characterization for taxonomic assignment and the potential establishment of a new species name.

growth promoting traits, all bioassays were performed three times for each strain.

## Screening for Indole-3-acetic acid (IAA) and indole related compounds production

Qualitative and quantitative analysis of IAA and IAA-related compound production by PSB isolates was determined by spectrophotometry in culture medium supplemented with tryptophan as IAA-precursor, using the method of Salkowski (Biswas et al., 2018). Firstly, bacterial isolates were overnight cultured in 5 mL of LB medium. Then, 20 µL of bacterial suspension (standardized to OD 600 nm = 0.7) were inoculated into 15 mL Falcon tubes containing 5 mL 10% LB liquid supplemented with tryptophan (5 mM). The resulting cultures were incubated for 5 days at 28°C on a rotary shaker (150 rpm) and cells from culture were separated by centrifugation (10,000 *g*, 20 min, 4°C). Salkowski reagent [2% of 0.5 M ferric chloride ($FeCl_3$) in 35% perchloric acid ($HClO_4$)] were mixed with the culture supernatant (1/1 v/v) and the mixture was incubated in the dark at

TABLE 2 Plant growth promoting traits of rhizoplane bacterial isolates.

| Rhizoplane PSB isolates | Species | Solubilization | | Production | | | | | Fixation | | Motility |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | P[a] | P ($\mu g\ mL^{-1}$)[b] | IAA | IAA ($\mu g\ mL^{-1}$)[b] | $NH_3$ | Siderophore | Biofilm | $N_2$ | Flagella | Pili |
| 7 | *Pseudomonas mohnii* | (+ + +) | 70.91 ± 2.17 | (−) | 20.60 ± 0.75 | (+) | (−) | (−) | (−) | (+) | (−) |
| 8 | *Novosphingobium resinovorum* | (+ − −) | 60.87 ± 0.77 | (−) | 6.82 ± 0.97 | (+) | (−) | (−) | (+) | (+) | (−) |
| 19 | *Pseudomonadales* sp. | (+ + +) | 70.01 ± 0.42 | (+) | 87.14 ± 0.84 | (+) | (−) | (+) | (+) | (+) | (−) |
| 23 | *Acinetobacter rhizosphaerae* | (+ + +) | 49.75 ± 0.33 | (+) | 48.85 ± 1 | (+) | (−) | (−) | (+) | (+) | (−) |
| 69 | *Achromobacter xylosoxidans* | (+ + +) | 82.56 ± 0.64 | (−) | 15.89 ± 2.05 | (+) | (−) | (−) | (−) | (+) | (−) |
| 82 | *Pseudomonas azotoformans* | (+ + +) | 39.13 ± 0.80 | (+) | 34.19 ± 3.01 | (+) | (+) | (−) | (−) | (+) | (−) |
| 90 | *Paenibacillus xylanexedens* | (+ + +) | 63.44 ± 0.66 | (+) | 69.56 ± 3.84 | (+) | (−) | (−) | (+) | (+) | (+) |
| 92 | *Pseudomonas* sp. | (+ − −) | 62.46 ± 0.03 | (+) | 51.80 ± 1.34 | (+) | (−) | (−) | (−) | (+) | (−) |
| 93 | *Pseudomonas* sp. | (+ + +) | 62.66 ± 0.02 | (−) | 11.52 ± 3.32 | (+) | (−) | (−) | (−) | (+) | (−) |
| 96 | *Brevibacillus* sp. | (+ − −) | 42.59 ± 0.45 | (−) | 6.15 ± 1.39 | (+) | (−) | (−) | (−) | (+) | (−) |
| 99 | *Paenibacillus purispatii* | (+ − −) | 260.01 ± 1.31 | (−) | 14.79 ± 1.55 | (+) | (−) | (−) | (−) | (+) | (−) |
| 132 | *Pseudomonas* sp. | (+ + +) | 65.07 ± 1.1 | (+) | 63.48 ± 1.33 | (+) | (+) | (−) | (−) | (+) | (−) |
| 133 | *Paenibacillus amylolyticus* | (+ − −) | 42.91 ± 0.45 | (+) | 330.27 ± 5.21 | (+) | (−) | (−) | (−) | (+) | (−) |
| 136 | *Stenotrophomonas maltophilia* | (+ + +) | 46.18 ± 0.87 | (−) | 10.14 ± 0.47 | (+) | (−) | (−) | (−) | (+) | (−) |
| 146 | *Bacillus thuringiensis* | (+ + +) | 63.64 ± 0.65 | (+) | 48.79 ± 2.41 | (+) | (+) | (−) | (−) | (+) | (−) |
| 153 | *Pseudomonas* sp. | (+ + +) | 68.66 ± 0.96 | (+) | 32.52 ± 0.8 | (+) | (−) | (−) | (−) | (+) | (−) |
| 149 | *Bacillus nitratireducens* | (+ − −) | 42.30 ± 0.29 | (+) | 53.30 ± 6.75 | (+) | (−) | (−) | (−) | (+) | (−) |
| 164 | *Pseudomonas fluorescens* | (+ − −) | 61.76 ± 0.54 | (−) | 3.23 ± 0.32 | (+) | (−) | (−) | (−) | (+) | (−) |
| 174 | *Pseudomonas fluorescens* | (+ + +) | 57.54 ± 0.6 | (−) | 7.25 ± 0.28 | (+) | (−) | (−) | (−) | (+) | (−) |
| 185 | *Pseudomonas* sp. | (+ + +) | 122.4 ± 0.44 | (+) | 39.90 ± 2.01 | (+) | (−) | (+) | (−) | (+) | (−) |
| 187 | *Paenibacillus polymyxa* | (+ + +) | 132.15 ± 0.21 | (+) | 251.58 ± 4.74 | (+) | (−) | (−) | (+) | (+) | (−) |
| 196 | *Brevibacterium* sp. | (+ + −) | 108.36 ± 0.9 | (+) | 242.03 ± 13.46 | (+) | (−) | (−) | (−) | (+) | (−) |
| 202 | *Brevibacillus* sp. | (+ + +) | 84.77 ± 0.11 | (−) | 10.35 ± 1.11 | (+) | (−) | (−) | (−) | (+) | (−) |
| 203 | *Bacillus* sp. | (+ + +) | 3.97 ± 0.06 | (+) | 35.50 ± 2.11 | (+) | (+) | (−) | (−) | (+) | (−) |

P, Phosphate solubilization test; IAA, Screening for Indole-3-acetic acid; $NH_3$, Ammonia production; $N_2$, Nitrogen fixation; (+): Positive for the trait; (−): Negative for the trait; (+ + +), (+ + −), and (+ − −): from strong to weak growth, respectively. [a]Isolate's responses after each of the three successive subcultures. [b]Values are the mean of three replicates independent assays ± standard error.





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room temperature for 20 min. The development of a pink color indicating IAA production. Estimation of IAA production was performed spectrophotometrically at 530 nm using a standard IAA concentration curve prepared in LB 50% containing serial dilutions of the synthetic indol-3-acetic acid (Sigma-Aldric, Oakville, ON, Canada).

### Siderophore production in agar medium

The chrome azurol S (CAS) assay, as described by Schwyn and Neilands (1987), was used to detect the siderophore production ability of PSB. In this colorimetric assay, siderophores sequester iron from the ternary complex CAS/iron (III)/hexadecyltrimethylammonium bromide (HDTMA), causing the release of the CAS dye and a consequent color change from blue to orange.

For CAS agar plate preparation, a liter of solution was prepared by dissolving 60.5 mg CAS in 50 mL glass-distilled water and combining it with 10 mL of iron (III) solution (1 mM $FeCl_3.6H_20$ in 10 mM HCl). This mixture was added to 72.9 mg of HDTMA in 40 mL of distilled water. The resulting dark blue CAS solution was autoclaved for 15 min and then added to agar medium/Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) mixture (30.24 g of PIPES in distilled water + salts solution +15 g of agar, pH adjusted to 6.8 using NaOH pellets before autoclaving) following the method by Louden et al. (2011).

Prior to inoculation, PSB isolates were cultured in 10% TSB (Tryptic Soy Broth, Becton Dickinson & Co., Franklin Lakes, NJ, USA) at 28°C for 48 h on a rotary shaker (150 rpm). The CAS plates were then spot-inoculated with PSB strains, with four bacterial strains per plate. The plates were observed for the development of an orange halo around the colonies after 7 days of incubation at 28°C. Each strain was tested in triplicate for both assays.

### Ammonia production

Estimation of ammonia production by PSB isolates was carried out in qualitative assays with peptone as described in Dutta et al. (2015). Aliquots of fresh overnight culture of isolates in TSA were inoculated into 10 mL tubes of 10% peptone water (peptone 10 g $L^{-1}$; NaCl 5 g $L^{-1}$; distilled water 1 L), then incubated at 28°C for 72 h. After incubation, 0.5 mL Nessler's reagent (10% $HgI_2$, 7%KI; 50% aqueous solution of NAOH 32%) was added to each tube. Appearance of brown to yellow color indicated positive test for ammonia production (Marques et al., 2010).

### Biofilm formation

Biofilm formation by isolates was assessed using the Crystal violet (CV) assay according to the method previously described by O'Toole (2011) with the following modifications proposed by Taktek et al. (2015). Inocula were prepared by growing bacteria overnight on a rotary shaker (180 rpm) at room temperature, in 25 mL of 10% TSB. Cells were collected and washed twice in 10 mL sterile saline (SS) (0.85% NaCl) after centrifugation (10,000 g, 5 min, 4°C) and re-suspended in SS. Flat bottom wells of sterile polystyrene 96-well microtiter plates (Costar, Corning Inc. Tewksbury, MA, USA) were filled with 100 µL of the modified NBRIP medium containing TCP. Then, each well was inoculated with 10 µL of a 48 h primary culture adjusted at an $OD_{600} = 0.7$. For these experiments, a non-inoculated TSB medium was used as the negative control. After 72 h of incubation at 28°C without agitation, the supernatant (i.e., medium and the non-adhering bacteria biofilm) were eliminated by simple inversion and each well was washed three times with 100 µL of sterile deionized water. The microplates were then air-dried for 30 min. The cells from the adhering biofilm were dyed with 100 µL of 0.1% crystal violet (CV) solution (30 min at room temperature). The excess of dye was removed by simple inversion and the biofilm was washed three times with running tap water at each wash. Then, the microplates were dried at room temperature during 10 min. Finally, bound CV was solubilized by adding 200 µL of 30% acetic acid solution and microtiter plates were incubated for 15 min. The absorbance in each well was sampled and the $OD_{530}$ was read on a microplate reader. The results obtained were then transformed into a quantification of biofilm formation by calculating the average absorbance of three replicates and dividing by the average absorbance of the blank (without bacteria). The results expressed as this ratio can be considered as non-dependent from the non-specific coloring of the surface of the wells of the microplates by the CV. Following this procedure, biofilm formation was considered to have occurred when the ratio is greater than two (Brian-Jaisson et al., 2014).

### In plate assays of nitrogen fixation

The Burks medium (HiMedia Laboratories, Kennett Square, PA, USA), containing inorganic salts along with carbon source but lacking nitrogen source, was used for detection of nitrogen fixing bacteria, able to fix nitrogen and grow when cultured on this nitrogen-free medium. Bacterial responses were observed after an incubation at 30°C for 7 days.

### Twitching and swarming motility tests

Twitching motility is exclusively facilitated by type IV pili, with repetitive extension and retraction movements leading to the translocation of the cell body. This motility is observed on solid surfaces, interfaces, or in mediums with moderate viscosities (1% agar). Swarming motility, on the other hand, is mediated by flagella in collaboration with type IV pili and exhibits a dendritic pattern of movement. It is observed along semisolid surfaces, such as 0.3–0.7% agar, while *in vitro* conditions with 1.5% agar and low humidity inhibit the swarming form of motility (Otton et al., 2017).

Both motility assays were conducted following previous descriptions (De Kievit et al., 2001; Ochoa et al., 2015; Otton et al., 2017) with some modifications. Briefly, strains were cultured overnight in TSB at 37°C. For twitching motility assays, 1 µL of each bacterial suspension was stabbed into TSB agar (1%) to the bottom of the plate. The plates were then incubated at room temperature for 48 h, and the motility zone was observed. For flagellar swarming motility assays, TSB medium supplemented with 0.3% agar was prepared. Subsequently, 1 µL of each bacterial suspension was inoculated onto the agar, and the plates were incubated at 28°C. The diameters of the swarming zones were observed after 10 h. Each strain was tested in triplicate for both assays.

## Statistical analysis

To assess the normality of the data, the Shapiro test was conducted using XLSTAT (version 2022.2.1.1318). Subsequently, the Kruskal-Wallis non-parametric test, supplemented with a post-hoc Dunn test, was employed to identify significant differences among means at a 5% probability level ($p \leq 0.05$). The experimental data provided represent

mean values obtained from three replicates, presented as the mean ± standard error for each treatment.

# Results

## Identification of PSB and additional plant growth promoting traits

### Rhizoplane PSB

A total of 210 isolates were obtained from root fragments collected from RP-rich soils in a mining area. From qualitative assays, 17% (35 isolates) of the isolates demonstrated clear TCP-solubilization halos on NBRIP solid medium, with diameters ranging from 0.5 to 4 cm. Through Sanger sequencing of the 16S rDNA, 24 PSB isolates were assigned to species using BLAST searches. These isolates were distributed among the bacterial phyla Pseudomonadota, Bacillota, and Actinomycetota (Table 1). Pseudomonadota and Bacillota dominated (with 14 and 9 isolates, respectively). Sequence comparisons of the 16S rDNA indicated similarities between 91 and 99% of known bacteria. Three families, Pseudomonadaceae, Bacillaceae, and Paenibacillaceae, dominated the bacterial community. Quantitative assays revealed varying P-solubilizing abilities, ranging from 39–260 $\mu g\,mL^{-1}$ (Table 2). The highest value was observed for isolate 99, which was identified as *Paenibacillus purispatii*.

Most of the bacterial isolates were assigned to the Pseudomonadota and were affiliated to four genera: *Pseudomonas* (ten isolates), *Stenotrophomonas* (two isolates) *Acinetobacter* (one isolate). One isolate was affiliated with the Pseudomonadota and was associated to the genus *Achromobacter* with more 99% sequence identity. The remaining isolate fell within the α-Proteobacteria and Burkholderiales order, and it was identified as *Novosphingobium* (Figure 2).

Among Bacillota, the alignment of the sequences showed clustering of isolates with species of the genus *Paenibacillus* (four isolates), *Bacillus* (three isolates), or *Brevibacillus* (two isolates). The Actinomycetota phylum was represented with only one isolate affiliated within the Micrococcales as *Brevibacterium* species.

Phenotypic characteristics of the 24 isolates were assessed for different PGP traits (Table 2). Thus, all the isolates were positive for ammonia production. The IAA production was observed for 13 PSB isolates (Table 2). It ranged from 32.52 to 330.27 $\mu g\,mL^{-1}$, with the isolates 133 (*Paenibacillus amylolyticus*), 187 (*P. polymyxa*) and 196 (*Brevibacterium*) showing the highest IAA production (330 ± 5.21, (Standard Error, SE), followed by 251.6 and 242 $\mu g\,mL^{-1}$), respectively. Only five PSB were positively screened for $N_2$ fixations (among them: one *Novosphingobium*, two *Paenibacillus*, one *Acinetobacter*), and four for siderophore capability (two isolates assigned to *Pseudomonas* and two as *Bacillus*) (Table 2). Two PSB among which one *Pseudomonas*, formed an important biofilm on an abiotic surface. The rhizoplane PSB mostly combined one additional PGP trait (nine isolates); six and eight PSB exerted, respectively, two and three additional capabilities. Phenotype of one PSB isolate (assigned to *Pseudomonas*) showed four additive PGP abilities (Table 2).

### Mycohyphospheric PSB

From a collection of 44 mycohyphospheric isolates (Table 3), 25 isolates (56.8%) sampled from maize, leek, potato and tomato were identified as PSB from qualitative and quantitative TCP solubilization assays (Table 4). Nine isolates were obtained from maize plants, seven from tomato plants, five from leek plants, and four from potato plants. The isolates showed varying levels of P solubilizing activity ranging from 24.2 to 175.7 $\mu g\,mL^{-1}$ and the isolate MI06 identified as *Paenibacillus typhae* exhibited the highest TCP solubilization activity (Table 4).

Of the selected PSB, 11 were affiliated to the Pseudomonadota phylum as shown in Table 3, seven in the Campylobacterota phylum with isolates displaying sequence identities with *Pseudoxanthomonas* (five isolates) or *Stenotrophomonas* (two isolates) (Figure 2). One strain was identified as β-Proteobacteria and Burkholderiales, close to the referenced *Cupriavidus necator*. In addition, two isolates grouped within the α-Proteobacteria class, related to Caulobacteraceae family and *Phenylobacterium* sp.

In addition, 12 isolates were affiliated to Bacillota, all being assigned to the Bacilli class. These isolates were mainly related to *Bacillus* (seven isolates), PTS08 sharing more than 99.5% identity with *Bacillus*. The other ones shared sequence identity with representatives of *Paenibacillus (two)*, *Priestia* (one) or *Lysinibacillus* (two) genus. The

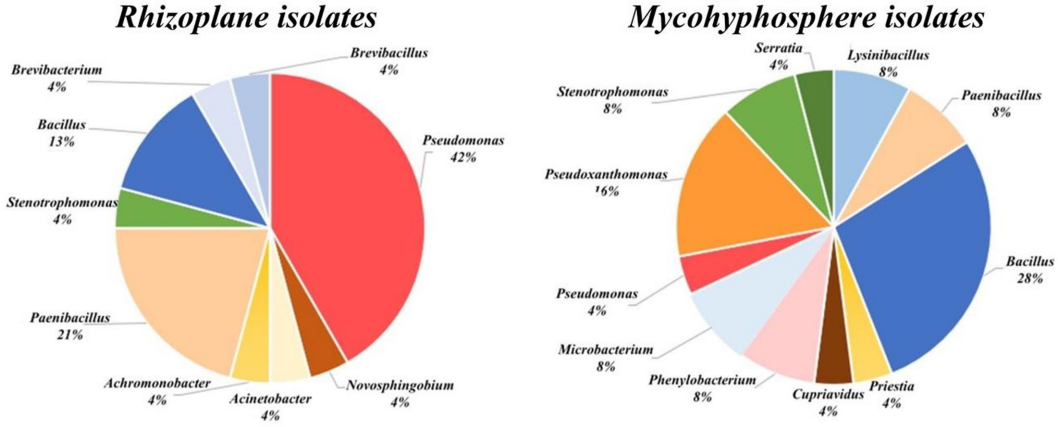


FIGURE 2
Taxonomic distribution of rhizoplane isolates (left panel) and mycohyphosphere isolates (right panel).

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TABLE 3 Taxonomic identifications of mycohyphosphere isolates.

| Hyphospheric PSB isolates | Sampled site | Seq. length (bp) | Identity (%) | Coverage (%) | Family | Species | Accession number |
|---|---|---|---|---|---|---|---|
| MI04 | Maize/i | 1,069 | 98% | 98% | Xanthomonadaceae | *Pseudoxanthomonas* sp. | OQ876740 |
| MI06 | Maize/i | 968 | 99% | 100% | Bacillaceae | *Lysinibacillus sphaericus* | OQ876739 |
| MI09 | Maize/i | 669 | 99% | 100% | Yersiniaceae | *Serratia inhibens* | OQ876738 |
| MI13 | Maize/i | 1,175 | 99% | 100% | Bacillaceae | *Paenibacillus typhae* | OQ876737 |
| MI14 | Maize/i | 1,028 | 99% | 100% | Bacillaceae | *Paenibacillus xylanexedens* | OQ876733 |
| MS03 | Maize/s | 1,083 | 99% | 100% | Xanthomonadaceae | *Pseudoxanthomonas* sp. | OQ876732 |
| MS09 | Maize/s | 851 | 99% | 100% | Xanthomonadaceae | *Stenotrophomonas maltophilia* | OQ876730 |
| MS11 | Maize/s | 1,055 | 99% | 99% | Microbacteriaceae | *Microbacterium oxydans* | OQ876728 |
| oMS04# | Maize/s | 1,164 | 97% | 99% | Xanthomonadaceae | *Stenotrophomonas maltophilia* | OQ876726 |
| Ps*# | Leek/s | 907 | 98% | 100% | Bacillaceae | *Bacillus thuringiensis* | OQ876725 |
| PS06 | Leek/s | 1,136 | 99% | 100% | Bacillaceae | *Lysinibacillus fusiformis* | OQ876724 |
| PS09 | Leek/s | 594 | 99% | 100% | Xanthomonadaceae | *Pseudoxanthomonas* sp. | OQ876717 |
| PS09b | Leek/s | 590 | 99% | 100% | Bacillaceae | *Bacillus wiedmannii* | OQ876715 |
| Psi07 | Leek/s | 909 | 99% | 99% | Caulobacteraceae | *Phenylobacterium* sp. | OQ876741 |
| PTS08 | Potatoe/s | 829 | 99% | 100% | Bacillaceae | *Bacillus thuringiensis* | OQ876743 |
| TI01 | Tomato/i | 1,038 | 99% | 100% | Bacillaceae | *Bacillus* sp. | OQ876707 |
| TI04 | Tomato/i | 1,108 | 99% | 98% | Caulobacteraceae | *Phenylobacterium* sp. | OQ876705 |
| TI05# | Tomato/i | 1,157 | 96% | 100% | Xanthomonadaceae | *Pseudoxanthomonas* sp. | OQ876742 |
| TS07 | Tomato/s | 1,037 | 99% | 100% | Bacillaceae | *Bacillus* sp. | OQ876745 |
| TS11 | Tomato/s | 1,079 | 99% | 100% | Bacillaceae | *Bacillus toyonensis* | OQ876747 |
| TS12 | Tomato/s | 1,138 | 98% | 99% | Burkholderiaceae | *Cupriavidus necator* | OQ876706 |
| TS18# | Tomato/s | 1,120 | 98% | 100% | Microbacteriaceae | *Microbacterium* sp. | OQ876704 |
| PTS17 | Potatoe/s | 870 | 99% | 100% | Bacillaceae | *Bacillus thuringiensis* | OQ876731 |
| PTS06# | Potatoe/s | 857 | 98% | 100% | Pseudomonadaceae | *Pseudomonas* sp. | OQ876746 |
| PTS03# | Potatoe/s | 767 | 97% | 100% | Bacillaceae | *Priestia megaterium* | OQ876744 |

(i): Plant supplemented with igneous RP; (s): Plant supplemented with sedimentary RP. #Indicates isolates with a percentage of identity below 98%, which is the generally accepted threshold for sequence similarity at the species level for bacteria (Yarza et al., 2014). These strains require thorough characterization for taxonomic assignment.

Actinomycetota phylum is represented with two *Microbacterium* isolates, one of which is related to *Microbacterium oxydans*.

Among the 25 mycohyphospheric PSB selected, all were identified as ammonia producers, while 20 isolates representing nine genera (with *Bacillus* being prevalent) displayed IAA production ranging from 41.4 to 963.9 $\mu g\,mL^{-1}$ (Table 4). The isolates MI14 (*P. xylanexedens*), MI13 (*P. typhae*) and PTS08-(*B. thuringiensis*) exhibited the highest IAA production (963.9, 566.6, and 425.9 $\mu g\,mL^{-1}$, respectively). Only 12 isolates showed nitrogen fixation, belonging to seven different genera. No isolate produced siderophore. Four isolates belonging to *Pseudoxanthomonas* (two isolates), *Cupriavidus* (one isolate), and *Lysinibacillus* (one isolate) exhibited biofilm formation.

All rhizoplane and mycohyphospheric isolates were found to be positive for swarming motility due to flagella. Pili motility was observed for one rhizoplane isolate (*Paenibacillus*) and three mycohyphospheric isolates assigned to *Stenotrophomonas*, *Pseudoxanthomonas* (Tables 3, 4).

## Distinct taxonomic profiles among PBS communities captured from the rhizoplane and mycohyphosphere samples

Interestingly, the two experimental setups produced distinct taxonomic profiles of PSBs, revealing both shared and unique taxa at the genus and species levels. Combining Sanger sequencing and phylogenetic analysis revealed that isolates from the rhizoplane and mycohyphospheric environments belonged to only three bacterial phyla: Pseudomonadota, Bacillota, and Actinomycetota (Tables 1, 3).

Four genera (*Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas*) were common to both bacterial collections, with isolates related to Bacillus and Paenibacillus (Bacillota) prevalent in both environments. In contrast, *Pseudomonas* and *Pseudoxanthomonas* were dominant in the rhizoplane and mycohyphosphere, respectively (Figure 2).

TABLE 4 Plant growth promoting traits of mycohyphosphere isolates.

| Mycoyphospheric PSB isolates | Species | Solubilization | | Production | | | | | Fixation | Motility | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | P[a] | P (μg mL$^{-1}$)[b] | IAA | IAA (μg mL$^{-1}$)[b] | $NH_3$ | Siderophore | Biofilm | $N_2$ | Flagella | Pili |
| MI04 | *Pseudoxanthomonas* sp. | (+ + +) | 59.46 ± 0.08 | (+) | 76.59 ± 6.5 | (+) | (−) | (−) | (+) | (+) | (−) |
| MI06 | *Lysinibacillus sphaericus* | (+ + +) | 175.69 ± 0.61 | (−) | 7.61 ± 0.29 | (+) | (−) | (−) | (−) | (+) | (−) |
| MI13 | *Paenibacillus typhae* | (+ + +) | 151.99 ± 0.22 | (+) | 566.67 ± 2.61 | (+) | (−) | (−) | (−) | (+) | (−) |
| Mi14 | *Paenibacillus xylanexedens* | (+ + +) | 65.23 ± 0.25 | (+) | 963.96 ± 8.05 | (+) | (−) | (−) | (+) | (+) | (−) |
| MS03 | *Pseudoxanthomonas* sp. | (+ + +) | 54.76 ± 0.22 | (+) | 139.11 ± 2.47 | (+) | (−) | (−) | (−) | (+) | (+) |
| MS09 | *Stenotrophomonas maltophilia* | (+ + +) | 98.28 ± 3.17 | (−) | 3.96 ± 0.53 | (+) | (−) | (−) | (+) | (+) | (−) |
| MS11 | *Microbacterium oxydans* | (+ − −) | 166.44 ± 0.17 | (+) | 73.27 ± 0.96 | (+) | (−) | (−) | (+) | (+) | (−) |
| oMS04 | *Stenotrophomonas maltophilia* | (+ + +) | 73.95 ± 0.09 | (+) | 126.32 ± 1.13 | (+) | (−) | (−) | (+) | (+) | (+) |
| Ps* | *Bacillus thuringiensis* | (+ + −) | 73.28 ± 0.30 | (+) | 60.3 ± 3.74 | (+) | (−) | (−) | (+) | (+) | (−) |
| PS06 | *Lysinibacillus fusiformis* | (+ − −) | 79.97 ± 0.37 | (+) | 110.83 ± 2.93 | (+) | (−) | (+) | (−) | (+) | (−) |
| PS09 | *Pseudoxanthomonas* sp. | (+ + +) | 64.16 ± 0.61 | (+) | 88.74 ± 6.83 | (+) | (−) | (−) | (−) | (+) | (−) |
| PS09b | *Bacillus wiedmannii* | (+ + −) | 67.41 ± 0.50 | (+) | 46.75 ± 2.62 | (+) | (−) | (−) | (−) | (+) | (−) |
| Psi07 | *Phenylobacterium* sp. | (+ + +) | 51.92 ± 0.53 | (+) | 80.21 ± 4.83 | (+) | (−) | (−) | (−) | (+) | (−) |
| PTS08 | *Bacillus thuringiensis* | (+ + +) | 24.15 ± 0.08 | (+) | 425.97 ± 5.85 | (+) | (−) | (−) | (−) | (+) | (−) |
| TI01 | *Bacillus* sp. | (+ + +) | 64.73 ± 0.70 | (+) | 97.51 ± 1.16 | (+) | (−) | (−) | (−) | (+) | (−) |
| TI04 | *Phenylobacterium* sp. | (+ + −) | 81.94 ± 0.36 | (+) | 107.09 ± 2.15 | (+) | (−) | (−) | (+) | (+) | (−) |
| TI05 | *Pseudoxanthomonas* sp. | (+ + −) | 48.23 ± 0.22 | (−) | 18.41 ± 0.95 | (+) | (−) | (+) | (+) | (+) | (−) |
| TS07 | *Bacillus* sp. | (+ + +) | 45.09 ± 0.15 | (+) | 49.74 ± 3.74 | (+) | (−) | (−) | (+) | (+) | (−) |
| TS11 | *Bacillus toyonensis* | (+ + +) | 41.56 ± 0.22 | (+) | 51.5 ± 1.15 | (+) | (−) | (−) | (+) | (+) | (−) |
| TS12 | *Cupriavidus necator* | (+ + +) | 133.59 ± 0.83 | (−) | 6.85 ± 2.37 | (+) | (−) | (+) | (+) | (+) | (−) |
| TS18 | *Microbacterium* sp. | (+ + −) | 99.09 ± 0.32 | (+) | 75.98 ± 1.38 | (+) | (−) | (−) | (+) | (+) | (−) |
| PTS17 | *Bacillus thuringiensis* | (+ + +) | 44.68 ± 0.07 | (+) | 41.40 ± 2.30 | (+) | (−) | (−) | (−) | (+) | (−) |
| PTS06 | *Pseudomonas* sp. | (+ + −) | 64.10 ± 0.77 | (−) | 21.12 ± 3.59 | (+) | (−) | (+) | (−) | (+) | (−) |
| PTS03 | *Priestia megaterium* | (+ + +) | 56.44 ± 0.77 | (+) | 128.2 ± 0.74 | (+) | (−) | (−) | (−) | (+) | (−) |
| MI09 | *Serratia inhibens* | (+ + −) | 170.05 ± 0.24 | (+) | 246.7 ± 8.69 | (+) | (−) | (+) | (+) | (+) | (+) |

P, Phosphate solubilization test; IAA, Screening for Indole-3-acetic acid; $NH_3$, Ammonia production; $N_2$, Nitrogen fixation; (+): Positive for the trait; (−): Negative for the trait; (+ + +), (+ + −), and (+ − −): from strong to weak growth, respectively. [a]Isolate's responses after each of the three successive subcultures. [b]Values are the mean of three replicates independent assays ± standard error.





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At the species level, five species (17%) were shared, namely *Bacillus thuringiensis*, *Paenibacillus xylanexedens*, Pseudomonas sp., Bacillus sp., and *Stenotrophomonas maltophilia* (Tables 2, 4). Isolates related to Microbacteriaceae, Caulobacteraceae, Burkholderiaceae, and Yersiniaceae were specifically identified among the mycohyphospheric isolate collection, displaying the largest taxonomic diversity at the family level. A representative of Moraxellaceae was exclusively identified among rhizoplane isolates.

# Discussion

## Prospecting PSB in little-explored habitats from RP-enriched environments

This study explores the potential of PSBs as bioinoculants to enhance the efficiency of low-solubility RP ore used directly in some countries as a cost-effective phosphate fertilizer in soils. Despite the widespread use of microbial-based P fertilizers, discrepancies in efficacy exist in the literature, likely due to environmentally and context-dependent factors influenced by complex interactions between soil P chemistry and microbiology (Ducousso-Detrez et al., 2022a). The study emphasizes the need for more mechanistic data and advocates actively developing new inoculants for diverse environmental conditions, selecting strains with high field success and minimal downstream impact. It suggests exploring unique or extreme habitats for prospecting novel microbial diversity. To achieve this goal, two distinct experimental setups were designed to isolate PSBs from specific environments enriched with RP. These setups targeted rhizoplane habitats within mining environments and mycohyphospheric habitats, both of which hold ecological significance.

The second experimental setup focused on the specificity of hyphosphere habitats. Studies suggest that AMF hyphae recruit their own soil microbiomes in their hyphosphere, distinct from those of the bulk soil or rhizosphere (Wang G. et al., 2023; Wang L. et al., 2023). However, the diversity, richness, and structure of hyphosphere microbiomes are only beginning to be understood (Emmett et al., 2021; Zhang et al., 2022; Basiru et al., 2023). Soil microbes colonizing the hyphosphere hold significant interest due to their potential functions, likely to contribute to or complement the specific functional capabilities of AMF in a particular context (Faghihinia et al., 2022; Zhang et al., 2022; Wang G. et al., 2023; Wang L. et al., 2023).

The last step (in bi-compartmented Petri dishes) was conducted to select AMF-associated bacteria growing along the hyphae of *R. irregularis*, i.e., mycohyphospheric bacteria from agronomic soils.

These two setups proved to be effective strategies to select PSB isolates. Particularly, our experimental setup confirms that two-compartment Petri dishes are a useful tool to target mycohyphospheric PSB, aligning with previous reports (Ordonez et al., 2016; Taktek et al., 2017).

Using the TCP assay, which remains prevalent in current literature despite being considered relatively weak for studying P solubilization capabilities of PSBs (Bashan et al., 2013), some root and mycohyphosphere isolates demonstrated promising results. They released solubilized P concentrations up to 260 and 175.6 $\mu g\,mL^{-1}$ in 7 days, respectively. In light of these findings, PSBs with high phosphate-solubilizing potential could be relevant as potential compounds for inoculant engineering and subsequent use in combination with low-availability P forms such as RP to promote plant growth.

## Rhizoplane PSB in RP-rich soils with high P availability from the mining area

Around 16% of the initially sampled rhizoplane isolates were identified as culturable PSB. According to Khan et al. (2007), phosphate-solubilizing microorganisms (Fungi and Bacteria) could make up to 50% of culturable populations, and their presence, abundance, and diversity are likely influenced by the plant species. Similarly, according to Goswami et al. (2016), estimates range from 20 to 40%. The differences in percentages observed in various studies may be attributed to various factors, including the selection of culture techniques. Notably, the selection and cultivation of PSB can be carried out from different steps of the *in vitro* culture process and performed on different culture media (Pikovskaya, 1948; Nautiyal, 1999), potentially favoring some taxa over others. Furthermore, it has been found that the rhizosphere contains a higher proportion of PSBs than the surrounding root-free soil (Mander et al., 2012; Nicolitch et al., 2016). Extensively, Spohn et al. (2020) established the relative abundance of PSB to be 24.4% in the rhizoplane (root surface), which was significantly higher than in the rhizosphere or on saprolite (a chemically weathered bedrock) where plants were growing (9.5 and 2.2%, respectively). This ecological data can obviously impact the percentage of the cultivable fraction among soil PSB. Nevertheless, many questions remain unanswered regarding the achievement of a consensus allowing more rigorous comparison of PSB studies in a relative manner. The choice of culture-dependent techniques, the development of culture methods for a larger number of bacterial taxa, the standardization of protocols (from sampling design to characterization of isolates and their sequencing, the comprehensive description of soil microbiomes, notably to further characterize the relation between taxonomy and functions), are still major challenges for the future of microbiome research and barriers to the replication of the same sampling strategies in different environments by different researchers. In a comparative approach, Stefani et al. (2015) employed both culture-independent methods, such as amplicon sequencing of soil DNA, and culture-dependent techniques to characterize bacterial communities in hydrocarbon-contaminated soils. Their findings indicated that these methods captured distinct microbial community fractions in soils. Notably, a significant proportion of taxa identified through culture-independent methods, including many of the most abundant taxa *in situ*, remained unrecovered by culture, despite the utilization of various media. Moreover, they observed only a 2% increase in bacterial species richness when isolation techniques were applied. These results highlight the fragmented nature of our understanding of soil microbial diversity, primarily due to biases introduced by the methods employed (for a comprehensive review, refer to Basiru et al., 2023). They underscore the need for ongoing efforts to explore and disentangle this diversity.

Interestingly, Spohn et al. (2020) also showed enrichment of PSB in the rhizosphere of plants grown on saprolites with low P availability compared to saprolites with a higher soluble P fraction. In light of these results, it is important to note that in our work, a significant proportion of PSB were successfully screened from the RP-rich soils where P availability was high (up to 339.5 mg $kg^{-1}$ P Olsen). Here, our

results raise some questions for the future: are the mechanisms classically associated with P solubilization and on which the *in vitro* selection of PSB is based (i.e., the release of organic acids) really contribute to soil P solubilization and to the high level of orthophosphate ions in the mining soils with elevated available P levels? Or do *in situ* PSB occurrences link to other functionalities in these soils [e.g., physicochemical mechanisms behind the phosphate solubilization trait could be involved in iron solubilization; indeed, in aerobic conditions and neutral pH, Fe is almost insoluble for plants (Schwab and Lindsay, 1983)]

Interestingly, a higher proportion of mycohyphospheric PSB was obtained compared to rhizoplane PSB (57 and 11% respectively). This outcome is notable, considering that the selection process for mycohyphospheric isolates could have significantly reduced the probability of selecting PSB isolates due to the multiple steps in the procedure and their inherent selectivity. Consequently, one could infer that the mycohyphospheric PSB isolation protocol is more efficient than the one for rhizoplane PSB isolation, particularly in terms of the proportion of PSB within the collection of cultivable PSB isolates. The differences in the quality and quantity of exudates from plant roots and AMF extraradical hyphae could differentially contribute to microbial community dynamics, symbiotic associations, soil structure, and chemical signaling in the rhizosphere. Ultimately, these factors may influence the isolation methods of microorganisms from these two biotopes (Zhang et al., 2022; Basiru et al., 2023).

## Diversity of culturable root and mycohyphospheric PSB isolated from RP-rich habitats

In this study, representatives of Pseudomonadota, Actinomycetota and Bacillota are dominant. Such data are in accordance with previously published results. Thus, Pseudomonadota, Actinomycetota, and, to a lesser extent, Bacillota have been described as ubiquitous in various soils worldwide (Delgado-Baquerizo et al., 2018). Besides, the taxonomic composition of bacterial communities from alkaline phosphate mine wastes showed that sequences belonging to Pseudomonadota and Actinomycetota, as well as the genera *Pseudomonas* and *Bacillus* were highly represented (Mghazli et al., 2021). Similarly, Pseudomonadota dominated the root-associated soils and bulk soil associated with Chinese mining sites (Ye et al., 2020). Furthermore, these three phyla are well-known in the literature to include PGPR and effective P-solubilizers (Mghazli et al., 2021). The growth-promoting abilities of *Pseudomonas* and *Bacillus* genera, which are widespread in various soils worldwide, are well documented and have been found to be effective and abundant P-solubilizers (Sharma et al., 2014; Kumar et al., 2016; Vacheron et al., 2016). Therefore, some of them are incorporated into the formulation of diverse microbial biofertilizers, available on the international market for farmers (Goswami et al., 2016; Kumari et al., 2019; Lobo et al., 2019). These findings are in accordance with the data of Yang et al. (2012), who classified 123 PSB, isolated from P-rich soils from a lake drainage area, into three bacterial phyla: Pseudomonadota (with some *Pseudomonas* representatives), Actinomycetota, and Bacillota (including *Bacillus* and *Brevibacillus*).

However, we must highlight an under-representation of Actinomycetota; only three Actinomycetota isolates were identified and referred to as genera *Microbacterium* (two mycohyphospheric isolates) or *Brevibacterium* (one root isolate). This phylum is ubiquitously distributed in a large range of soils (Pierzynski et al., 2005; Qin et al., 2016). Moreover, a large number of Actinomycetota exhibit PGP traits, with several being P-solubilizers (Hamedi and Mohammadipanah, 2015), either as free-living bacteria or endophytic bacteria (Qin et al., 2017; Chen et al., 2019). Similarly, Yang et al. (2012) only identified three isolates as Actinomycetota among the 123 PSB they selected. It is likely due to the culture-dependent procedures which were not optimal for Actinomycetota. For example, the effectiveness of TCP-based medium for assessing the *in vitro* capability of bacteria for solubilization may be suboptimal in certain taxa (Bashan et al., 2013, 2014).

The collection also comprises isolates associated with less common taxa (e.g., Microbacteriaceae, Caulobacteraceae, Burkholderiaceae, Yersimiaceae), expanding our understanding of PSB in RP-rich environments. These promising results are expected to contribute to the advancement of environmentally friendly fertilization practices compared to current methods.

## Multifunctionality of PSB isolates for inoculant formulation

Our study demonstrated that the selected PSB possessed additional and diverse PGP abilities. Ammonia production emerged as the most prevalent PGP trait among the isolates, with all tested isolates showing a positive result. This trait is acknowledged for its role in enhancing plant growth by providing available nitrogen to the host plant, thereby promoting root and shoot elongation (Hayat et al., 2010; Kandjimi et al., 2015). Furthermore, approximately 58.3 and 80% of rhizoplane and mycohyphospheric isolates, respectively, demonstrated the ability to produce IAA after incubation with tryptophan as the auxin precursor. IAA production is associated with increased root elongation, lateral root formation, and root hairs, potentially enhancing the efficiency of the plant's root system for water and nutrient uptake (Jain and Patriquin, 1985). Consistent with these findings, some reports suggest that PSB may exert a more significant effect on root traits (such as root biomass, diameter, length, surface, or volume) than rhizosphere phosphate solubilization alone (Elhaissoufi et al., 2020), highlighting the importance of selecting isolates with both IAA production and PSB traits.

Notably, all isolated bacteria exhibited motility, adding another interesting feature likely to facilitate rhizoplane colonization.

# Conclusion

Addressing P deficiency in croplands, especially those utilizing phosphate-based fertilizers, has spurred interest in microorganisms capable of converting insoluble P into bioavailable forms. We conducted two independent experiments in distinct habitats to capture PSBs, resulting in diverse PSB communities. These findings, demonstrate the efficacy of both protocols in selecting a broader cultivable PSB biodiversity, and hold promise for more environmentally friendly fertilization practices. Future investigations should incorporate diverse cultivation media and techniques to reduce potential biases and capture bacterial diversity closer to that observed in natural habitats (Chen and Liu, 2019). Additionally, investigations

on bacterial taxonomic diversity, community structure, and functions in the mining area [using sequencing and phylogenetic analysis of the 16S rRNA gene combined with OMICS technologies (Mghazli et al., 2021)] would be relevant for identifying taxa and functions involved in P cycling in native P mining environments.

Also, further greenhouse and field studies will be essential in the future to test the dual use of mineral nutrient and microbial resources (individually or in polymicrobial formulations, multi-species, and pluri-functional) for agronomic purposes. Exploring PSB from P-rich soils could aid in developing sustainable microbe-assisted rehabilitation strategies for derelict phosphate mine lands. Our data may enhance phytoextraction strategies, utilizing efficient soluble-P producers, or phytoremediation efforts in phosphate mining wasteland soils. Further research into the molecular mechanisms of PSB evolution and adaptation to complex environments, such as RP-rich mining soils, is a pertinent avenue for exploration.

## Data availability statement

The accession numbers for the 16S rDNA sequences of all isolates utilized in this study are presented in Tables 1, 3 for rhizoplane and mycohyphosphere isolates, respectively.

## Author contributions

AD-D: Formal analysis, Methodology, Writing – original draft. ZL: Methodology, Writing – review & editing. JF: Supervision, Writing – review & editing. AL-H: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. MH: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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# Interactions between halotolerant nitrogen-fixing bacteria and arbuscular mycorrhizal fungi under saline stress

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**Background and aims:** Soil salinity negatively affects crop development. Halotolerant nitrogen-fixing bacteria (HNFB) and arbuscular mycorrhizal fungi (AMF) are essential microorganisms that enhance crop nutrient availability and salt tolerance in saline soils. Studying the impact of HNFB on AMF communities and using HNFB in biofertilizers can help in selecting the optimal HNFB-AMF combinations to improve crop productivity in saline soils.

**Methods:** We established three experimental groups comprising apple plants treated with low-nitrogen (0 mg N/kg, N0), normal-nitrogen (200 mg N/kg, N1), and high-nitrogen (300 mg N/kg, N2) fertilizer under salt stress without bacteria (CK, with the addition of 1,500 mL sterile water +2 g sterile diatomite), or with bacteria [BIO, with the addition of 1,500 mL sterile water +2 g mixed bacterial preparation (including *Bacillus subtilis* HG-15 and *Bacillus velezensis* JC-K3)].

**Results:** HNFB inoculation significantly increased microbial biomass and the relative abundance of beta-glucosidase-related genes in the rhizosphere soil under identical nitrogen application levels ($p < 0.05$). High-nitrogen treatment significantly reduced AMF diversity and the relative abundance of beta-glucosidase, acid phosphatase, and urea-related genes. A two-way analysis of variance showed that combined nitrogen application and HNFB treatment could significantly affect soil physicochemical properties and rhizosphere AMF abundance ($p < 0.05$). Specifically, HNFB application resulted in a significantly higher relative abundance of *Glomus-MO-G17-VTX00114* compared to that in the CK group at equal nitrogen levels.

**Conclusion:** The impact of HNFB on the AMF community in apple rhizospheres is influenced by soil nitrogen levels. The study reveals how varying nitrogen levels mediate the relationship between exogenous HNFB, soil properties, and rhizosphere microbes.

KEYWORDS

salt stress, nitrogen fertilization, halotolerant nitrogen-fixing bacteria, arbuscular mycorrhizal fungi, plant growth-promoting rhizobacteria

# 1 Introduction

Approximately 20% of the world's arable land is currently at risk of salinity, and this percentage is steadily increasing by 10% each year, posing a considerable challenge to agricultural production and contributing to land degradation (Farooq et al., 2021). Apple trees are highly resistant to salinity and alkali stress, making them the preferred fruit-tree species for the efficient development and utilization of saline-alkali land in the Yellow River Basin. However, nitrogen deficiency is a major cause of restricted apple growth in saline soils. Typically, crop yield is improved with the application of nitrogen. However, excessive nitrogen application does not always result in a continuous yield increase; it reduces nitrogen-use efficiency and leads to environmental problems (Grassini et al., 2013; Faostat, 2016). Thus, it is important to consider that using inorganic nitrogen fertilizers can increase nutrient amounts and soil salinity, which may damage plants instead of promoting growth (Jha et al., 2012). In apple cultivation on saline-alkali lands, one potential solution to address this issue is to develop efficient, sustainable, and environmentally friendly biological nitrogen fertilizers that can partially replace chemical nitrogen fertilizers (Chen et al., 2023a).

Many studies have confirmed the importance of microbial inoculants in achieving higher crop yields, improving crop quality and soil fertility, and deepening our understanding of the mechanisms of interactions between certain bacterial and plant strains in specific ecosystems (Primieri et al., 2021; Liu et al., 2022). However, there are still some problems with the application of nitrogen-fixing bacteria under natural conditions: (1) several nitrogen-fixing bacterial strains struggle to colonize saline soils for extended periods, resulting in unstable nitrogen fixation effects. Additionally, there has been limited research on the screening methods, application, and mechanism of action of salt-tolerant nitrogen-fixing bacteria, also known as halotolerant nitrogen-fixing bacteria (HNFB). (2) While several studies have focused on the growth-promoting effects of exogenously inoculated plant growth-promoting rhizobacteria (PGPR) on host plants (Thirkell et al., 2020), only a few have examined the synergistic effects of PGPR with other rhizosphere microorganisms on saline soils and crops, especially beneficial flora with unique abilities. In recent years, there has been a trend toward developing compound bacterial fertilizers comprising two or more bacterial strains instead of single-strain fertilizers, and a shift from fuzzy decision support systems to clear decision support systems. For example, in maize stems, xylem selectively recruited conserved microorganisms dominated by γ-proteobacteria, and the combination of *Klebsiella* var*iicola* MNAZ1050 and *Citrobacter* sp. MNAZ1397 increased nitrogen accumulation in maize by 11.8% (Zhang et al., 2022).

Halotolerant PGPRs with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity can reduce plant ethylene accumulation, prevent oxidative stress, promote plant growth, and regulate the rhizosphere microbial community structure. Consequently, halotolerant PGPRs have become a valuable microbial resource for promoting crop growth in saline-alkali soils (Ji et al., 2022a; Li et al., 2023). Among the beneficial microorganisms in saline soils, ascomycetous fungi (AMF) can form a symbiotic relationship with most crops, improving soil nutrient acquisition, promoting plant growth and water absorption, and initiating defense responses in host plants (Bennett and Groten, 2022). Although AMF may not prove to be a "sustainable savior" in agroecosystems (Thirkell et al., 2017), they have the potential to help crops assimilate nutrients (Thirkell et al., 2020). Currently, there is a lack of research on HNFB-mediated AMF community responses.

Changes in the core strains in the rhizosphere of crops can alter the types and quantities of metabolites and affect the interaction network of the entire community (Coyte et al., 2015). For example, Niu et al. (2017) constructed a synthetic community composed of seven bacteria and found that in the absence of *Enterobacter cloacae*, the abundance of *Brevibacterium parvum* increased, while other species disappeared from the community, demonstrating the importance of key species in the microbiome. Therefore, analyzing the composition of core microorganisms is necessary for accurately regulating the rhizosphere microbial community, improving microbial community function, and elucidating the mechanisms of microbial community-plant interactions (Hogle et al., 2023). However, because chemical nitrogen fertilizers and nitrogen-fixing bacteria coexist in the agricultural sector, they may interact with each other, with uncertain consequences for the soil–microbe system (Tian et al., 2017); whether the coexistence of chemical nitrogen fertilizers and nitrogen-fixing bacteria affects the core strains remains unknown.

In this study, we inoculated the rhizosphere of apple trees in saline land with a compound flora composed of two salt-tolerant strains that can stably colonize saline land, exhibit ACC deaminase activity, and demonstrate high efficiency in synergistic nitrogen fixation. Additionally, we examined the effects of compound inoculation on the apple plants and the rhizosphere AMF communities under three nitrogen application levels. We proposed and tested the following two hypotheses: (1) the AMF community in the rhizosphere of apple trees in saline-alkali soil is unique, and excessive nitrogen application has a negative effect on the structure and function of the AMF community; (2) exogenous HNFB positively affects the structure and function of the AMF community by influencing one or more core rhizosphere AMF species.

# 2 Materials and methods

## 2.1 HNFB strains and culture media

The microbial inoculant consisted of a mixture of *Bacillus subtilis* HG-15 and *Bacillus velezensis* JC-K3 strains. The 16S rDNA sequences of these two strains were deposited in the NCBI database under accession number MN689681 and MT605169. Both strains have been

shown to possess efficient nitrogen fixation ability, antagonistic activity, and other growth-promoting characteristics in our previous studies (Ji et al., 2021, 2022b). The nitrogen fixation activity of the HG-15 strain is 24.30 ± 0.75 mg N/g glucose, while that of the JC-K3 strain is 30.25 ± 0.42 mg N/g glucose. The ACC deaminase activity of the HG-15 strain is 14.816 ± 0.965 μmol/(mg h), while that of the JC-K3 strain is 18.10 ± 0.97 μmol/(mg h). Luria–Bertani liquid medium was used as the seed and fermentation medium. When the spore formation rate in the fermentation liquid exceeded 95%, diatomite sterilized at 121°C for 20 min was added at a concentration of 10% to the fermented liquid. The bacteria were allowed to adsorb onto the diatomite. The suspension was then centrifuged at 3,100 × *g* for 20 min. The supernatant was discarded, and the sediment was stored at −40°C for 48 h before being placed in a lyophilizer (Labconco FreeZone® Plus 4.5 L; Kansas City, MO, USA) and treated at −48°C and 9 Pa for 48 h (Ji et al., 2020). The densities of the HG-15 and JC-K3 strains in the resulting solid microbial agents were $451 \times 10^8$ CFU/g and $498 \times 10^8$ CFU/g, respectively. The two bacterial preparations were diluted to a concentration of $20 \times 10^8$ CFU/g with sterile diatomite, and then mixed in a 1:1 ratio for later use.

## 2.2 Experimental design

The experiment was conducted in the Weifang Economic Development Zone (119°3′30″E, 36°48′14″N), Shandong Province, China, in 2022. A four-year-old dwarf rootstock M9T337 grafted Fuji apple (Red Fuji) plant was selected as the test material [electrical conductivity (EC) = 671 s/cm, pH = 7.6, 14.51 g/kg soil organic matter, 59.25 mg/kg soil available nitrogen, 21.46 mg/kg soil available phosphorus (by Olsen P test), and 122.17 mg/kg soil exchangeable potassium]. Before planting, all seedlings were fertilized with urea (46% N) at three different concentrations: 0 mg N/kg (N0, low nitrogen level), 200 mg N/kg (N1, normal nitrogen level), and 300 mg N/kg (N2, high nitrogen level), which corresponded to 0, 240, and 360 kg N/ha of field fertilizer (urea), respectively. Simultaneously, 10 g of potassium sulfate (containing 50% $K_2O$) and 17 g of calcium superphosphate (containing 14% $P_2O_5$) were applied per plant. Apple plants with strong and uniform growth were selected and planted in pots in mid-March, with one plant per pot. The pots and soil used in this experiment were not sterilized. After 14 days of plant colonization, the three nitrogen application levels were established: without bacteria (CK, with the addition of 1,500 mL sterile water +2 g sterile diatomite) or with bacteria (BIO, with the addition of 1,500 mL sterile water +2 g mixed bacterial preparation). This resulted in a total of six groups, each containing 12 pots. The experimental plants were arranged randomly, while the other experiments were conducted according to standard field procedures. Samples were taken at the flower bud morphological differentiation stage in July 2022. This experiment was repeated thrice.

## 2.3 Rhizosphere soil sampling and analysis

Soil pH and EC values were analyzed using digital pH (FE20) and EC (FE930) meters (Mettler Toledo, Switzerland), respectively. The soil-water ratios used for analysis were 1:2.5 and 1:5. The organic matter content of the soil was determined using the method described by Aj and Black (1934). The total nitrogen content in the soil (soil N) was determined using the Bremner (2009) method.

Microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) contents were determined using the chloroform fumigation-$K_2SO_4$ extraction method (Brookes et al., 1985; Vance et al., 1987), while microbial biomass phosphorus (MBP) content was determined using the chloroform fumigation-$NaHCO_3$ extraction method (Brookes et al., 1982).

## 2.4 DNA extraction and polymerase chain reaction (PCR)-based amplification

Soil genomic DNA was extracted from 0.5 g of soil using a FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. The DNA quality was examined using 1.0% agarose gel electrophoresis, and the DNA concentration was quantified using a NanoDrop 2000 UV–Vis spectrophotometer (Wilmington, USA) (Zeng et al., 2021). Nested PCR was conducted to amplify specific fragments of the AMF 18S rRNA gene. The first PCR reaction consisted of a 20-μL mixture containing 1 μL of genomic DNA (approximately 10 ng), 2 μL of 2.5 mM dNTPs, 0.4 μL of FastPfu DNA Polymerase (5 U/μL), 0.4 μL of each primer [10 μM; AML1 (5′-ATCAACTTTCGATGGTAG GATAGA-3′)/AML2 (5′-GAACCCAAACACTTTGGTTTCC-3′) primer pair], 4 μL of 5-fold Fastpfu DNA Buffer (Takara, Dalian, China), and molecular-grade water. The products from the first PCR (approximately 10 ng used as the template) were then amplified in a second PCR reaction using the primers AMV4.5NF (5′-AAGCT CGTAGTTGAATTTCG-3′) and AMDGR (5′-CCCAACTA TCCCTATTAATCAT-3′), following the same protocol as the first PCR step. The thermal cycling conditions for both PCR steps were as follows: initial denaturation at 95°C for 3 min, 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 45 s, and final elongation at 72°C for 10 min. The PCR products were extracted using 2% agarose gels, purified with an AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA USA) according to the manufacturer's protocol, and quantified with a QuantiFluor ST instrument (Promega, Madison, WI, USA).

## 2.5 Illumina MiSeq and bioinformatics analyses

Quantified and purified PCR products were sent to Majorbio BioPharm Technology Co. Ltd. (Shanghai, China) for sequencing using the Illumina MiSeq PE300 platform (San Diego, CA, USA). The raw sequences were deposited in the NCBI Sequence Read Archive (SRA) database (Accession ID PRJNA999929). The forward and reverse raw sequences were merged using FLASH (Mago and Salzberg, 2011) by overlapping paired-end reads with a required overlap length of >10 base pairs (bp) and quality controlled using Trimmomatic software (Bolger et al., 2014). Low-quality sequences (average quality score < 20) containing ambiguous bases, sequences without valid primer or barcode sequences, and sequences with a read length < 50 bp were excluded. The permitted maximum error ratio of the overlapping

sequences was 0.2, which was used as the basis for screening overlapping sequences.

Non-repeating sequences were then extracted, and individual sequences that did not repeat were removed using Usearch 7.0 (Edgar, 2013). The sequences were subsequently clustered into operational taxonomic units (OTUs) with a 97% similarity cut-off using the QIIME software (Caporaso et al., 2010). After sequences were clustered, the taxonomy of each OTU was classified from the domain level to the OTU level using the RDP Classifier algorithm against the MaarjAM database (Maarjam 081) (ÖPik et al., 2010), with a default confidence threshold of 0.7.

## 2.6 Real-time quantitative reverse transcription PCR (qRT-PCR) analysis

The PCR products were purified and then ligated into a pMD18 vector using a pMD™ 18-T Vector Cloning Kit (TaKaRa Bio Inc.). Plasmid extraction and purification were performed using a MiniBEST Plasmid Purification Kit Ver. 4.0 (TaKaRa Bio Inc.). The concentration and purity of the plasmid were determined using a NanoDrop 2000 microspectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After determining the plasmid copy number, the preparation was serially diluted to prepare $10^1$ to $10^8$ copies. A standard curve ($R^2 = 0.99$), plotting the logarithm of the initial amount of template DNA as the abscissa and the Ct value of each diluted sample during the PCR reaction as the ordinate, was used to establish an amplification efficiency of 90–100%. The primers used for quantitative PCR (qPCR) amplification of the nitrogen-fixing bacteria were PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA), while AM fungi were amplified using the AMV4.5NF and AMDGR primers. qPCR amplifications were performed using an ABI 7900 (USA) fluorescence quantitative PCR thermocycler using 15-μL reaction systems containing 7.5 μL × 2 SYBR Premix Ex Taq II, 0.3 μL × 50 ROX Reference Dye, 0.6 μL 10 μmol·$L^{-1}$ pre-primer, 0.6 μL 10 μmol·$L^{-1}$ post-primer, 2 μL DNA template, and 4.0 μL double-distilled water ($ddH_2O$). The amplification program comprised an initial pre-denaturation at 95°C for 30 s, followed by 40 cycles of chain cleavage at 95°C for 5 s, annealing at 62°C for 30 s, extension at 72°C for 60 s, and signal acquisition at 83°C for 10 s. All samples were analyzed in triplicate.

## 2.7 Statistical analyses

Data analysis was performed using IBM SPSS 19.0 (IBM, Armonk, NY, USA). The plant and soil parameters followed a normal distribution, and Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare differences among plant parameters ($p < 0.05$). Interaction between nitrogen application and bacterial addition was detected using a two-way ANOVA. Redundancy analysis (RDA) was conducted to examine the relationships between the relative abundance of fungal and AMF taxa and the chemical properties of soil samples, using Canoco 4.5.1 (Microcomputer Power, Ithaca, NY, USA). The non-parametric factorial Kruskal–Wallis sum-rank test of the LEfSe tool was used to identify and detect the characteristics of groups exhibiting significant differences in abundance. LEfSe utilizes linear discriminant analysis (LDA) to estimate the effect of the abundance of each component (species) on the differences.

# 3 Results

## 3.1 Effects of nitrogen fertilizer and exogenous HNFB on soil chemical properties

Among the different nitrogen application treatments, the MBP and MBC were significantly higher in the BIO + N1 treatment group than in the BIO + N0 group (by 28.08 and 6.25%, respectively) and the BIO + N2 group (by 36.85 and 82.68%, respectively) ($p < 0.05$). The MBN content in the BIO + N1 treatment group was significantly higher than in the BIO + N2 treatment group (by 51.23%) ($p < 0.05$). The EC and pH values of the BIO + N0 and BIO + N2 treatment groups were significantly higher than those of the BIO + N1 group ($p < 0.05$). For the BIO treatment, as the concentration of applied nitrogen increased, MBN and MBP first increased and then decreased. Moreover, the MBC/MBN ratio decreased gradually, with values of 8.24, 7.27, and 6.02 for the three nitrogen application levels, respectively, (Supplementary Figure S1).

The MBC of the CK + N2 group was significantly lower than that of the CK + N0 group (by 39.07%) and the CK + N1 group (by 38.97%) ($p < 0.05$). The MBN of the CK + N1 group was significantly higher than that of the CK + N0 group (by 24.61%) and the CK + N2 group (by 112.68%) ($p < 0.05$). The EC of the CK + N2 group was significantly higher than that of the CK + N0 group (by 3.13%) and the CK + N1 group (by 9.65%) ($p < 0.05$). In the CK treatment groups, MBN first increased and then decreased with increasing nitrogen application levels. The MBC/MBN ratio exhibited a trend of initially decreasing and then increasing, with values of 8.46, 6.78, and 8.81 for the three nitrogen application levels, respectively. Nitrogen application did not result in significant differences in MBP or pH in the CK treatment groups (Supplementary Figures S1C,E). Under the same nitrogen level, the MBP, MBC, and MBN levels in the BIO group were significantly higher than those in the CK group ($p < 0.05$). Additionally, the EC level in the CK group was significantly higher than that in the BIO group ($p < 0.05$). There was no significant difference in the pH levels between the BIO and CK groups. The soil N content of the BIO + N0 group was significantly higher than that of the CK + N0 group ($p < 0.05$) (Supplementary Figure S1).

Regardless of HNFB inoculation, excessive nitrogen application had a negative impact on MBN and MBC in the rhizosphere soil, further exacerbating salt stress. N1 application significantly increased the MBC and MBP content in the rhizosphere soil, while reducing the degree of salt stress. Inoculation with HNFB also significantly increased MBN, MBC, and MBP levels in the rhizosphere soil under both N0 and N1 conditions, leading to a reduction in salt stress. Under excessive nitrogen application, HNFB significantly increased MBC and MBP in the rhizosphere soil. Under low-nitrogen conditions, HNFB significantly increased the total nitrogen content in the rhizosphere soil. The results of the two-way ANOVA further confirmed that the nitrogen application level and bacterial treatment had significant effects on the physical and chemical properties of the soil and showed interaction effects on the MBP, MBC, and EC (Table 1).

## 3.2 Effects of nitrogen fertilizer and exogenous HNFB application on AMF community composition in the apple rhizosphere

In total, 365,930 valid AMF sequences (average length, 215 bp) were obtained, accounting for 98.86% of the original sequences and covering most of the AMF community in the rhizosphere soil (Supplementary Figure S2). Based on a 97% similarity analysis, 60 OTUs were classified from the effective AMF sequences, including one phylum, three families, three genera, and 20 species. There were 23 common OTUs among the six treatments, 35 common OTUs in the BIO groups, and 23 common OTUs in the CK groups. The results showed that HNFB increased 12 OTUs in the apple rhizosphere (Figure 1). Therefore, excessive nitrogen application was not instrumental in increasing AMF species in the rhizosphere soil and HNFB inoculation enriched the AMF species in the rhizosphere soil under N0 and N1 levels.

Inoculation with exogenous HNFB increased the rhizosphere AMF community richness (Simpson, ACE and Chao index, Table 2) under N0 conditions and maintained rhizosphere AMF community richness under N1 and N2 conditions. After inoculation with exogenous HNFB, excessive nitrogen application significantly reduced AMF richness and diversity compared to those at low or normal nitrogen levels (Table 2). Two-way analysis further confirmed that nitrogen fertilizer and HNFB application significantly affected the AMF richness of the rhizosphere soil, and there was an interaction with the AMF richness. However, nitrogen fertilizer and HNFB had no significant effect on or interaction with the AMF diversity (Supplementary Table S1). The results of qRT-PCR revealed a gradual decline in the abundance of nitrogen-fixing bacteria in soil with an increase in nitrogen application rate. In addition, the abundance of

TABLE 1 Interaction analysis of nitrogen application levels and HNFB treatment.

| Factor | MBC | | MBN | | EC | | MBP | | pH | | Soil N | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* | *F* | *p* |
| BIO | 1218.518 | 0 | 32.215 | 0 | 420.963 | 0 | 149.756 | 0 | 0.011 | 0.918 | 4.379 | 0.058 |
| N | 952.109 | 0 | 15.569 | 0 | 70.994 | 0 | 22.995 | 0 | 10.626 | 0.002 | 8578.062 | 0 |
| BIO*N | 62.264 | 0 | 0.131 | 0.879 | 5.299 | 0.022 | 18.729 | 0 | 1 | 0.397 | 0.061 | 0.941 |

*F* and *p* values represent the results of the interaction analysis between nitrogen application levels and bacterial treatment (two-way ANOVA). HNFB, halotolerant nitrogen-fixing bacteria; MBN, microbial biomass nitrogen; MBC, microbial biomass carbon; MBP, microbial biomass phosphorus; EC, electrical conductivity; N, nitrogen; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3).

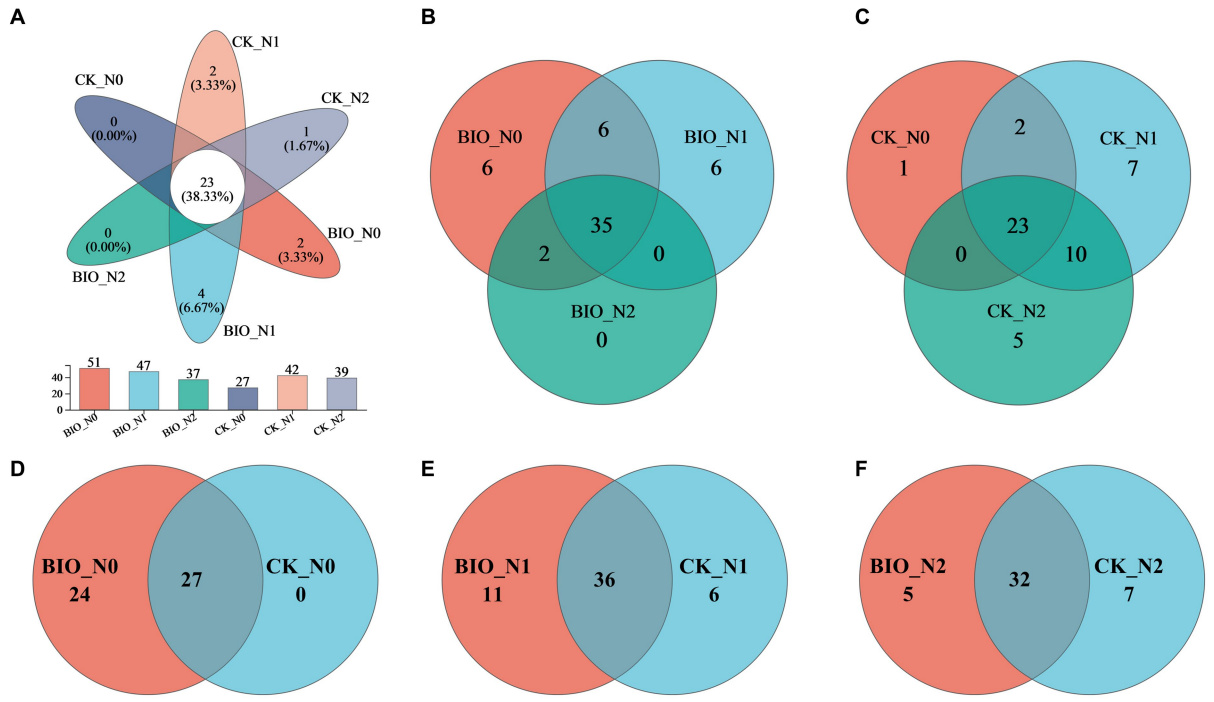


FIGURE 1
Venn diagrams depicting the AMF community structure in the different treatment samples at the OTU level. OTU, operational taxonomic unit. AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

AMF was significantly lower at N0 and N2 than at N1 ($p < 0.05$), and under the same level of nitrogen application, the abundances of nitrogen-fixing bacteria and AMF in the inoculated HNFB group were significantly higher than those in the CK group ($p < 0.05$) (Supplementary Figure S3).

The top 10 most abundant AMF are shown in Figure 2. We found that the main AMF genus was *Glomus*. In detail, *Glomus-Glo7-VTX00214*, *Glomus*-sp.-*VTX00304*, *Glomus-viscosum-VTX00063*, and *Glomus*-sp.*VTX00301* were the dominant species in each treatment group (Figure 2; Supplementary Figure S4). Among the CK groups, the relative abundance of *s__Glomus-Wirsel-OTU16-VTX00156* in the N0 group was 15.48%, which was significantly higher than that in the N1 (2.68%) and N2 (0.81%) groups ($p < 0.001$) (Figure 3A; Supplementary Figures S4B,D,F). Among the BIO groups, the relative abundance of *Glomus-MO-G17-VTX00114* was 4.56% in the N1 treatment group, which was significantly higher than that in the N2 (3.72%) and N0 (1.41%) groups ($p < 0.05$) (Figure 3B;

TABLE 2 Diversity index of AMF in the apple rhizosphere soil samples from different treatment groups.

| Sample | Sobs | Chao | Ace | Shannon | Simpson |
|---|---|---|---|---|---|
| BION0 | 38.00 ± 13.00 | 41.83 ± 7.65 | 51.82 ± 7.36 | 2.37 ± 0.36 | 0.15 ± 0.04 |
| BION1 | 36.00 ± 3.00 | 36.61 ± 3.76 | 38.27 ± 5.09 | 2.21 ± 0.12 | 0.17 ± 0.02 |
| BION2 | 30.33 ± 1.53 | 31.78 ± 1.58 | 31.95 ± 1.48 | 1.96 ± 0.04 | 0.23 ± 0.02 |
| CKN0 | 24.00 ± 0.00 | 24.61 ± 0.35 | 28.5 ± 5.09 | 1.98 ± 0.02 | 0.19 ± 0.00 |
| CKN1 | 34.00 ± 4.58 | 38.50 ± 9.76 | 42.45 ± 16.86 | 2.12 ± 0.12 | 0.17 ± 0.02 |
| CKN2 | 27.33 ± 2.89 | 28.33 ± 4.04 | 28.52 ± 4.14 | 2.00 ± 0.18 | 0.18 ± 0.05 |
| N treatment | 0.277 | 0.105 | 0.091 | 0.141 | 0.078 |
| Microbial treatment | 0.042 | 0.035 | 0.077 | 0.120 | 0.940 |

N treatment indicate significant differences between the nitrogen application level groups for the same treatment (one-way ANOVA, $p < 0.05$). Microbial treatment indicate significant differences between the results of the different treatments for the same nitrogen application level (two-sided *t*-test). AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

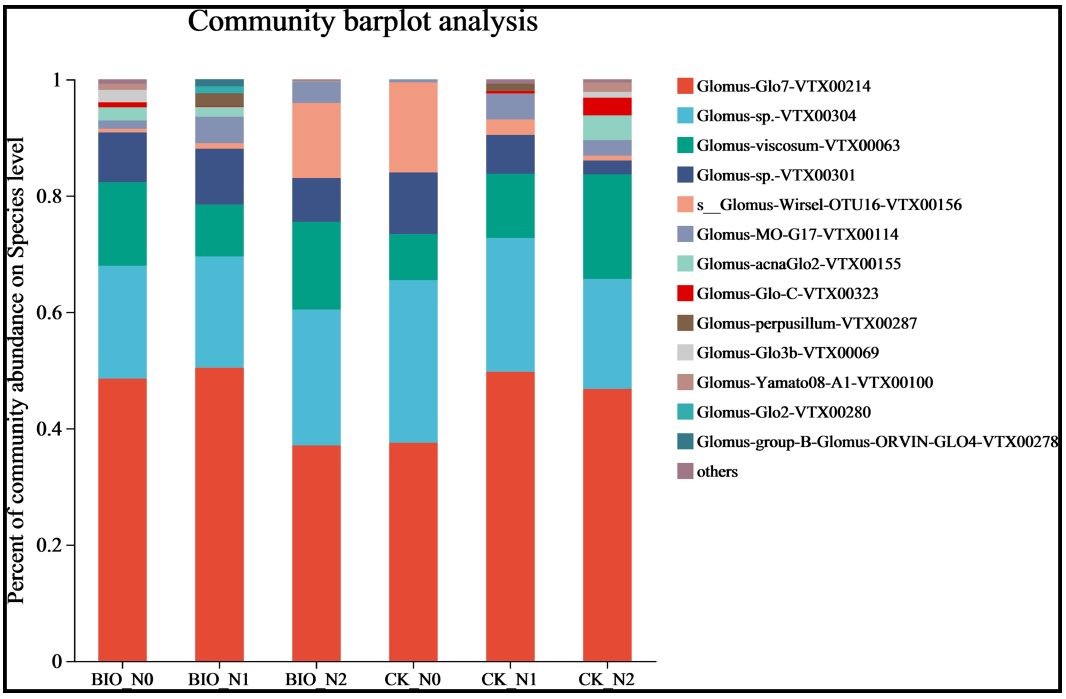


FIGURE 2
The relative abundance of AMF in the apple rhizosphere soil across the different treatment groups at the species level. AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

Supplementary Figures S4A,C,E). The relative abundance of *s__Glomus-Wirsel-OTU16-VTX00156* in the BIO group increased with increasing levels of applied nitrogen. Furthermore, the relative abundance of this fungus in the BIO + N0 group was significantly lower than that in the CK groups (Figure 3C) ($p < 0.001$). For the N0 and N2 treatment groups, the relative abundance of *Glomus-MO-G17-VTX00114* in the BIO group was significantly higher than that in the CK group (Figures 3C,D) ($p < 0.05$). The relative abundance of *Glomus-MO-G17-VTX00114* decreased after excess nitrogen application with or without exogenous HNFB inoculation (Figures 3A,B) ($p < 0.05$).

## 3.3 Effects of nitrogen fertilizer and exogenous HNFB application on AMF community function in the apple rhizosphere

The PICRUSt2 software was used to predict the function of the microbial community detected in the samples based on the amplicon sequencing results, and the enzyme types produced by the flora in the different treatment groups and their relative levels were determined (Supplementary Table S2). The relative abundances of beta-glucosidase and acid phosphatase in the BIO + N1 group were significantly higher

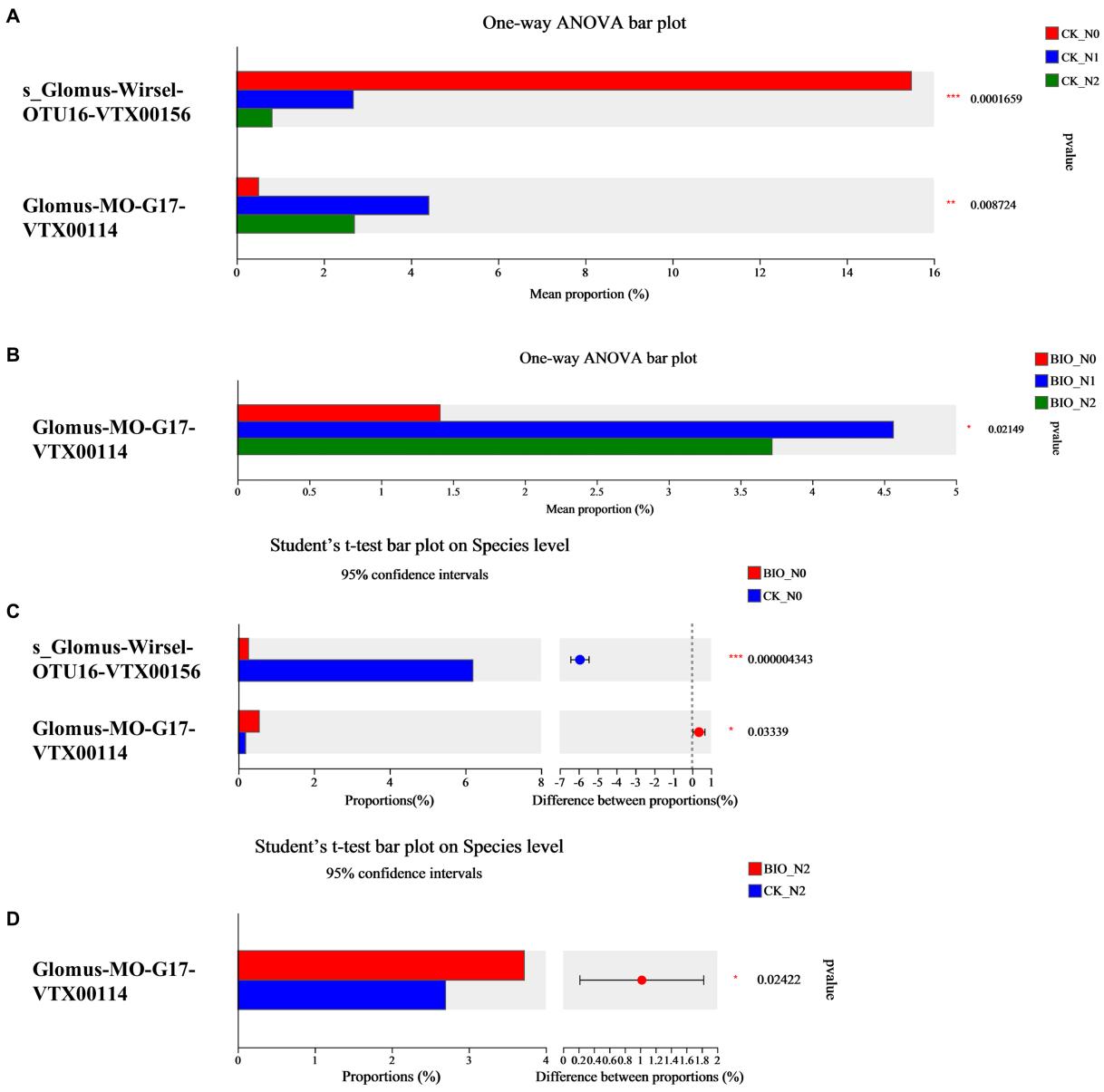


FIGURE 3
The significant differences in the relative abundance of AMF in the apple rhizosphere soil from different treatment groups at the species level. AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

than those in the BIO + N0 and BIO + N2 groups (Figure 4A) ($p < 0.05$). The relative abundance of beta-glucosidase in the CK + N1 group was significantly lower than that in the CK N0 and N2 groups (Figure 4A) ($p < 0.05$). Acid phosphatase levels in the BIO group first increased and then decreased with increasing nitrogen application levels, and the BIO + N1 group had significantly higher levels than those in the BIO + N2 and BIO + N0 groups ($p < 0.05$). Nitrogen application in the CK group had no significant effect on the relative abundance of acid phosphatases (Figure 4B).

In contrast, in the BIO group, the relative abundances of alkaline phosphatase and urease increased with increasing nitrogen levels. The N2 group had significantly higher levels compared to the N0 and N1 groups (Figure 4C) ($p < 0.05$). There was no significant difference in the relative abundances of alkaline phosphatase and urease between the CK + N1 and CK + N2 groups. However, they were significantly higher than those in the CK + N0 group (Figures 4C,D) ($p < 0.05$). Urease levels in the BIO + N0 group were significantly higher compared to the BIO + N1 and N2 groups ($p < 0.05$), whereas urease levels in the CK + N0 group were significantly lower compared to the CK + N1 and N2 groups (Figure 4D) ($p < 0.05$). In conclusion, excessive nitrogen application reduced the relative abundance of beta-glucosidase, acid phosphatase, and urease, regardless of whether HNFB was inoculated. However, when HNFB was inoculated, the relative abundance of alkaline phosphatase significantly increased with increasing nitrogen application levels.

Under the same nitrogen application level, the relative abundance of beta-glucosidase in the BIO group was significantly higher than that in the CK group ($p < 0.05$) (Figure 4A). The relative abundance of acid phosphatase in the BIO + N1 group was significantly higher than that in the CK + N1 group ($p < 0.05$); however, there was no significant difference due to HNFB inoculation at the N0 and N2 levels (Figure 4B). The relative abundances of alkaline phosphatase and urease in the CK + N0 and N2 groups were significantly higher than those in the BIO + N0 and N2 groups ($p < 0.05$), and there were no significant differences between the CK + N1 and BIO + N1 groups (Figures 4C,D).

Therefore, inoculation with exogenous HNFB significantly increased the relative abundance of beta-glucosidase at the N0 level but significantly reduced the abundance of alkaline phosphatase and urease. The relative abundances of beta-glucosidase and acid phosphatase significantly increased at the N1 level, and the relative

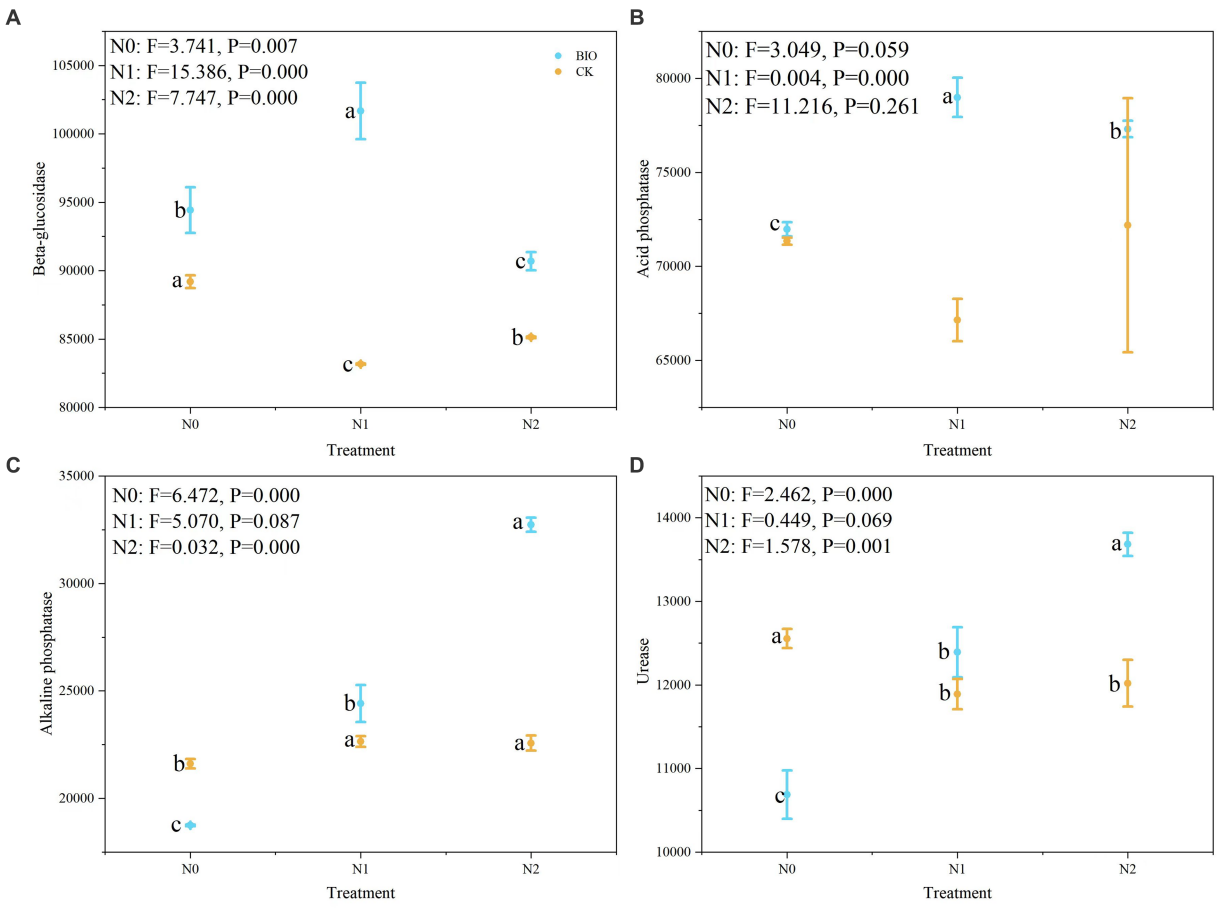


FIGURE 4
Relative levels of enzymes produced by the microbial community in the different treatment groups. The relative levels of **(A)** beta-glucosidase, **(B)** acid phosphatase, **(C)** alkaline phosphatase, and **(D)** urease in the rhizosphere after different treatments were detected using PICRUSt2 software. AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

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abundances of beta-glucosidase, alkaline phosphatase, and urease significantly increased at the N2 level.

## 3.4 Correlation analysis of environmental factors

We conducted an analysis of the variance inflation factor (VIF). The VIF values of the environmental factors MBP (VIF = 4.59), MBN (VIF = 5.90), pH (VIF = 1.48), EC (VIF = 7.70), and soil N (VIF = 1.53) were less than 10 and could be used for RDA. However, the VIF of MBC was 19.63, which was greater than 10, indicating collinearity with other environmental factors. Therefore, MBC was removed from the RDA to ensure an accurate assessment of the effect of soil physical and chemical factors on structural latitude biodiversity.

Significant differences in microbial structure were observed after different nitrogen treatments and the addition of HNFB. By combining environmental factor analysis, we found that EC and pH were positively correlated with *Glomus-viscosum-VTX00063* and *s__Glomus-Wirsel-OTU16-VTX00156*. Soil N and MBP were positively correlated with *Glomus-MO-G17-VTX00114*, while MBN was positively correlated with *Glomus*-sp.*VTX00304* cells (Figure 5A). The results of the correlation analysis of the environmental factors were consistent with those of the RDA. The clustering relationship between EC and pH was similar, with EC being significantly negatively correlated with *Glomus-MO-G17-VTX00114* ($p < 0.05$). The pH showed a significant negative correlation with *Glomus-perpusillum-VTX00287* ($p < 0.01$), *Glomus-Glo2-VTX00280* ($p < 0.05$), and *Glomus-group-B-Glomus-ORVIN-GLO4-VTX00278* ($p < 0.05$) levels. Soil N was significantly positively correlated with *Glomus-MO-G17-VTX00114* ($p < 0.01$) (Figure 5B).

PERMANOVA was used to interpret the correlation between different environmental factors and the AMF community structure in various samples. A permutation test was employed to determine the statistical significance of the division. The results revealed that EC, soil N, and MBP significantly influenced the samples ($R^2 = 0.677$, $p = 0.001$) (Figure 6). Both the BIO + N0 and BIO + N2 groups, as well as the BIO + N1 and BIO + N2 groups, were significantly impacted by MBP ($p < 0.05$) and MBC ($p < 0.05$). Similarly, the CK + N0 and CK + N2 groups were significantly affected by EC ($p < 0.05$) and MBN ($p < 0.05$). Similarly, the BIO + N1 and CK + N1 groups were significantly affected by EC ($p < 0.05$) and MBN ($p < 0.05$). Additionally, the BIO + N2 and CK + N2 groups were significantly affected by EC ($p < 0.05$), MBN ($p < 0.05$), and MBC ($p < 0.05$). The pH did not have an overall significant effect, nor did it differ significantly between groups. Therefore, nitrogen treatments primarily influenced the AMF community structure in the rhizosphere soil by regulating the content of MBP and MBC, following inoculation with exogenous HNFB. In the absence of inoculation with exogenous HNFB, nitrogen primarily affected the AMF community structure by regulating the EC and MBN content in the rhizosphere soil. Under the N1 and N2 treatments, significant differences in the AMF community structure between the BIO and CK groups were mainly attributed to differences in EC and MBN.

This study examined the collinear model of AMF related to nitrogen fertilizer and HNFB application by constructing two molecular ecological networks. In the AMF co-occurrence network of the CK and BIO samples, *Glomus-Glo7-VTX00214* played a vital role (Figures 7A,B). Consistent with the results of the flora composition depicted in Figure 2, *Glomus-Glo7-VTX00214* had the highest relative abundance in each treatment, and its relative abundance in the BIO group was greater than that in the CK group under the N0 and N1

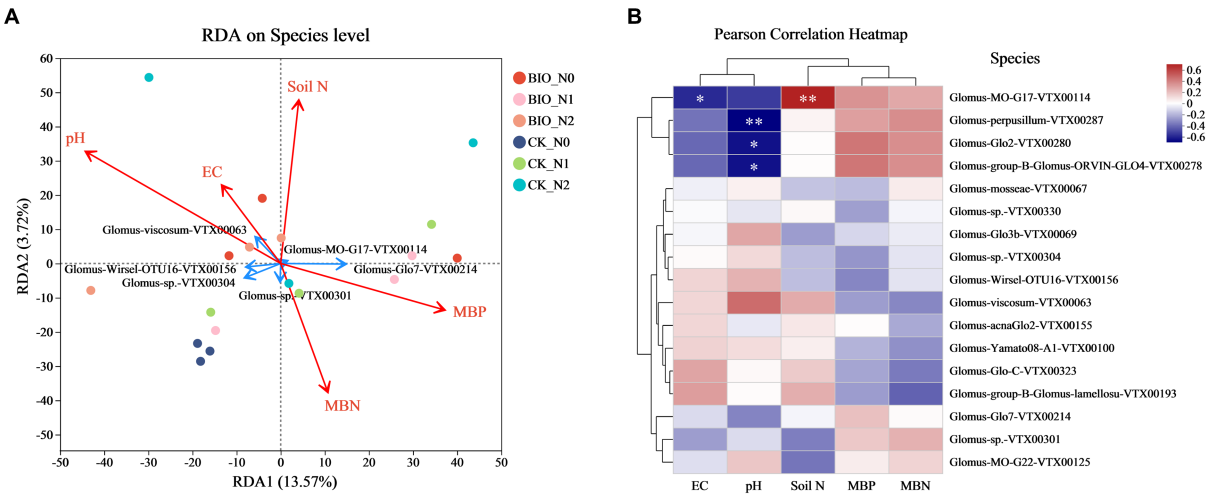


FIGURE 5
**(A)** Redundancy analysis of the AMF community based on Bray-Curtis distances. Different colored points represent samples from the different treatments. The closer the points of the two samples are, the more similar their species compositions. The red arrows indicate environmental factors, and the blue arrows indicate the dominant species. **(B)** Correlation between different environmental factors and AMF species. The X-axis and Y-axis represent environmental factors and species, respectively, and the right legend shows the color interval of the different R values. The left and upper sides represent the clustering trees for the species and environmental factors, respectively. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. This analysis is based on a weighted UniFrac matrix. AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

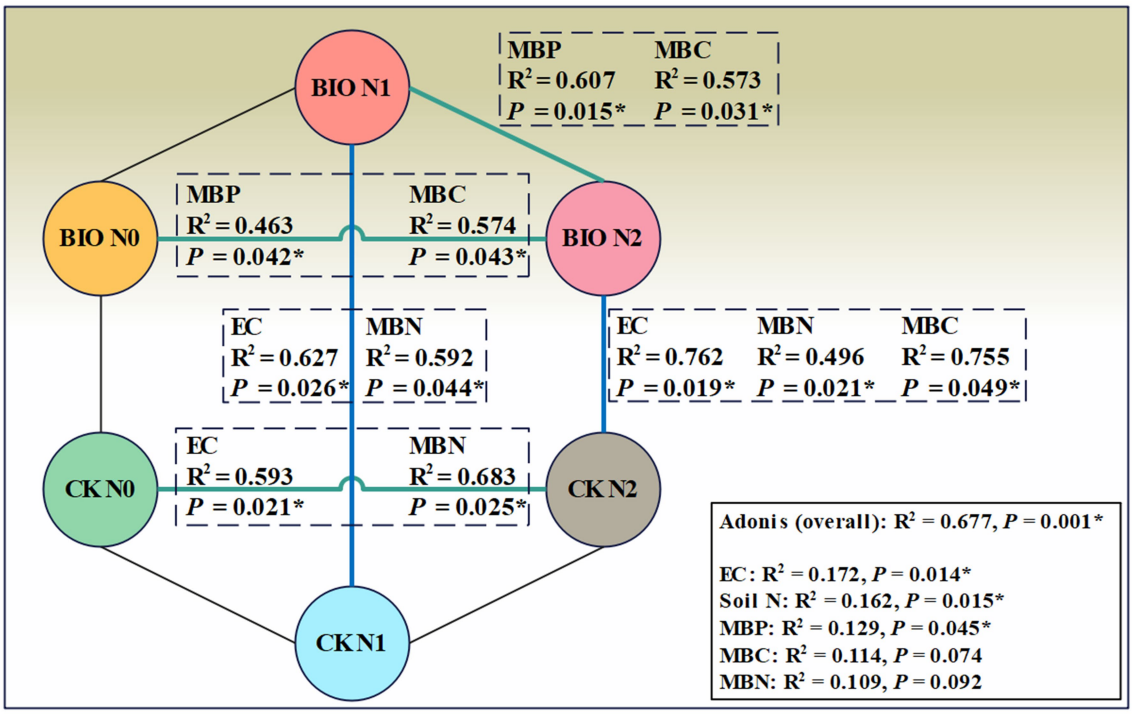


FIGURE 6
PERMANOVA analysis of the AMF community in the six treatment groups. AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

treatments. Furthermore, the results from the random forest analysis further demonstrated that *Glomus-Glo7-VTX00214* was the most crucial predictor of the AMF structure in the apple rhizosphere soil (Figure 7C).

# 4 Discussion

## 4.1 Differences between the effects of nitrogen application levels and that of exogenous HNFB inoculum on soil physical and chemical properties

In our results, nitrogen application led to significant changes in soil MBC and MBN content. Most notably, excessive N application reduced MBC and MBN regardless of whether HNFB was inoculated (Supplementary Figures S1A,B). The main reason underling this change may be that excessive nitrogen can increase nitrogen uptake by crops, but it also alters soil nitrogen effectiveness (Cairney, 2011), affecting nitrogen absorption and reabsorption by plants (Ostonen et al., 2011) and the exchange of metabolites between roots and microorganisms. The competition between plants and microorganisms for available nutrients, such as MBC and MBN, under salt stress was intensified, thereby reducing the available MBC and MBN (Ganeshamurthy and Reddy, 2015). Furthermore, exogenous HNFB significantly increased microbial biomass in rhizosphere soil under both low and normal nitrogen conditions and reduced salt stress. We speculate the main reasons for the above finding is that inoculation with PGPR can increase the number and diversity of rhizosphere microorganisms, improve microbial nitrogen fixation, promote metabolite exchange, and enhance ion-transport efficiency between roots and rhizosphere microorganisms, thereby increasing nutrient-use efficiency (Zhang et al., 2022).

We found that after HNFB inoculation, although all soil MBC/MBN ratios were > 6, these ratios decreased with increasing nitrogen application levels (Supplementary Figure S1). On one hand, this indicates that fungi still play a beneficial role in the rhizosphere soil, ensuring relatively stable soil carbon sequestration capacity (Chowdhury et al., 2011; Morrissey et al., 2013). On the other hand, excessive nitrogen application further reduces the MBC/MBN ratio and leads to a shift in the apple rhizosphere soil flora from fungi to bacteria. However, in the control group without HNFB inoculation, the soil MBC/MBN ratio increased with increasing nitrogen application levels (Supplementary Figure S1). Therefore, this finding suggests that an improved HNFB composition might increase the presence of synergistic fungal species and mitigate the adverse effects of the soil microbial biomass on the C/N imbalance caused by a purely bacterial flora.

Consistent with previously reported findings (Silva et al., 2013; Dynarski and Houlton, 2017), in our results, we observed a decrease in the abundance of nitrogen-fixing bacteria in response to increased nitrogen application rates (Supplementary Figure S3A). The main reason underlying this observation may be that nitrogen levels are negatively correlated with the number of nitrogen-fixing bacteria and nitrogen-fixing activity. Lower levels of nitrogen application have been shown to be more conducive to the nitrogen-fixing activity of nitrogen-fixing microorganisms (Sarathchandra et al., 1988; Bailey et al., 2002; Bagyaraj, 2010). These findings indicate that the nitrogen

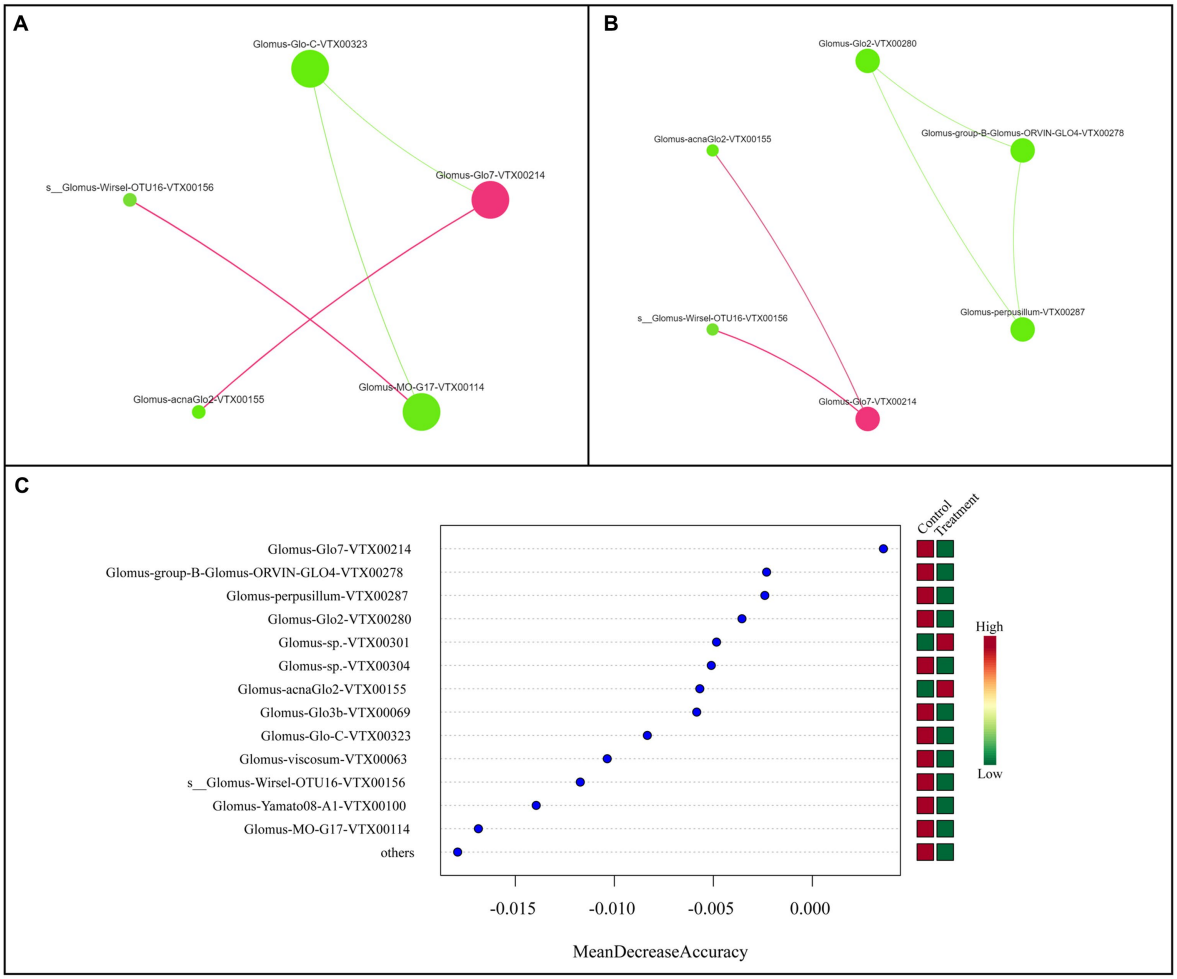


FIGURE 7
Colinear network analysis and random forest model showing the relationship between key AMF species. Collinear network analysis of the **(A)** CK and **(B)** BIO groups. **(C)** Random forest model, wherein the ordinate is the species and the abscissa is the measured value of species importance. AMF, ascomycetous fungi; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

application rate can be controlled within a reasonable range to ensure a high amount and activity of microbial nitrogen fixation in saline-alkali soil.

Phosphorus is a limiting factor that affects the activity of nitrogen-fixing microorganisms. However, both low and excessive nitrogen application could not assist microbial agents in increasing MBP, which significantly increased ($p < 0.05$) under normal nitrogen application levels (Supplementary Figure S1C). Under the same nitrogen application conditions, MBP in the CK group was significantly lower than that in the BIO group, which further confirms that HNFB plays a beneficial role in improving soil microbial vigor and nutrient content when applied to saline soil (Primieri et al., 2021; Liu et al., 2022). The interaction between nitrogen application and exogenous HNFB on MBP further confirms the critical role of the balance between nitrogen and phosphorus in maintaining soil microbial activity (Table 1). qRT-PCR results revealed that the abundance of arbuscular fungi was significantly higher under N1 conditions than under N0 and N2 conditions and was higher under N2 conditions than under N0 (Supplementary Figure S3B). The same trend was observed for MBP treated with HFNB inoculation. Although it is unclear how native AMF communities respond to diminishing soil phosphorus effectiveness, AMF can obtain phosphorus via exoenzymes (Meeds et al., 2021). Therefore, the effects of nitrogen application and exogenous HNFB on AMF community structure are also important factors influencing phosphorus accumulation in the microbial biomass.

Generally, when there is a high input of nitrogen, it can enhance soil nitrification, which leads to an increase in the release of $H^+$ during nitrogen transformation and soil acidification (Wang et al., 2023). However, our results showed that the rhizosphere soil pH in the BIO + N1 group was significantly lower than that of the BIO + N0 and BIO + N2 groups. On the other hand, there were no significant differences among the other groups and treatments (Supplementary Figure S1E). Therefore, applying excessive amounts of urea in saline soils may not promote nitrification in the soil, and it is difficult to significantly affect soil pH by simply adjusting the amount of nitrogen applied. In contrast, when HNFB was inoculated

under normal nitrogen application, it reduced the soil pH. This suggests that normal nitrogen application helps maintain a higher HNFB-plant biochemical response.

## 4.2 Differences in the effects of nitrogen application and that of HNFB inoculum on the AMF community

In nitrogen-deficient ecosystems, plants increase their nitrogen use efficiency (NUE) to meet growth needs (Li et al., 2016). However, excessive nitrogen application inhibits the symbiotic relationship between plants and microorganisms (Laws and Graves, 2005), including AMF (Panneerselvam et al., 2023). The effects of nitrogen treatment and HNFB on AMF community composition and abundance during short-term fertilization can help explain changes in soil physicochemical properties and enzyme activities. Low nitrogen treatment, due to the lack of carbon return from the host, reduces fungal colonization in plants, particularly AMF (Irving et al., 2022). This may be why N1 had the highest AMF abundance and N0 had the lowest abundance in the CK group (Table 2). In contrast, the AMF community richness and diversity in the BIO group were higher than those in the CK group at the same nitrogen application level, suggesting a more sustainable low-input agricultural cropping system, consistent with a previous study (Primieri et al., 2021).

In the BIO group, there was no significant difference in AMF diversity between the treatment without nitrogen application and the N1 treatment. However, the diversity in the N0 group was significantly higher than that in the N2 group (Table 2). The negative effect of excess nitrogen on the rhizosphere AMF community in the apple rhizosphere aligns with the findings of previous studies (Zeng et al., 2021). Hence, there is compelling evidence to suggest that long-term and/or increased application of exogenous HNFB under salt stress conditions can effectively improve crop rhizosphere soil microbial ecology, positively influencing AMF community structure and diversity. The bacterial members of exogenous HNFB are indispensable for maintaining functional stability.

Similarly, nitrogen and exogenous HNFB caused changes in the AMF community composition (Figures 2, 5). The dominant relative abundance of *s__Glomus-Wirsel-OTU16-VTX00156* in the CK + N0 group decreased significantly with increased nitrogen content and HNFB inoculation (Figure 2; Supplementary Figure S4). This suggests that this strain is better adapted to low-nitrogen conditions. It is likely that exogenous HNFB and this strain have a mutually inhibitory relationship, and the combination of HNFB and this strain may reduce the effect of bacterial action.

## 4.3 Effects of nitrogen application and exogenous HNFB inoculum on AMF community function

Previous studies have found that a sufficient nitrogen source is conducive to the synthesis of phosphatases (Braun et al., 2010), particularly alkaline phosphatases (Chen et al., 2023b). Our results demonstrate that the activities of beta-glucosidase, alkaline phosphatase, acid phosphatase, and urease were significantly affected by the nitrogen application level and exogenous HNFB (Figure 6). Nitrogen fertilizer can increase soil β-glucosidase and alkaline phosphatase activities while reducing urease activity. This contradicts the results of the β-glucosidase and urease assessments conducted in this study (Figure 4). One possible reason is that the number, diversity, and richness of microorganisms in salt-stressed soil are lower compared to those in regularly cultivated land (Chen et al., 2021). The microbial community structure in the rhizosphere soil is influenced by crop root metabolites and differs significantly from that in the topsoil (Wang et al., 2021). Under the N1 treatment, nitrogen-fixing bacteria expedite the conversion of soil substances, enhance metabolism in plant roots, promote shedding, and increase soil organic matter content. As a result, enzymatic reaction substrates and enzyme activity are elevated (George et al., 2006). An increase in alkaline phosphatase levels may be associated with an increase in MBC content as nitrogen application levels rise. MBC is a major predictor of the abundance of microorganisms carrying phoD and is positively correlated with alkaline phosphatase activity (Luo et al., 2019). The decrease in urease activity can be attributed to the inhibition of released urea ($NH_4^+$) hydrolysis products, as urease is involved in the nitrogen release process and $NH_4^+$ is considered an end product of urease. Microbial communities determine the relative abundance of enzyme-encoding genes and influence their expression. Therefore, significant differences in soil enzyme activity may be related to differences in microbial biomass and composition.

Exogenous HNFB, nitrogen application, indigenous flora, and plants are closely linked, and focusing solely on HNFB or nitrogen application may not provide a deeper understanding of the process of soil phosphorus transformation under real-world production conditions (Smith and Read, 2008). When soil phosphorus effectiveness is low, AMF can accelerate soil organic phosphorus mineralization and inorganic phosphorus activation by secreting phosphatases and organic acids (Fujii et al., 2012; Fan et al., 2019). For example, nitrogen application enhances acid phosphatase activity, increases the participation in ester-phosphate bond hydrolysis in soil organic phosphorus, and releases orthophosphate for plant uptake. Moreover, organic acids can release orthophosphate from the medium and other readily decomposable forms of soil inorganic phosphorus by chelating iron and aluminum (Lin et al., 2020). However, once AMF infects plant roots, if there is a change in the AMF community structure, the polyphosphate absorbed by the hyphae outside the roots is degraded into orthophosphate (Solaiman et al., 1999). This degradation promotes soil phosphorus transformation and plant phosphorus absorption.

## 4.4 The positive response of key AMF species to exogenous HNFB

The triple symbiotic system, consisting of nitrogen-fixing bacteria, AMF, and crops, synergistically promotes crop biomass and nitrogen fixation. This growth promotion effect is significantly superior to that of rhizobia, AMF, and crop symbionts (Primieri et al., 2021). Previous studies have shown that AMF contributes more to host growth under stressful conditions compared to normal conditions (Feng et al., 2020). Our results also confirm that AMF can establish ineffective interactions with exogenous HNFB and highlight the presence of core

strains and auxiliary functions that have important synergistic effects. This synergy resembles that reported for other crops such as *Glycine max* (Wang et al., 2011), *Sibiraea angustata* (He et al., 2021), and *Amorpha canescens* (Larimer et al., 2016), and it may play a crucial role in sustainable low-input agricultural planting systems (Artursson et al., 2006). However, these effects depend on the specific combination of species involved in the interaction, specifically HNFB and/or AMF. A meta-analysis has even reported conflicting results, contradicting the hypothesis that AMF and nitrogen-fixing bacteria always exhibit synergistic effects in different plants (Larimer et al., 2010). This suggests that the occurrence of any synergistic effect may depend on the properties of the microsymbionts involved, as well as the spatial and temporal scales, the host, and/or the environmental conditions. The findings of our study provide a theoretical reference for the interaction between HNFB and AMF, with an emphasis on the dominance of *Bacillus*.

AMF can form a complex mycelial network with plants, affecting plant growth and stress resistance. *Glomus* is the dominant genus of the AMF of many soil types, and it was the only one genus was identified in this study. Of course, this result is not unique. For example, more than 740,000 valid sequences were obtained from 77 citrus root samples, 99% of which were of *Glomus*; furthermore, *Glomus-MO-G17-VTX00114* was confirmed to be a key species to ensure the stability of the AMF community in the rhizosphere and improve crop stress resistance (Song et al., 2015). Accordingly, our study further confirmed that the relative abundance of *Glomus-MO-G17-VTX00114* would be reduced by excessive nitrogen application (Figures 3A,B) and could be increased by HFNB application (Figures 3C,D). Moreover, *Glomus-Glo7-VTX00214* showed potential as an auxiliary HNFB strain in the apple rhizosphere AMF. Therefore, these results suggest an effective way to improve the relative abundance of key AMF species in the apple rhizosphere. Our proposed explanations are: (1) salt stress exerts a filtering effect on AMF (Rath et al., 2018), and *Glomus* has a significant advantage in terms of salinity tolerance or competitiveness; (2) plant variety is the most important factor affecting the composition of rhizosphere microorganisms (Edwards et al., 2023). Among AMF, *Glomus* may have an extremely close interaction with the root system of the examined apple plant, specifically selected for its variety. Simultaneously, several application studies have found that *Glomus* can be used to promote the growth of saline-alkali crops (Ouziad et al., 2006; Jahromi et al., 2008; Latef and He, 2014; Bharti et al., 2016; Amir and Samira, 2023) and indicate that the *Glomus* species has great potential for application in saline-alkali land agricultural production.

In this study, the relative abundance of *Glomus-MO-G17-VTX00114* for the N1 treatment in the BIO and CK groups was significantly higher than that in the N0 and N2 treatment groups. Generally, nitrogen fertilization is more beneficial to crops and the environment; this strain likely forms a synergistic relationship with the two nitrogen-fixing bacteria that were inoculated together to improve the rhizosphere AMF community structure and salt tolerance in apple trees in saline land (Figure 3). *Glomus-Glo7-VTX00214* was confirmed to play an important role in the AMF community composition and was the most important predictor (Figure 6). This strain can be used as an auxiliary strain to improve the structural and functional stability of the core flora. Here, nitrogen application was performed to increase the accuracy of the results. Moreover, the results of the flora composition and key species assessments were based on accurately identified and named dominant strains, ensuring that key strains could be isolated and cultured. Given the key role of AMF in plant productivity, nutrient cycling, and ecosystem responses to global change, a deeper understanding of the cross-scale coupling between plants and AMF diversity will reduce the uncertainty of ecosystem consequences when predicting species gains and losses (Fei et al., 2021). Therefore, the results of this study provide an important reference for the construction of a multitrophic synthetic HNFB with a more reasonable structure and greater diversity of species.

In general, our results showed that adding inorganic nitrogen alone weakened the function of AMF, whereas inoculation with HNFB stimulated its activity, thereby improving the salt and alkali resistance of plants. Excessive nitrogen application has a negative effect on the structure and function of the AMF community, which is worse than that under low nitrogen conditions. The core and auxiliary strains, determined through complex treatment and conditions in field experiments, are crucial references for the artificial synthesis of HNFB strains that have the ability to stably colonize saline-alkali land and exhibit a stronger synergistic effect. Thus, these results provide an overall "landscape" of the apple-AMF association in agricultural settings in major apple-production areas under different N application levels in China. They should serve as a guide for the future development of major apple-adapted AMF as potential bio-fertilizers.

## 5 Conclusion

This study revealed the uniqueness of the AMF community in the apple rhizosphere in saline-alkali soil and indicated that the AMF community was significantly affected by different N application levels and HNFB inoculation. This is evidenced by following findings. Inoculation with exogenous HNFB promoted soil nutrient accumulation and alleviated salt stress; simultaneously, exogenous HNFB increased the richness of rhizosphere AMF communities in low-nitrogen conditions and maintained their richness under both normal and high-nitrogen application conditions. However, excessive nitrogen application significantly reduced the richness and diversity of AMF after exogenous HNFB inoculation, highlighting the ecological risk associated with excessive nitrogen application in saline soil for the AMF community. Furthermore, in the apple rhizosphere AMF, the core strains *Glomus-MO-G17-VTX00114* and *Glomus-Glo7-VTX00214* exhibited potential as core and auxiliary HNFB strains, respectively. The new strain combination analyzed in this study is expected to be developed into a multitrophic HNFB with a more balanced structure and a greater variety of species. Considering the importance of AMF for crop growth in saline soil, it is essential to understand the response mechanisms of the apple and rhizosphere microorganisms to nitrogen application and exogenous HNFB for the sustainable management of apple agriculture in saline-alkali soil. Although this study did not accurately determine the appropriate amount of nitrogen fertilizer for apple tree growth in saline land, it provides data for exploring the relationship between exogenous HNFB-plant-AMF communities under different nitrogen application levels in complex habitats. This study could contribute to enhancing the salt tolerance of crops in saline land and reducing the reliance on chemical nitrogen fertilizers.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

## Author contributions

CJ: Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. YG: Conceptualization, Methodology, Writing – original draft. HZ: Data curation, Writing – original draft. YZ: Formal analysis, Software, Writing – original draft. ZX: Data curation, Investigation, Writing – original draft. JL: Funding acquisition, Software, Writing – original draft. JZ: Formal analysis, Software, Writing – original draft. ZL: Data curation, Writing – original draft. HC: Formal analysis, Funding acquisition, Writing – original draft. KL: Funding acquisition, Methodology, Project administration, Writing – original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1288865/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Effects of different applied nitrogen levels and HNFB on the chemical properties of rhizosphere soil. The **(A)** MBN, **(B)** MBC, **(C)** MBP, **(D)** EC, **(E)** pH, and **(F)** soil N content in the different treatment groups is indicated. The F and *p* values represent significant differences in the results of different treatments within the same nitrogen application level (Student's *t*-test); lowercase letters indicate significant differences in different nitrogen application levels for the same treatment (one-way ANOVA, $p < 0.05$). HNFB, halotolerant nitrogen-fixing bacteria; MBN, microbial biomass nitrogen; MBC, microbial biomass carbon; MBP, microbial biomass phosphorus; EC, electrical conductivity; N, nitrogen; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

SUPPLEMENTARY FIGURE S2
Rarefaction and Shannon curves of rhizosphere microorganisms in different treatment groups at the OTU level. OTU, operational taxonomic unit; CK, control wherein the three nitrogen application levels were set up without bacteria; BIO, treatment wherein the three nitrogen application levels were set up with bacterial inoculation (*Bacillus subtilis* HG-15 + *Bacillus velezensis* JC-K3); N0, low nitrogen level; N1, normal nitrogen level; N2, high nitrogen level.

SUPPLEMENTARY FIGURE S3
Abundances of nitrogen-fixing bacteria **(A)** and arbuscular mycorrhizal fungi **(B)** in the apple rhizosphere soil of different treatment groups.

SUPPLEMENTARY FIGURE S4
Relative abundance (percentage) of AMF species in the apple rhizosphere soil from different treatment groups.

SUPPLEMENTARY TABLE S1
Two-way ANOVA for assessing the effect of nitrogen fertilizer and HNFB on diversity indices of AMF communities.

SUPPLEMENTARY TABLE S2
Statistical analysis results of KEGG functional abundance in AMF communities.

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# Modifying soil bacterial communities in saline mudflats with organic acids and substrates

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**Aims:** The high salinity of soil, nutrient scarcity, and poor aggregate structure limit the exploitation and utilization of coastal mudflat resources and the sustainable development of saline soil agriculture. In this paper, the effects of applying exogenous organic acids combined with biological substrate on the composition and diversity of soil bacterial community were studied in moderately saline mudflats in Jiangsu Province.

**Methods:** A combination of three exogenous organic acids (humic acid, fulvic acid, and citric acid) and four biological substrates (cottonseed hull, cow manure, grass charcoal, and pine needle) was set up set up on a coastal saline mudflat planted with a salt-tolerant forage grass, sweet sorghum. A total of 120 kg ha$^{-1}$ of organic acids and 5,000 kg ha$^{-1}$ of substrates were used, plus two treatments, CK without application of organic acids and substrates and $CK_0$ in bare ground, for a total of 14 treatments.

**Results:** No significant difference was found in the alpha diversity of soil bacterial community among all treatments ($p \geq 0.05$), with the fulvic acid composite pine needle (FPN) treatment showing the largest increase in each index. The beta diversity differed significantly ($p < 0.05$) among all treatments, and the difference between citric acid–grass charcoal (CGC) and CK treatments was greater than that of other treatments. All treatments were effective in increasing the number of bacterial ASVs and affecting the structural composition of the community. Citric acid–cow manure (CCM), FPN, and CGC treatments were found to be beneficial for increasing the relative abundance of *Proteobacteria*, *Chloroflexi*, and *Actinobacteria*, respectively. By contrast, all treatments triggered a decrease in the relative abundance of *Acidobacteria*.

**Conclusion:** Among the 12 different combinations of exogenous organic acid composite biomass substrates applied to the coastal beach, the CGC treatment was more conducive to increasing the relative abundance of the salt-tolerant bacteria *Proteobacteria*, *Chloroflexi* and *Actinobacteria*, and improving the community structure of soil bacteria. The FPN treatment was more conducive to increase the species diversity of the soil bacterial community and adjust the species composition of the bacterial community.

KEYWORDS

**mudflat soil, organic acid, biological substrate, bacterial community structure, soil microbial enhancement**

## Introduction

As indispensable decomposers in the soil ecosystem, microorganisms not only participate in important material transformation and nutrient cycling processes in soil but also regulate nutrient uptake by aboveground plants (Gu et al., 2022). Soil microorganisms are strongly influenced by extreme soil environments when utilizing metabolic activities to alter soil physicochemical properties (Dong et al., 2022), and their community structure, spatial distribution, and biodiversity are regulated by the effects of various factors such as soil nutrients, hydrometeorological conditions, salinity, vegetation type, and land-use practices (Valéria et al., 2021). Poor physical structure, nutrient deprivation and high basal salinity of coastal beach soils inhibit soil microbial activity and affect microbial community diversity (Albdaiwi et al., 2019; Zhao et al., 2022). The main pressure on microbial survival in coastal mudflat soils is salinity, and microbial abundance and activity are negatively correlated with salinity (Siddikee et al., 2011; Wu et al., 2015; Singh, 2016). Related studies have shown that soil fungi are more resistant to salinity stress than soil bacteria due to their chitin cell walls and different energy generation systems from bacteria (Strickland and Rousk, 2010; Rath et al., 2016). In a study by Sun et al. (2021) on the abundance and vertical distribution of soil microbiomes in coastal saline soils under different amelioration measures, it was similarly found that soil bacteria had a more pronounced response to salt concentration, with significantly higher abundance than fungi and archaea. In addition, soil bacteria are involved in the ecological processes of material decomposition and nutrient cycling, and their metabolic activities play an important role in promoting the mineralization of organic matter and humus formation in mudflat soils, which is one of the main factors in restoring and maintaining the productivity of saline soils (Bardgett and van der Putten, 2014; Xiang et al., 2021), so bacterial communities are to a large extent determines the sustainable productivity of beach agroecosystems (Bender et al., 2016).

Plant root exudates in mudflat soils with organic acids as the main components induce and enrich the mass reproduction of some bacterial communities, thus changing the composition and diversity of rhizospheric bacterial communities and then triggering changes in the structure and function of bacterial communities in saline alkali soil (Pei et al., 2017; Zheng et al., 2018). Exogenously applied organic acid–based biological substrates can cause directional changes in the internal structure and composition of the community through their unique physicochemical properties and nutrient composition; they take advantage of bacterial preferences for different living environments and energy substances, thus promoting the ripening of mudflat soil (Mao et al., 2022). Related studies have shown that organic acids and their composite products can not only provide a large amount of nutrients for soil microorganisms, improve soil microbial community diversity, but also improve soil microenvironment through the formation of soil aggregates, providing independent habitats for different microbial groups (Compant et al., 2009). Humic acid compound fertilizer has a sustained effect on microbial community changes at different plant growth stages, and the promotional effect on the increase in the abundance of beneficial fungal and bacterial communities differs at various times (Liu et al., 2019). Humic acid-rich organic amendments have a significant promotional effect on the increase in the abundance of microorganisms involved in the process of carbon and nitrogen cycling, such as *aerobic_ammonia_oxidation*, *aerobic_chemoheterotrophy*, and *nitrification*, and they have an important effect on the functional diversity of microorganisms (Guo et al., 2022). Compared with humic acid, fulvic acid has a lower molecular weight, and higher oxygen and lower carbon make fulvic acid contain more acidic functional groups (especially carboxyl groups), which will be more favorable to reduce soil salinity and promote soil microbial uptake and utilization (Wang, 2008; Zhang and Katayama, 2012; Martinez et al., 2013). Citric acid can enhance the carbon source metabolism ability of soil microbial communities, strengthen carbon turnover efficiency in saline alkali soil (Su et al., 2022), and enhance microbial activity. These advantages affect the internal composition of bacteria and fungi and promote their secretion of extracellular enzymes, thereby facilitating the improvement of soluble nutrient conversion efficiency in saline soils (Macias-Benitez et al., 2020).

Changes in soil physicochemical properties triggered by biological substrate application, such as changes in soil organic carbon, soil pH, and electrical conductivity, indirectly drove adjustments in the structure of soil bacterial and fungal communities, enhanced the relative abundance of bacteria and fungi associated with available nitrogen and phosphorus transformations, and reduced the proportion of pathogenic genera in soil (Ding and Li, 2022; Wang Z. H. et al., 2022). These adjustments have a positive effect on soil structure improvement and microecological environment promotion (Wang Z. H. et al., 2020). Wang Z. J. et al. (2022) showed that the application of organic materials, such as cow manure, biochar, and straw, effectively increased the bacterial abundance and community diversity of coastal saline alkali soils, and they also showed different transformation directions in the structural composition of the bacterial community. Acid-modified biochar significantly increased the relative abundance of *Acidobacteria*; as well as the number of bacteria in specific families, such as Pseudomonadaceae and Sphingobacteriaceae, which have strong ecological connections with C, N, and P cycling and organic matter degradation, and had a strong effect on mitigating soil salinity stress and neutralizing soil pH (Soothar et al., 2021). Pine forest litter exhibited differences in fungal and bacterial biomass, structure, and functional diversity and significantly affected the content and activity of soil enzymes (Vuong et al., 2020; Picariello et al., 2021).

Although domestic and international research involved the influence of different types of biological substrates or organic acid addition on soil microorganisms in saline alkali areas, the utilization mode is mostly the addition of a single organic material. However, studies have found that natural biological substrates such as plant litter, livestock manure, and agricultural by-products have an important impact on improving soil structural stability and accelerating salt leaching (Farrell et al., 2011; Chen et al., 2019). The groove-like microstructure of pine needles has good water collection properties, which helps the uniform distribution and adhesion of exogenous organic acids on its surface, and the proportion of soluble nutrients in pine needles is large, which can rapidly increase the content of quick-acting nutrients in the soil, and its salinity reduction in saline soil remediation process has an obvious effect on the reduction of salts. The study of Kusvuran et al. (2021) showed that cow dung and humic acid application can reduce the salinity of soil by reducing the damaging effects of salt stress by decreasing the uptake of $Cl^-$ and $Na^+$ with enhancing the uptake of $K^+$ and $Ca^{2+}$, and that a

decrease in malondialdehyde content had a favorable effect on alleviating the oxidative stress response of the crop.

The composite application of organic acids and bio-based materials, with their unique physical structure and chemical function, effectively slowed down the leaching process of organic acid components, overcame the effects of soil salinity and other adversities in a short period of time, increased the saline and alkaline nutrient reservoirs, accelerated the decomposition of the organic matter in the soil and the bio-based materials, and released the necessary nutrients for soil microbial uptake and utilization (Wang, 2021; Xiao et al., 2022). If scientifically applied to mudflat soils not only promotes the growth and metabolism of soil bacteria, but also positively influences bacterial community diversity (Wang et al., 2015; Matteo et al., 2022). However, at present, relatively few studies have been conducted on the regulation of bacterial community composition and diversity in saline alkali soils by organic material addition. Moreover, the effect of the combination of exogenous organic acids and biological substrates on the bacterial community structure in mudflat has not been reported. In this study, we chose the salt-tolerant forage sweet sorghum with strong resistance and good palatability as the test material (Hu et al., 2017), sampled the soil during the tasseling period of sweet sorghum, and analyzed the effects of different exogenous organic acid composite biobased treatments on the structural characteristics of soil bacterial communities through high-throughput sequencing technology, and derived the alpha diversity and beta diversity indices based on the sequencing results, and analyzed the effects on the soil bacterial communities diversity under different The effects of the treatments on the diversity of soil bacterial communities were analyzed, and the species composition and differences of soil bacteria in different treatments were comparatively analyzed through species taxonomic level annotation. The purpose of this study is to explore the advantageous combinations of different organic acids and biological substrates to enhance the abundance of soil salt-tolerant microorganisms, to improve the status quo of microbial barrenness in coastal saline soils, and to provide scientific basis for the microecological improvement of soils in coastal mudflat areas.

# 1 Materials and methods

## 1.1 Experimental site

The experiment was carried out in a coastal saline soil in the strip mud reclamation area of Snare Town, Dongtai City, Jiangsu Province, China (N 32°50′01″, E 120°56′43″). The soil type in this area is silty loam and contains 20.5% sand, 8.0% clay, and 71.5% silt (0–20 cm). The specific physicochemical properties are shown in Table 1. The area has a subtropical monsoon maritime climate with an average annual temperature of 16.3°C and an average annual precipitation of 1,024 mm, which mainly occurs in summer (Wang et al., 2023; Yang et al., 2023).

## 1.2 Experimental design

A field experiment was conducted from June 2021, to September 2021, with a total of 14 treatments set up: (1) HCM, supplemented with humic acid and cow manure; (2) HPN, supplemented with humic acid and pine needle; (3) HCH, supplemented with humic acid and cottonseed husk; (4) HGC, supplemented with humic acid and grass charcoal; (5) FCM, supplemented with fulvic acid and cow manure; (6) FPN, supplemented with fulvic acid and pine needle; (7) FCH, supplemented with fulvic acid and cottonseed hull; (8) FGC, supplemented with fulvic acid and grass charcoal; (9) CCM, supplemented with citric acid and cow manure; (10) CPN, supplemented with citric acid and pine needle; (11) CCH, supplemented with citric acid and cottonseed hull; (12) CGC, supplemented with citric acid and grass charcoal; (13) CK, without the addition of organic acids and biomasses; and (14) $CK_0$, bare ground treatment. All treatments were replicated three times, and the application rates of organic acid and biomass were 120 and 5,000 kg ha$^{-1}$, respectively, in all treatments. The sources and properties of organic acid materials are shown in Table 2. Cow manure was aerobically composted and fermented for about 40 days after wet and dry separation. Cottonseed husk was made from residual husk scraps of edible mushroom culture material. Grass charcoal was prepared from the accumulation of incompletely decomposed plant residues fermented in an overly wet and suspicious natural environment. Pine needles referred to those that had accumulated on the soil surface of lacebark pine forests and were collected after natural weathering and decomposition. The specific physical and chemical properties of these biomass materials are shown in Table 3.

The study was conducted in a plot test with a split-zone design and a plot arrangement of 10 m$^2$ (4 m long and 2.5 m wide). The soil tillage layer (0–20 cm) was rototilled before the start of the experiment. In addition to the land preparation, drainage ditches (30 cm deep and 40 cm wide) were opened between the plots so that these ditches were connected to the drainage pipes at the edges of the fields to avoid seedling damage caused by waterlogging in the fields. A double layer of mulch was placed along a vertical depth of 0–30 cm close to the edge position around each plot to stop the migration of water salts, nutrients, and other substances

TABLE 1 Basic physical and chemical properties of primitive soil.

| Items | Values | Items | Values |
|---|---|---|---|
| FC (%) | 23.05 ± 0.16 | OM (g kg$^{-1}$) | 8.77 ± 0.02 |
| BD (g cm$^{-3}$) | 1.47 ± 0.04 | TN (g kg$^{-1}$) | 0.45 ± 0.01 |
| pH | 9.16 ± 0.01 | TP (g kg$^{-1}$) | 0.52 ± 0.02 |
| $EC_{1:5}$ (us cm$^{-1}$) | 492.00 ± 4.73 | AN (mg kg$^{-1}$) | 22.18 ± 0.13 |
| SS (g kg$^{-1}$) | 3.22 ± 0.04 | AP (mg kg$^{-1}$) | 14.18 ± 0.02 |

FC, field water capacity; BD, soil bulk density; $EC_{1:5}$ electrical conductivity of 1:5 soil:water extract; SS, soluble salt; OM, organic matter; TN, total nitrogen; TP, total phosphorus; AN, alkali-hydrolyzed nitrogen; AP, available phosphorus.

TABLE 2 Sources and basic properties of organic acids.

| Organic acid | Solubility | Structure | Appearance | pH | C (%) | H (%) | O (%) | N (%) |
|---|---|---|---|---|---|---|---|---|
| Humic acid | Slightly soluble in water | High molecular polymer | Black powder | 6.82 ± 0.02 | 37.91 ± 0.24 | 3.46 ± 0.03 | 25.43 ± 0.24 | 0.61 ± 0.01 |
| Fulvic acid | Soluble in water, acid, and alkali | High molecular polymer | Brown powder | 5.26 ± 0.03 | 28.35 ± 0.18 | 4.23 ± 0.02 | 41.18 ± 0.17 | 3.24 ± 0.02 |
| Citric acid | Highly soluble in water | Low molecular weight | White granule | 1.47 ± 0.01 | 36.72 ± 0.10 | 4.77 ± 0.03 | 55.30 ± 0.19 | 0.18 ± 0.01 |

FC, field water capacity; BD, soil bulk density; $EC_{1:5}$ electrical conductivity of soil:water extract at 1:5 ratio, SS, soluble salt; OM, organic matter; TN, total nitrogen; TP, total phosphorus; AN, alkali-hydrolyzed nitrogen; AP, available phosphorus. Humic acid and fulvic acid are provided by Xinjiang Shengda Party Biotechnology Co., Ltd (Xinjiang, China) and citric acid is produced by Weifang Ensign Industry Co (Shandong China).

TABLE 3 Sources and basic properties of biomass materials.

| Biological substrates species | pH | Electrical conductivity (μs $cm^{-1}$) | Organic matter (g $kg^{-1}$) | Total nitrogen (g $kg^{-1}$) | Total phosphorus (g $kg^{-1}$) | Alkali-hydrolyzed nitrogen (mg $kg^{-1}$) | Available phosphorus (mg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| Cottonseed hull | 7.30 ± 0.03 | 1682.00 ± 15.52 | 297.91 ± 3.58 | 11.85 ± 0.63 | 2.95 ± 0.01 | 728.85 ± 8.24 | 776.70 ± 8.98 |
| Pine needle | 5.38 ± 0.02 | 1308.00 ± 6.66 | 407.85 ± 5.54 | 10.20 ± 0.09 | 0.66 ± 0.01 | 1374.46 ± 24.71 | 79.73 ± 1.87 |
| Grass charcoal | 5.08 ± 0.03 | 841.00 ± 9.07 | 244.12 ± 3.22 | 8.46 ± 0.02 | 0.79 ± 0.01 | 693.22 ± 16.13 | 45.82 ± 1.75 |
| Cow manure | 7.70 ± 0.03 | 1416.00 ± 8.14 | 345.79 ± 4.91 | 9.69 ± 0.04 | 2.36 ± 0.02 | 477.55 ± 6.69 | 519.76 ± 8.53 |

Cottonseed hull, pine needle, and grass charcoal were purchased from Shijiazhuang Nongyou Biotechnology Co. Ltd., and cow manure was provided by Jurong Lantian Bishui Biotechnology Co., Ltd. $EC_{1:5}$, electrical conductivity of soil:water extract at 1:5 ratio; OM, organic matter; TN, total nitrogen; TP, total phosphorus; AN, alkali-hydrolyzed nitrogen; AP, available phosphorus. The three bio-based materials were purchased from Shijiazhuang Nongyou Biotechnology Co (Hebei, China).

between the plots. The base fertilizer application was 300 kg $ha^{-1}$ of compound fertilizer (15% N, 15% P, and 15% K), which was applied during land preparation. The follow-up fertilizer was applied two times at 30 and 60 days after seedling emergence with 75 kg $ha^{-1}$ of urea (46.4% N). The organic acid pellets (or powder) were mixed evenly with the biomass material and then spread into the plots and pulled back and forth with a rake to mix evenly into the topsoil. The sweet sorghum variety was Big Kahuna, which was planted in June 2021 via strip sowing with a row spacing of 40 cm and a sowing depth of 2 cm. The seedlings were set to a specification standard of 25 cm apart during the three-leaf period. During the growing season, timely prevention and elimination were carried out in conjunction with the occurrence of diseases, insects, and weeds in the field. Owing to the abundant rainfall during the growing season, no irrigation was carried out for moisture management, and the water in the field was removed in a timely manner.

## 1.3 Soil sampling methods

During the heading stage of sweet sorghum (90 days after emergence), soil samples were collected using the five-point method ("S" distribution) at a depth of 0–20 cm. The collected soil at five points was mixed evenly as a sample, with three replications for each treatment. After impurities were removed, the soil samples were placed in sterile sealed bags and quickly brought back to the laboratory in an ice box. The soil samples were mixed well and passed through a 2 mm-sterile sieve, and 5–10 g was taken and stored in sterile EP tubes in a −80°C refrigerator for soil microbial determination and analysis.

## 1.4 Soil DNA extraction, PCR amplification, and library construction

First, 0.5 g soil sample stored in a −80°C refrigerator was weighed, and nucleic acid was extracted using an OMEGA D5625-01 Soil DNA Kit (OmegaBio Tek, Norcross, GA, United States). The extracted soil DNA was detected by 0.8% agarose gel electrophoresis and quantified by a UV spectrophotometer. Then, the highly variable V3–V4 region of the bacterial 16S rRNA gene (Wang Z. et al., 2020), which was about 468 bp in length, was selected for sequencing conducted by Nanjing Personal Gene Technology Co., Ltd. (Nanjing, China). PCR amplification was performed using the following bacterial 16S rDNA V3–V4 region specific primers: 338\u00B0F (55′-ACTCCTACGGGGAGGCAGCA-3′) and 806 R (5′-GACTACHVGGGTWTCTAAT-3′). The PCR amplification program was as follows: pre-denaturation at 98°C for 30 s; 26 cycles of 98°C denaturation for 15 s, annealing at 50°C for 30 s, extension at 72°C for 30 s; and maintain at 72°C for 5 min. The PCR amplification products were detected by 2% agarose gel electrophoresis, and then the target fragments were recovered using an Axygen gel recovery kit (Soothar et al., 2021). The recovered products were subjected to fluorescence quantification with a Quant-iT PicoGreen dsDNA Assay Kit as the fluorescence reagent and a microplate reader (BioTek, FLx800, Agilent, United States) as the quantification instrument. Libraries were constructed using Illumina's TruSeq Nano DNA LT Library Prep Kit (San Diego, CA, United States). On the basis of the fluorescence quantification results, each sample was mixed in the corresponding proportion in accordance with its sequencing quantity requirements, and machine sequencing and subsequent data processing were performed.

### 1.5 Data statistical analysis

The original sequence of the microbiome was processed using QIIME 2 (2019) software (Bolyen et al., 2019), and the merged ASV feature sequence was compared with the reference sequence in Silva database to obtain specific taxonomic information of ASV. Data analysis and image rendering were mainly carried out using QIIME 2 and R software packages (version 3.2.0). QIIME 2 software was used for the following purposes: to draw sparse curves to evaluate whether the current sample size could reflect the real situation of the changes in the structural characteristics of the bacterial community; to calculate the alpha diversity indices, such as Chao1, Shannon, and Faith's PD, and the Good's coverage of the soil bacterial community; and to present the soil bacterial community abundance, diversity, and coverage in the form of box plots. Beta diversity was analyzed on the basis of Bray–Curtis distance to characterize the developmental distances between soil bacterial communities by nonmetric multidimensional scaling (NMDS) and statistically tested using permutational multivariate analysis of variance (PERMANOVA). The number of unique versus shared ASVs between different treatments was counted using petal plots generated by Venn Diagram in R package. MetagenomeSeq analysis was used to statistically compare the abundance of taxonomic groups at the phylum and genus levels between treatments, and linear discriminant analysis (LDA effect size, LEfSe) was used to compare different signatures of species at each taxonomic level between treatments.

## 2 Results

### 2.1 Effects of exogenous organic acids and biological substrates on the alpha diversity of soil bacteria in mudflat

The sparse curves of soil bacterial sequences under different treatments of exogenous organic acids and biological substrates are shown in Figure 1. The number of ASVs in each treatment first increased sharply with sequencing depth and then gradually flattened out. This finding indicated that the sequencing depth of the samples met the requirements of the analysis, and it is sufficient to cover most of the bacterial information in the samples, which can truly reflect the structural characteristics of bacterial communities in soil under different treatment conditions.

The effect of exogenous organic acids and biological substrates on the alpha diversity of soil bacterial community is shown in Figure 2. The average value of Chao1 index in descending order was FPN, FCM, CCM, FGC, FCH, CCH, CPN, HPN, CGC, HCM, HCH, HGC, CK, and $CK_0$ treatments. The Chao1 index in the treatments of exogenous organic acids and biological substrates was higher than that in CK and $CK_0$ treatments, indicating that the abundance of soil bacterial community under all treatments improved to varying degrees. The Shannon index in descending order was FPN, FCH, CPN, FCM, CCM, HPN, HCM, HGC, CCH, HCH, FGC, CK, CGC, and $CK_0$ treatments. The bacterial diversity under treatments of exogenous organic acids and biological substrates, except CGC treatment, was higher than that of CK treatments. This finding indicated that the combination of citric acid and grass charcoal was not conducive for increasing the number of primitive bacterial species type in mudflat soils, whereas the other materials were favorable in increasing the community diversity. Faith's PD index showed that all treatments with different exogenous organic acids and biological substrates contributed to the enhancement of the genetic diversity of bacterial community, with the FPN treatment having the highest effect. The Good's coverage index of each treatment showed that the species coverage was in the range of 97.95–98.55%, which indicated that the experimental sampling was reasonable, and the sequencing volume was sufficient. Furthermore, the detection results can represent the real change rule of species diversity in bacterial communities.

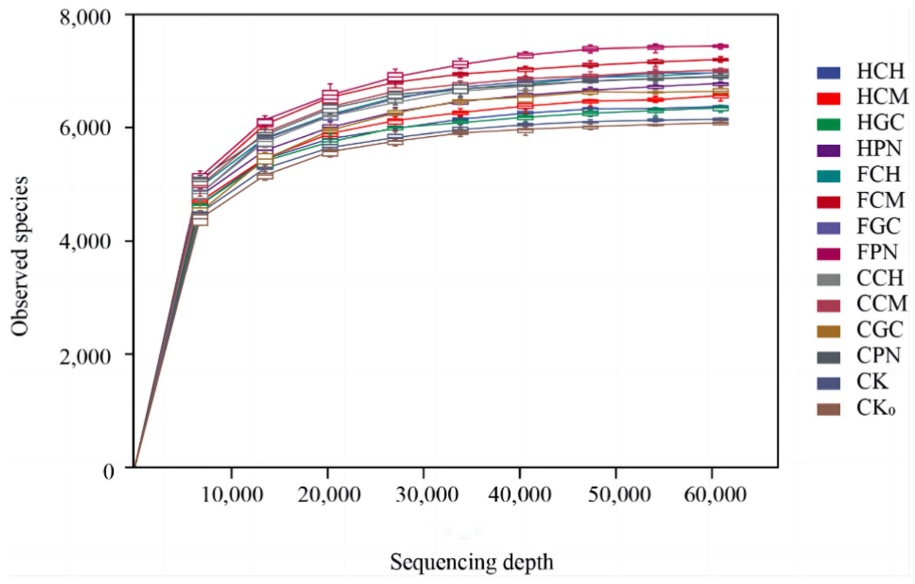


FIGURE 1
Rarefaction curves of soil bacterial sequences from different treatments.

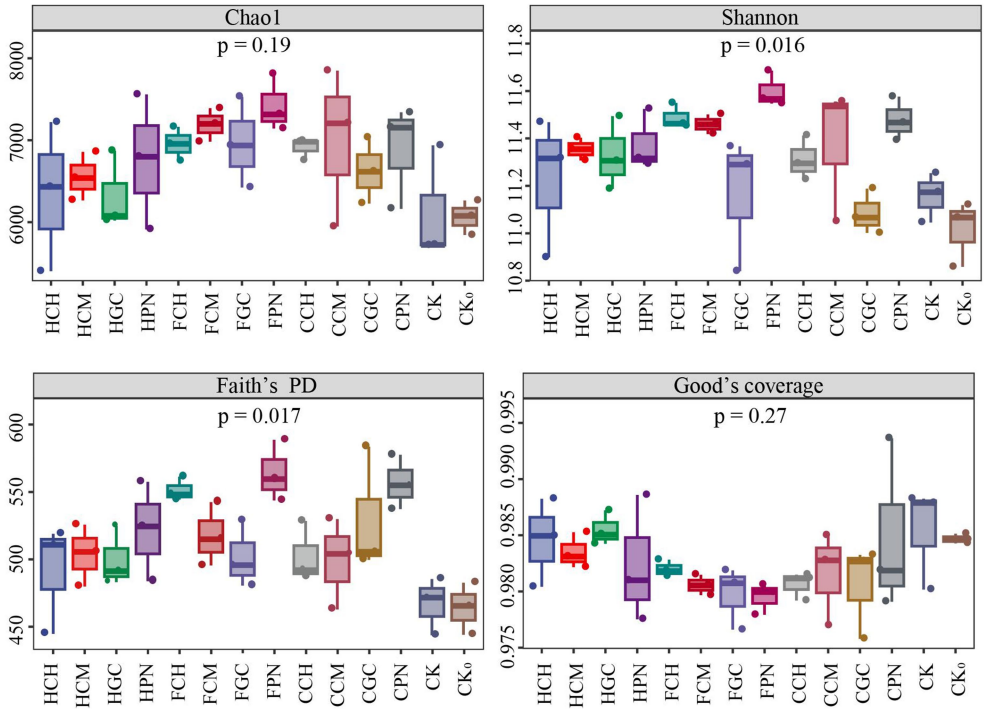


FIGURE 2
Effects of different treatments on the alpha diversity of bacterial communities in mudflat soil.

## 2.2 Effects of exogenous organic acids and biological substrates on the beta diversity of soil bacteria in mudflat

The NMDS analysis of soil bacterial community structure based on the abundance of ASVs is shown in Figure 3A. The stress value of the NMDS results was 0.168, indicating that the results of NMDS analysis can accurately reflect the true distribution of the data, and the closer the distance between the two points in the figure, the smaller the difference between the two bacterial communities. The distance among HCH, HGC, FCM, FGC, CCM, CGC, and CPN treatments was farther away than that in CK treatment, indicating that the difference in soil bacteria between them and CK treatment was more significant. Meanwhile, the distance among HCM, HPN, FCH, FPN, $CK_0$, CCH, and CK treatments was relatively far away, indicating that the bacterial community structure was different under the above treatments compared with under CK treatment. However, the comprehensive effect on the changes in soil bacterial community structure was relatively small. In addition, the distance between CGC treatment and all other treatments was far, indicating that the structural composition of the soil bacterial community in CGC treatment was significantly different from those in other treatments. PERMANOVA, which is based on distance matrices, serves as a further computational test for NMDS analysis. It can determine specific differences between different treatments through pairwise comparisons. Figures 3B,C show the inter-group difference analysis of PERMANOVA based on CK and $CK_0$ treatments compared with other treatments ($p = 0.001$). As shown in Figure 3B, the treatments that had the largest distances from CK treatments were CGC, HCH, FCM, CCM, FGC, HGC, and CPN treatments ($p < 0.001$). Figure 3C shows that the treatments with a large difference from $CK_0$ treatment were CGC, CCM, FGC, HCH, CPN, HGC, and FCM treatments ($p < 0.001$). The calculation result was consistent with the distribution pattern presented by NDMS analysis, effectively verifying the significant difference in beta diversity among different treatments. These findings indicated that each exogenous organic acid composite and biological substrate had different influences on the structural composition of the bacterial community in mudflat soils.

## 2.3 Effect of exogenous organic acids and biological substrates community composition in mudflat soil

As shown in Figure 4, the top 10 bacterial phyla ranked in terms of relative abundance in soil bacterial communities at the phylum level were *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Firmicutes*, *Planctomycotes*, *Nitrospirae*, and *Chlamydiae*. The soil bacterial community of different treatments was dominated by *Proteobacteria*, which accounted for 42.31–48.62% of the total number of bacteria, followed by *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*, which accounted for 11.51–18.94%, 8.7–16.62%, and 6.4–10.44%, respectively. The bacteria whose relative abundance was in the range of 1–5% in different treatments were *Bacteroidetes*, *Gemmatimonadetes*, *Firmicutes*, *Planctomycotes*, and *Nitrospirae*, which accounted for 2.51–4.48%, 1.86–3.46%, 1.82–3.73%, 0.86–1.95%, and 1.08–1.96%, respectively, of the total bacterial population. Compared with that in CK treatment, the relative abundance of *Acidobacteria* in the treatment of exogenous organic acids and biological substrates decreased overall in the range of 3.37–47.62%, whereas the relative abundance of *Firmicutes*, *Planctomycotes*, and

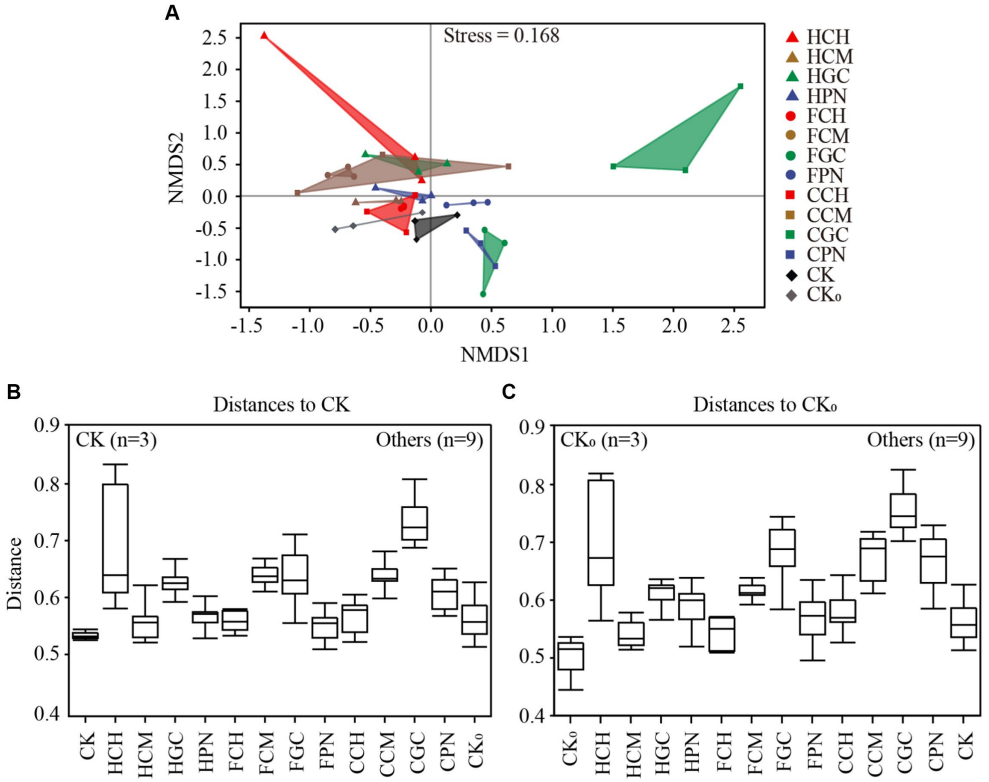


FIGURE 3
Analysis of group differences in bacterial communities of mudflat soil under different treatments. **(A)** shows the nonmetric multidimensional scaling analysis of soil bacterial communities in different treatments; **(B,C)** show the permutational multivariate analysis of variance of bacterial communities.

*Chlamydiae* significantly increased. FCM, CCM, HPN, FGC, and CCH treatments were conducive to the increase in the relative abundance of *Proteobacteria*; HCH, CCM, CPN, and FGC treatments caused a decrease in the relative abundance of *Chloroflexi*; and CPN, HGC, FCH, HCM, and FPN treatments facilitated an increase in *Actinobacteria*. The main bacteria in mudflat soil had different responses to the treatment of exogenous organic acids and biological substrates. Figure 4B shows the clustering relationship between treatments and bacterial communities at the phylum level. FPN and HGC, CCH and CCM, CPN and FCH, HCH and FCM, and CK and $CK_0$ treatments were clustered to one another. This finding suggested that these treatments had similar effects on the soil bacteria, thereby showing a similar community composition. In addition, CGC treatment showed strong positive correlations with *Chloroflexi*, *Gemmatimonadetes*, and *Bacteroidetes* and strong negative correlations with *Acidobacteria*, *Planctomycotes*, and *Chlamydiae*. FGC treatment had a negative correlation with *Gemmatimonadetes*, CCH treatment had a negative correlation with the *phylum Actinobacteria*. FCM treatment had a positive correlation with *Chlamydiae*. The trends in the influence of various treatments with exogenous organic acids and biological substrates on the species composition and structural distributions of soil bacteria varied considerably.

Figure 5A shows that the ranking of the top 10 genera in soil bacterial community abundance at the genus level was as follows: *Subgroups_6*, *Subgroups_10*, *SBR1031*, *NB1-j*, *Subgroup_17*, *A4b*, *KD4-96*, *MND1*, *Desulfuromonas*, and *Hailiangium*. Among them, the relative abundance of *KD4-96* and *MND1* in HCM treatment was the highest, with increases of 79.11 and 66.89%, respectively, compared with that in CK treatment. The relative abundance of *SBR1031* and *A4b* in CGC treatment was the highest, with increases of 56.56 and 43.86%, respectively, compared with that in CK treatment. The genera *Subgroup_6* in FCH treatment and *Hailiangium* in CCM treatment exhibited the highest levels, with increases of 4.39 and 36.96%, respectively, compared with that in CK treatment. As shown in Figure 5B, HPN and FCM, FGC and CPN, and FCH and CK treatments were clustered to each other, indicating that the community structures showed a similarity. CGC treatment showed a strong positive correlation with *SBR1031* and *A4b* and a negative correlation with *MND1*, *Subgroup_6*, and *Subgroup_10*. CCM treatment showed a strong positive correlation with *Hailiangium*, CPN treatment showed a negative correlation with *Desulfuromonas*, and HGC treatment showed a negative correlation with *Subgroup_ 17*. These findings suggested that the dominant species at the genus level in mudflat soils under different treatments of exogenous organic acids and biological substrates differed considerably.

## 2.4 Analysis of soil bacterial ASVs and significant bacterial differences in treatments of exogenous organic acids and biological substrates

Figure 6 shows that except for HCM and HGC treatments, all treatments of exogenous organic acids and biological substrates

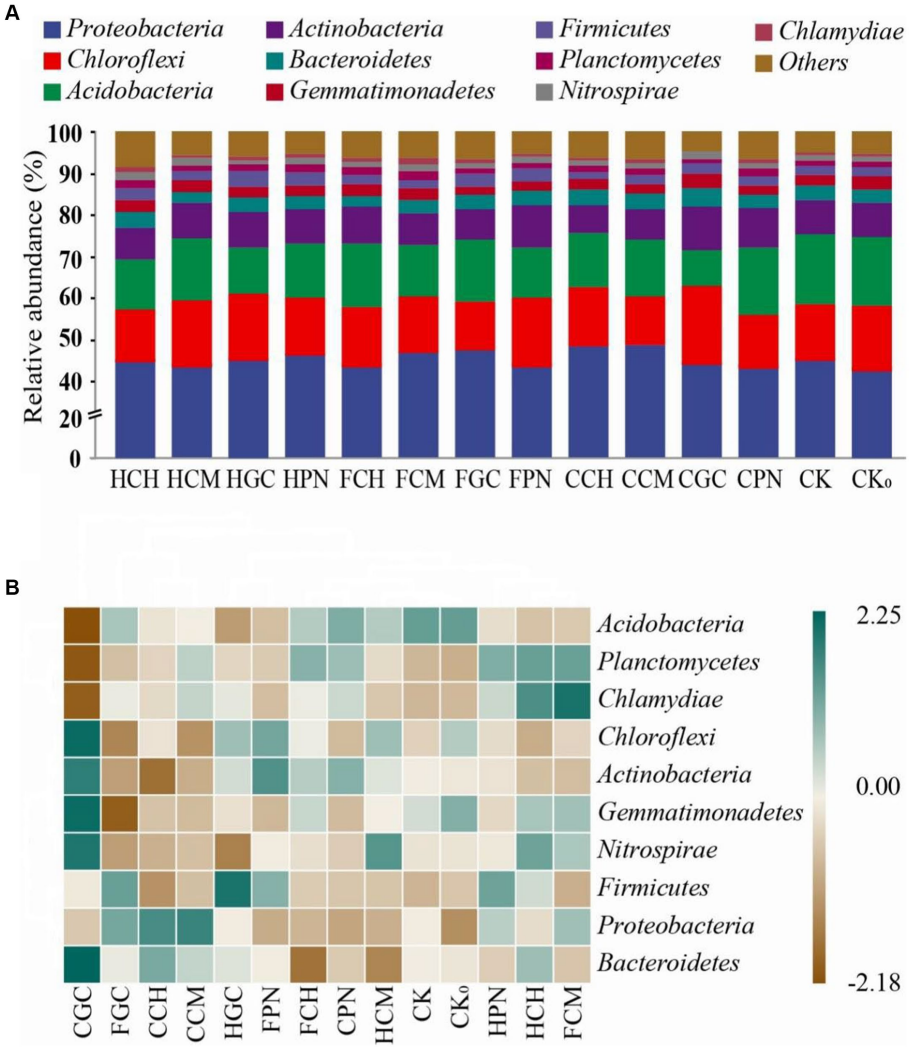


FIGURE 4
Community composition of top 10 bacterial phyla in terms of relative abundance in mudflat soil of different treatments. **(A)** shows the relative abundance of soil bacterial phyla in different treatments; **(B)** shows the clustering relationships among bacterial communities. The different color block sizes in **(A)** correspond to the relative abundance of each phylum; the blue and brown colors in **(B)** blue represent positive and negative correlations, respectively.

improved the soil bacterial community compared with CK treatment, and the number of ASVs of the 14 treatments in descending order was as follows: 7205, 5,903, 5,525, 5,218, 5,120, 5,100, 4,910, 4,476, 4,295, 4,092, 4,074, 3,997, 3,938, and 3,796 in CGC, CPN, CCM, FCM, FPN, HCH, FGC, CCH, FCH, HPN, CK, HGC, $CK_0$, and HCM treatments, respectively. The number of shared ASVs was 878. The enhancement of soil bacterial ASV by different treatments of exogenous organic acids and biological substrates varied significantly, with CGC, CPN, CCM, FCM, FPN, HCH, FGC, CCH, FCH, and HPN treatments showing increases of 76.85, 44.89, 35.62, 28.08, 25.68, 25.18, 20.52, 9.87, 5.42, and 0.44%, respectively, compared with CK treatment. On the contrary, HGC, $CK_0$, and HCM treatments had decreases of 1.89, 3.34, and 6.82%, respectively.

The LEFSe of significantly different bacteria in the bacterial community of mudflat soil treated with exogenous organic acids and biological substrates was analyzed at different taxonomic levels, and the results are shown in Figure 7. A total of 33 significantly different bacteria were found among the 14 treatments, The LEFSe of significantly different bacteria in the bacterial community of mudflat soil treated with exogenous organic acids and biological substrates was analyzed at different taxonomic levels, and the results are shown in Figure 7. A total of 33 significantly different bacteria were found among the 14 treatments. At the genus level, the FCM treatment contained the highest number of characterized genera with significant differences, seven (*OPB41*, *Bacteroidetes_vadinHA17*, *Blvii28_wastewater_sludge_group*, *SJA_15*, *Desulforhabdus*, *Desulfococcus*, *Desulfoprunum*); FCM (*bacteriap25*, *TRA3_20*, *JTB23*, *subgroup_2*, *AT_s3_28*, *Subgroup_22*) and $CK_0$ (*Subgroup_9*, *S085*, *Dadabacteriales*, *Turicibacter*, *Amb _16S_1323*, *Gaiella*) contain six characterized genera; HCH (*Latescibacteria* and *Enhygromyxa*), HPN (*cvE6* and *Sulfurifustis*), FGC (*Pseudomonas* and *Bdellovibrio*), CPN (*0319_6G20*, *Aquicella*), CK (*Coxiella* and *Subgroup_10*) contained two characteristic genera. HCM, HGC, FCH, FPN, CCH contained one characteristic genus, *MND1*, *S085*, *Pseudorhizobium*, *Hydrogenispora*,

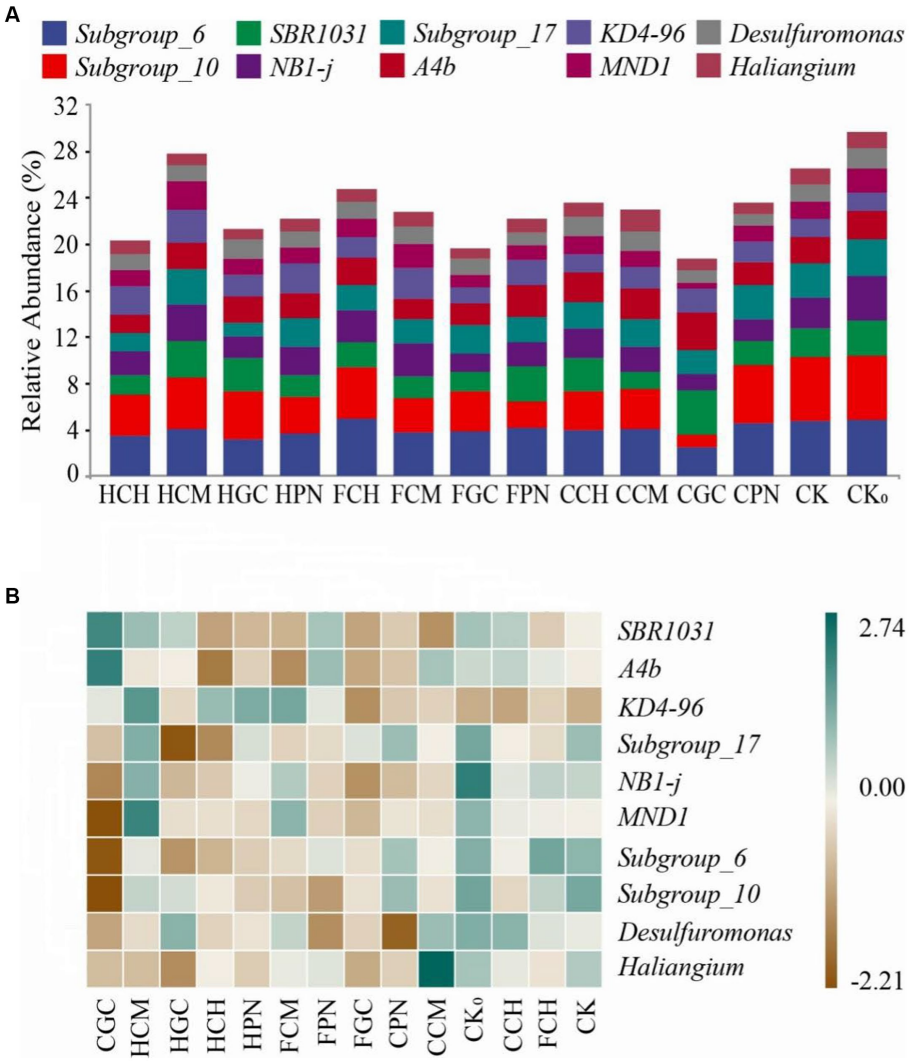


FIGURE 5
Community composition of top 10 bacterial genera in terms of relative abundance in mudflat soil of different treatments. **(A)** shows the relative abundance of soil bacterial genera in different treatments; **(B)** shows the clustering relationship between bacterial communities and treatments. The different color block sizes in **(A)** correspond to the relative abundance of each genus; the blue and brown colors in **(B)** blue represent positive and negative correlations, respectively.

*1013_28_CG33*; CCM No characteristic genera containing significant differences were detected.

# 3 Discussion

## 3.1 Effects of exogenous organic acids and biological substrates on bacterial community diversity in mudflat soil

Soil bacterial community is sensitive to changes in physicochemical factors, and it has a certain preference for organic materials from different sources. Therefore, after long-term improvement of soil by using organic materials, some bacteria could gradually enrich and lead to a change in the composition and diversity of the original bacterial community (Canellas et al., 2015). The present study showed that the alpha diversity of bacterial community richness and genetic diversity under the treatment of different exogenous organic acids was higher than those under CK and $CK_0$ treatments, but the difference was not significant. The results of beta diversity showed that the bacterial community structure under different treatments changed significantly, which was similar to the change rule of Shi et al. (2018), who used organic acids to improve microbial community structure in saline soil. Low molecular weight organic acids in the soil participate in soil formation, promote mineral dissolution, and change soil physicochemical properties, thereby alleviating the toxic effects of elements such as metals on plants or soil microorganisms (Lima et al., 2009; Gao et al., 2015). It can also mediate interactions between plants and soil microorganisms, increase soil enzyme activity and microbial activity, promote the formation of aggregates, and accelerate soil nutrient cycling to improve the soil ecosystem. It was found that exogenously applied organic acids can adjust the acid – base and redox conditions of soil tillage using acidic functional groups, adsorb soil saline ions through chelation, and

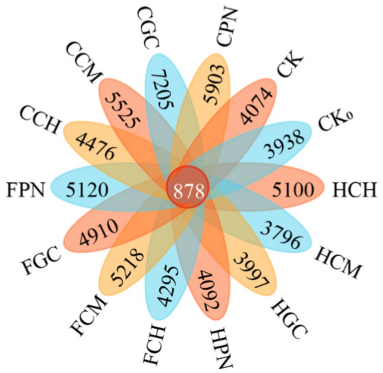


FIGURE 6
ASV numbers of bacterial community in mudflat soil under different treatments. HCH, humic acid–cottonseed hull composite; HCM, humic acid–cow manure composite; HGC, humic acid–grass charcoal composite; HPN, humic acid–pine needle composite; FCH, fulvic acid–cottonseed hull composite; FCM, fulvic acid–cow manure composite; FGC, fulvic acid–grass charcoal composite; FPN, fulvic acid–pine needle composite; CCH, citric acid–cottonseed hull composite; CCM, citric acid–cow manure composite; CGC, citric acid–grass charcoal composite; CPN, citric acid–pine needle composite; CK without the addition of organic acids and biological substrates; $CK_0$, bare area. The same for the following.

enhance the solubility and mobility of phosphorus, thus promoting the enhancement of effective nutrients in saline soils (Chatterjee et al., 2015). Consequently, it may be due to the addition of exogenous substances to improve the soil microbial survival of the adverse environmental conditions, such as soil structure, pH, aeration and permeability, and topsoil nutrient composition changes (Yang et al., 2023), the improvement of the adverse environmental conditions makes most of the bacterial community's more easy to grow and reproduce, which adjusts the species and number of various bacterial groups in the soil, resulting in the bacterial community development direction differences, changing the diversity of the community. However, short-term application cannot easily have a significant effect on bacterial community diversity due to the relatively stable structure of organic materials (Wu et al., 2013).

The bacterial community in this study had a positive response to the addition of citric acid complex, with an increase in bacterial relative abundance and community diversity. Similarly, Bao et al. (2022) used exogenous citric acid to improve the removal effect of polycyclic aromatic hydrocarbons (PAHs) in contaminated soil. Meanwhile, the fulvic acid component of the composite was more effective in increasing the diversity of the bacterial community of cultivated soils than citric acid, similar to the study results of Li et al. (2021) and Sun et al. (2023) on fulvic acid organic fertilizers. The alpha diversity of the bacterial community was the highest in FPN treatment, followed by CCM. The main reason for the difference in the response of soil bacterial community in mudflat to fulvic acid–pine needles and citric acid–cow manure is that fulvic acid is highly soluble in alkaline environments and has a microporous structure accessible to microorganisms, thus attracting a large number of bacteria attached to the surface of the soil colloid to come close to it (Zhang et al., 2020). When combined with pine needles with high nutrient balance and total amount, fulvic acid can adapt to the feeding preferences of different kinds of microorganisms, with a wide range of energy supply, which is conducive to the formation of microbial diversity (Wang et al., 2011). Meanwhile, the high C/N ratio of citric acid can provide abundant and available carbon sources for bacteria, thus promoting the life activities and nutrient turnover of soil bacteria (Chen et al., 2008). Cow manure contains more cellulose and lignin, which can be slowly decomposed by specific bacterial communities in aerobic or anaerobic soil environments, thereby supporting the increase in its population (Meng et al., 2019). In summary, the addition of exogenous organic acids and biological substrates provides a relatively independent microenvironment and sufficient nutrients for the life activities of different energy-type bacteria, thereby playing a regulatory role in the species composition and functional characteristics of bacterial communities. Furthermore, it has a significant effect on the overall diversity of bacterial communities.

## 3.2 Effect of exogenous organic acids and biological substrates community composition in mudflat soil

In this study, the main bacteria with relative abundance greater than 5% in the bacterial community of mudflat soil were *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*, with *Proteobacteria* having absolute dominance in all treatments, consistent with the results of most studies on bacterial diversity in saline alkaline soil environments (Canfora et al., 2014; Abdallah et al., 2018; Ayantha et al., 2018). The highest relative abundance of *Proteobacteria* in CCM treatment may be due to the fact that the increased level of soil humus caused by the application of fermented cow manure positively affected the increase in the number of *Proteobacteria*, a group of eutrophic bacteria, and resulted in a relative decrease in the proportion of nutrient-poor bacteria in the soil (Liu et al., 2021). Moreover, a large number of *Proteobacteria* contributed to soil carbon storage through the synthesis of microbial mucilage and polysaccharides that contribute to the stabilization of aggregates in mudflat soils (Pascual et al., 2018). The high relative abundance of *Chloroflexi* in FPN treatment was attributed to the higher nitrogen content of fulvic acid and pine needles than other materials; such a high content attracts the migration and colonization of *Chloroflexi* to the tillage layer, facilitates nitrification of the soil in the root zone, and replenishes the nitrogen deficit (Zhao et al., 2014). The highest relative abundance of *Actinobacteria* in CGC treatment was attributed to its ability to produce various extracellular hydrolases, which degrade and convert exogenous organic matter in the soil into soluble phosphorus, nitrogen, potassium, and other components and play an important role in the mineralization of organic matter. The addition of citric acid and grass charcoal was beneficial to the propagation and metabolic activities of *Actinobacteria*, similar to the effect of organic materials on the microbial community of alkalized soil in the study of Liang et al. (2023). In the present study, the treatment of organic acids and biological substrates resulted in a decrease in the relative abundance of native *Acidobacteria* in saline alkali soil. Given that *Acidobacteria* is an oligotrophic bacterium, nutrients have an important influence on its lifestyle, and excess nutrients reduce its activity and thus lead to a decrease in abundance (Ward et al., 2009).

The dominant genera of soil bacteria in this study belong to *Acidobacteria*, *Chloroflexi*, and *Proteobacteria*, respectively. These genera are more tolerant in alkaline soils, with certain degradation functions for complex compounds. The increase in the relative abundance of these genera is favorable to the nutrient cycling and

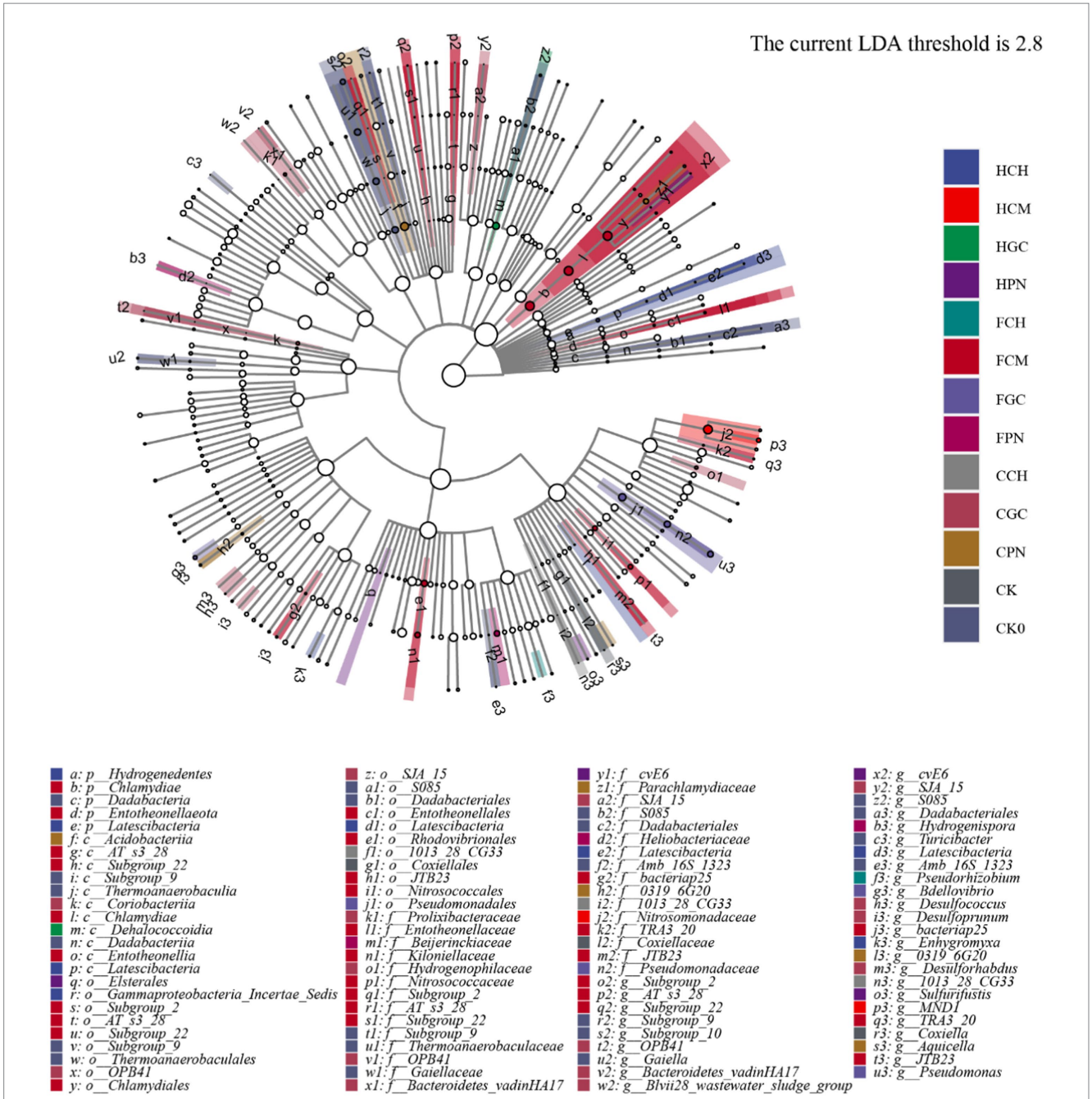


FIGURE 7
LEFSe analysis of significantly different bacteria in mudflat soil among different treatments. The branches from the inner circle to the outer circle show the taxonomic rank relationships of soil bacterial communities at phylum, class, order, family, and genus levels. The node size corresponds to the mean relative abundance of the taxon. The hollow nodes represent taxa with insignificant between-treatment differences. The colored nodes indicate that these taxa have significant between-treatment differences and that the abundance is high in the treatment represented by the color.

supply of plant rhizosphere soil, and they belong to the bacterial communities that widely exist in pristine saline and alkaline soils and have a high application value for saline and alkaline soil remediation (Liu et al., 2016). The results of the present study showed that the signature differential bacterial species and quantities of bacterial communities varied among different treatments of organic acids and biological substrates, because the physicochemical properties of the exogenous materials are an important driver of the variability in the structure of the soil bacterial community. These properties not only influence the relative abundance of dominant species of the bacterial community but also utilize the trophic relationships of bacteria to promote the emergence of bacterial endemics, in agreement with the findings of Jung and Choi (2020). In summary, the addition of exogenous organic acids and biological substrates can cause changes in the original bacterial community composition of mudflat soil. However, this change can take a long time to maintain to form a stable bacterial community structure. The effect of exogenous organic acids and biological substrates on the specific function of soil bacterial community needs to be further explored, and the available bacterial resources of mudflat soil need to be further revealed.

## 4 Conclusion

The results of the study on the effect of exogenous organic acid composite biological substrates additions on the structural characteristics of bacterial communities in beach soils showed that different treatments of exogenous organic acids and biological substrates had no significant effect ($p \geq 0.05$) on bacterial alpha diversity, but FPN treatment showed a slight increase in the alpha index values compared with CK treatment. Significant differences ($p < 0.05$) were found in the beta diversity of bacterial communities among all treatments, with the greatest difference found in CGC treatment. CCM, FPN, and CGC treatments increased the relative abundances of *Proteobacteria*, *Chloroflexi*, and *Actinobacteria*, respectively, thus playing important roles in nutrient cycling and supply in mudflat soils.

## Data availability statement

The datasets presented in this study can be found at https://www.ncbi.nlm.nih.gov under accession number SRP498511.

## Author contributions

XYL: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. LZ: Writing – original draft. RY: Conceptualization, Resources, Writing – original draft. HW: Supervision, Validation, Writing – review & editing. XBL: Methodology, Supervision, Writing – review & editing. WX: Supervision, Validation, Writing – review & editing. HY: Supervision, Writing – review & editing. YS: Methodology, Supervision, Writing – review & editing. JL: Writing – review & editing. ZS: Funding acquisition, Project administration, Resources, Visualization, Writing – review & editing, Writing – original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

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# Plant growth-promoting rhizobacteria enhance active ingredient accumulation in medicinal plants at elevated $CO_2$ and are associated with indigenous microbiome

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**Introduction:** Plant growth-promoting rhizobacteria (PGPR) and elevated $CO_2$ ($eCO_2$) have demonstrated their individual potential to enhance plant yield and quality through close interaction with rhizosphere microorganisms and plant growth. However, the efficacy of PGPR under $eCO_2$ on rhizosphere microbiome and, ultimately, plant yield and active ingredient accumulation are not yet fully understood.

**Methods:** This study investigated how the medicinal plant *Pseudostellaria heterophylla* (*P. heterophylla*) and its rhizosphere microbes respond to PGPR (*Bacillus subtilis* and *Pseudomonas fluorescens*) at $eCO_2$ (1,000 ppm).

**Results and Discussion:** It was found that the yield and active ingredient polysaccharides accumulation in the tuber of *P. heterophylla* were significantly increased by 38 and 253%, respectively. This promotion has been associated with increased root development and changes in the indigenous microbial community. Metagenomics analysis revealed a significant reduction in pathogenic *Fusarium* abundance in the rhizosphere. Potential biocontrol bacteria *Actinobacteria* and *Proteobacteria* were enriched, especially the genera *Bradyrhizobium* and *Rhodanobacter*. The reshaping of the rhizosphere microbiome was accompanied by the upregulation of biological pathways related to metabolite biosynthesis in the rhizosphere. These modifications were related to the promotion of the growth and productivity of *P. heterophylla*. Our findings highlighted the significant role played by PGPR in medicinal plant yield and active ingredient accumulation when exposed to $eCO_2$.

KEYWORDS

**PGPR, elevated $CO_2$, active ingredient, microbial community, biological pathways**

# 1 Introduction

Administration of plant growth-promoting rhizobacteria (PGPR) is an important and environmentally friendly method to achieve sustainable crop production (Bhattacharyya and Jha, 2012; Gray and Smith, 2005; Lugtenberg and Kamilova, 2009). The mechanisms associated with plant promotion commonly reported include direct promotion via the production of phytohormones and/or mobilization of nutrients in the soil, such as phosphorus (Liu et al., 2016; Richardson et al., 2009), and indirect promotion via inducing systemic resistance to biotic and/or abiotic stress (Akbar et al., 2022). While an increasing number of studies identified that biological amendments modified the composition and functionality of the rhizosphere microbial community, resulting in enhanced plant growth and health (Deng et al., 2022; Guo et al., 2024; Tao et al., 2023). The specific way in which PGPR affect soil microbiome varies depending on the PGPR strains, plant species, and environmental factors.

Recently, studies have revealed that the native rhizobacteria (Sun et al., 2022), fungi (Tao et al., 2023), and predatory protists (Guo et al., 2024) can be recruited of PGPR and cooperate as partners to enhance plant development (Sun et al., 2022; Zhang et al., 2023). These changes in indigenous organisms are indirectly influenced by elevated atmospheric $CO_2$ ($eCO_2$) (Kohler et al., 2009; Williams et al., 2018; Zytynska et al., 2020). Studies showed that the effects of $eCO_2$ on microorganisms in the rhizosphere had large variability, and can be both positive (Jin et al., 2022) and negative (Li et al., 2022). Microbial growth, including bacteria, fungi, arbuscular mycorrhizal fungi, and actinomycetes can be stimulated under $eCO_2$ (Lee and Kang, 2016). However, the high $eCO_2$ may suppress the soil microbes and reduce their biomass (Xiao et al., 2017). These opposite effects happened through plant-mediated and soil-mediated mechanisms (Paterson et al., 1997; Montealegre et al., 2002). The phenotypic traits of PGPR related to plant growth, such as hormone indole-3-acetic acid secretion in *Pseudomonas* strains, are changed under 1,000 ppm conditions, contributing to establishing a developed root system that efficiently absorbs water nutrients from the soil (Sun et al., 2022; Tarnawski et al., 2006; Yu et al., 2016). The influenced root growth and rhizodeposition, in turn, potentially manipulate the indigenous microbial populations and activities (Redondo-Gómez et al., 2022; Sadowsky and Schortemeyer, 1997; Zak et al., 2000). The effects of $eCO_2$ on microbes are also linked to the soil nutrient availability. For instance, under $eCO_2$, reduced soil nitrogen availability resulting from enhanced plant nutrient uptake led to a decrease in microbial biomass (Butterly et al., 2016). Increased microbial biomass under $eCO_2$ was associated with increased dissolved organic carbon in the soil (Hu et al., 2020). The functionality of recruited microorganisms is associated with competitive interactions (Wei et al., 2015) and secondary metabolism manipulation in the soil (Zhang et al., 2023). For example, bacteria strains with the ability to produce 6-hydroxypentadecanedioic acid (Cai et al., 2021) and antibiotics (Zhang et al., 2022), as well as the ability to increase peptide synthetase gene abundance (Deng et al., 2022) were enriched to protect against pathogen invasion. However, despite the understanding that PGPR and elevated $CO_2$ are manipulations that can influence the composition and functioning of rhizosphere microbiomes for plant growth. It is unclear if these manipulations could enhance treatment efficacy or not when plants grow in a changing climate scenario. Regarding this, a better understanding of how additive PGPR impacts plant root development and indigenous soil microbiomes under $eCO_2$ conditions is necessary to support more effective application strategies to improve host plant development.

The tuberous root of *Pseudostellaria heterophylla* (*P. heterophylla*) Rupr. & Maxim, also known as crown prince ginseng, has been a common traditional Chinese medicine for nearly 300 years. The below-ground tubers contain bioactive components, and tuber extractions are the most used materials for treating coronavirus disease 2019 in clinical practices (Alam et al., 2021; Ren et al., 2021; Wu et al., 2019). However, the tubers of *P. heterophylla* have a high incidence of *Fusarium* wilt infection, a disease caused by the soil pathogen *Fusarium oxysporum* (Chen et al., 2021; Liu et al., 2020). *Fusarium* wilt poses an ever-increasing threat to agriculture, causing significant declines in plant yields, such as banana, tomato, and medicinal plants, ranging from 20 to 90% annually (Strange and Scott, 2005; Tao et al., 2023; Yuan et al., 2022). As PGPR plays an essential role in pathogen control, they manipulate microbial resource competition networks (Wei et al., 2015), bacterial community diversity (Hu et al., 2016), and organism interactions (Huo et al., 2018). Although these studies revealed the importance of PGPR in pathogen suppression, how PGPR impacts the fungal genera *Fusarium* species presence under $eCO_2$ and how these manipulations influence active ingredient accumulation in medicinal plants remains largely unknown.

The objectives of this study include (1) to investigate the differences in plant trait indicators; (2) to study the differences in soil microbiome composition and biological pathways; and (3) to reveal the relationship between plants and soil microbiome. *P. heterophylla* was grown in two separate chambers and inoculated with two types of PGPR strains (*Bacillus subtilis* and *Pseudomonas fluorescens*) under ambient $CO_2$ and $eCO_2$ conditions, respectively. The growth and productivity of *P. heterophylla* were monitored and evaluated by measuring its shoot, root, tuber yield, and active ingredient content. We first monitored the composition and diversity of the indigenous bacterial communities and the density of wilt pathogen *Fusarium* in the rhizosphere. Based on these results, we conducted the analysis to identify the changes in the key metabolites' biosynthesis biological pathways related to the microbial community shift.

# 2 Materials and methods

## 2.1 Plant material and growth conditions

The experiments were conducted in two climate-controlled chambers in the Department of Civil and Environmental Engineering at The Hong Kong University of Science and Technology (HKUST) (22.3°N 114.2°E), from January to May 2022. The study used the *P. heterophylla* cultivar as the experiment plant material. Seed tubers were surface sterilized with 5% (v/v) sodium hypochlorite and washed four times with sterilized distilled water. The washed seed tubers were sown in a pot (8 cm diameter, 17 cm depth) filled with 1.7 kg soil collected from Bijie, Guizhou Province, China (Kong et al., 2021). Soil water condition (60% field capacity) was monitored with soil moisture probes and tensiometers (Ng et al., 2022a). The pots were watered every 2 days with deionized water. The basic physicochemical properties of soil are summarized in Table 1.

The growth conditions are presented in Supplementary Figure S1. One of the chambers was set with an elevated $CO_2$ concentration ($eCO_2$), and the other was maintained at the average ambient $CO_2$ concentration ($aCO_2$). $CO_2$ was supplied to the chamber via a

TABLE 1 Soil physicochemical properties after harvest.

| Treatment | pH | EC (mS/cm) | C (μg/g) | N (μg/g) | P (μg/g) | IAA (μg/g) |
|---|---|---|---|---|---|---|
| aCK | 5.47 ± 0.03d | 1.43 ± 0.01c | 198.27 ± 4.07b | 166.55 ± 19.68a | 175.34 ± 9.81b | 197.34 ± 25.00c |
| aBS | 5.81 ± 0.03a | 1.14 ± 0.01e | 208.00 ± 6.00b | 113.48 ± 1.60c | 140.40 ± 17.01c | 283.35 ± 25.81b |
| aPF | 5.83 ± 0.02a | 1.01 ± 0.02f | 220.38 ± 5.43a | 164.22 ± 22.02ab | 145.89 ± 25.95bc | 349.39 ± 17.04a |
| eCK | 5.67 ± 0.01c | 1.49 ± 0.03b | 220.26 ± 4.20a | 143.08 ± 3.66b | 242.39 ± 24.59a | 223.17 ± 12.78c |
| eBS | 5.49 ± 0.10d | 1.59 ± 0.03a | 207.10 ± 7.00b | 171.34 ± 3.81a | 214.12 ± 24.94ab | 305.86 ± 14.67b |
| ePF | 5.70 ± 0.02b | 1.20 ± 0.02d | 226.31 ± 3.81a | 169.48 ± 10.22a | 175.76 ± 19.66b | 278.17 ± 13.42b |

a: ambient $CO_2$ condition (400 ppm). e: elevated $CO_2$ condition (1,000 ppm). CK, control group without bacterial inoculation; BS, *Bacillus subtilis*-inoculated group; PF, Pseudomonas fluorescence-inoculated group; EC, electricity conductance. C: total organic carbon. N: total nitrogen. P: bioavailable phosphorus. IAA: indole-3-acetic acid. Data presented are the mean ± standard deviation ($n = 3$). Different letters beside the data indicate the significance of differences among treatments ($p < 0.05$).

compressed $CO_2$ tank connected to a pipe within the chamber at the beginning of the experiments. Both chambers were set to a long-day photoperiod of 16 h:8 h, light: dark, at 23.5 ± 3°C. The light intensity was set to 79.198 mW/nm, and the relative humidity was maintained at 60 ± 10% (Chen et al., 2021).

## 2.2 Experiment treatments

The experiments were conducted in two independent chambers, one with $eCO_2$ 1,000 ± 50 ppm (IPCC, 2022 predicted level) monitored and controlled using a remote sensor and an environmental controller (BETC-B2, Netherlands). The other one was with $aCO_2$ concentration of approximately 400 ppm. In each chamber, plants were performed with 3 treatments: control groups (CK), a *B. subtilis*-inoculated group (BS), and a *P. fluorescens*-inoculated group (PF). All treatments were CK group grown under $aCO_2$ (aCK) and $eCO_2$ (eCK), BS group grown under $aCO_2$ (aBS) and $eCO_2$ (eBS), PF group grown under $aCO_2$ (aPF) and $eCO_2$ (ePF), respectively. To conduct the PGPR inoculation, the bacterial strains *B. subtilis* subsp. (GDMCC 1.372) and *P. fluorescens Migula* (GDMCC 1.782) were selected based on our previous study, and their beneficial traits were assessed (Ng et al., 2022b). The bacteria cultures were grown on nutrient agar for routine use and were maintained in Luria-Bertani broth with 15% glycerol at −80°C for long-term storage. The inoculated volume was 15 mL/pot of diluted PGPR solution at a concentration of 10^8 CFU/mL in the BS and PF groups during plant growth at 186-h intervals to ensure their colonization (Yadav et al., 2014). Due to the BS and PF inoculants being diluted with 0.9% NaCl solution, the same volume of 0.9% NaCl solution was inoculated into the CK group at the same frequency as the BS and PF groups. Each treatment contained 3 pots, and each pot contained 16 seed tubers at the beginning of the cultivation. During cultivation, the tuber production of *P. heterophylla* varied among individual plants. After harvest, the plants that exhibited tuber formation were counted into the replicates (12–19). Plants that did not form tubers and those that died due to reasons, such as germination failure and disease during the 150-day cultivation period were excluded from the count.

## 2.3 Plant growth, productivity, and disease incidence

To evaluate the growth changes in *P. heterophylla*, the shoot characteristics, including leaf area, number of leaves per plant, and shoot height, were monitored at five different time points after its germination: 60, 75, 90, 105, and 120 days. The leaf area was obtained with the software Image J (Abràmoff et al., 2004). Leaves numbers were counted and shoot height was measured with a ruler. Furthermore, we measured the photosynthetic parameters with the three fully expanded uppermost leaves of each plant. Stomatal conductance ($g_s$) and soil plant analysis development (SPAD) were measured using a Leaf Porometer (SC-1, United States) and a chlorophyll meter (SLY-C, China), respectively. After 150 days of growth, plants were harvested and washed with deionized water (Supplementary Figure S2). Then, the dry weights of shoot, root, and tubers were recorded to calculate the ratio of shoot dry weight to height ($W/H_{shoot}$), specific root length (SRL), tuber dry biomass, and harvest index (HI) based on Equations 1–4, respectively (Roberts, 2020; Bell and Fischer, 1994):

$$\text{Ratio of shoot dry weight to height (g/cm)} = \frac{\text{Dry weight of shoot}}{\text{Shoot height}} \quad (1)$$

$$\text{Specific root length (cm/g)} = \frac{\text{Root length}}{\text{Dry weight of root}} \quad (2)$$

$$\text{Tuber dry biomass (mg / plant)} = \text{Dry biomass of tubers per plant} \quad (3)$$

$$\text{Harvest index} = \frac{\text{Tuber dry biomass}}{\text{Total dry biomass per plant}} \quad (4)$$

The maximum widths and lengths of the tubers were measured to present the morphology according to Hong Kong Chinese Materia Medica Standard (2020).

To assess the tuber quality, extraction and analysis of the active compounds comprised of polysaccharides (Hong Kong Chinese Materia Medica Standard, 2020), saponins (Ng et al., 2022a), and heterophyllin B (Zheng et al., 2019) were conducted. All tuber samples were dried at 65°C up to dryness via the oven-drying method and crushed to powder. To measure the polysaccharides, 0.5 g of the tuber powder was extracted with 30 mL of Milli-Q water in a water bath for 60 min. The polysaccharides content was measured using the anthrone-sulfuric acid colorimetric method at 625 nm with a UV–visible spectrophotometer (Lambda 950, Perkin Elmer, United States). To measure the saponins content, 0.1 g of dried powder was extracted with 40 mL methanol at 60°C for one hour. The saponin content was

estimated using ginsenoside Rb1 as the reference standard. The absorbance of the solution was measured at 560 nm using a UV–visible spectrophotometer (Lambda 950, Perkin Elmer, United States). To measure the heterophyllin B content, 0.5 g of dried powder was ultrasonicated in 25 mL of ethanol for 45 min and filtered through a membrane (0.45 μm). high-performance liquid chromatography (HPLC) analysis was performed on a 1,200 series HPLC system (Agilent, United States). The one-point external standard method was used to calculate the content of heterophyllin B.

To calculate the disease incidence, the number of *P. heterophylla* tubers with wilt observation of disease symptoms, including wilting and dry brown rot, and the total number of *P. heterophylla* tubers after harvest in each treatment were counted. Each plant had one tuber, thus, the total number of tubers was 12–19 in all treatments. Disease calculation follows the below Equation 5.

$$\text{Tuber disease incidence (\%)} = \frac{\text{Number of tubers with wilt observation}}{\text{Number of total tubers}} \times 100\% \quad (5)$$

## 2.4 Quantification of coupled effects

To assess the coupled effects of PGPR and $eCO_2$, the independent action concept is used (Lasch et al., 2020). This method is applied to components with different modes of action, so the coupling effects can be calculated from the response of the individual components (Foucquier and Guedj, 2015). In the current study, the predicted effects' values were calculated with Equation 6 (Alassane-Kpembi et al., 2017):

$$\text{Coupled (A+B) predicted value} = (\text{mean value A} + \text{mean value B}) - (\text{mean value A} \times \text{mean value B}) \quad (6)$$

A is the effects of PGPR, and B is the effects of $eCO_2$. The combination index (CI) is used to distinguish the different effects. CI is defined in Equation 7:

$$CI = \frac{\text{Coupled (A+B) predicted value}}{\text{Coupled (A+B) measured value}} \quad (7)$$

The empirical threshold of $CI < 0.9$ is set as the threshold indicates synergistic effects (synergism in PGPR and $eCO_2$). $0.9 \leq CI \leq 1.1$ is set to indicate the additive effects, and $CI > 1.1$ is set to indicate antagonistic effects (Chou, 2006).

## 2.5 Soil sampling, DNA extraction, and metagenome sequence analysis

To collect the rhizosphere soil, *P. heterophylla* plants were carefully uprooted from each pot. The rhizosphere soil was obtained by gently shaking off loosely attached soil around the roots and collecting the soil tightly attached to the roots (Akbar et al., 2022). Subsequently, the collected soil samples were then homogenized by pot. Each treatment had three pots, and therefore, the rhizosphere soil sample had three replicates per treatment. The soil samples were sieved through a 2-mm mesh, and a portion of each sample was stored at −80°C for total DNA extraction. The remaining soil was air-dried and used for nutrient and indole-3-acetic-acid (IAA) determination following Ng et al. (2022b) and Yan et al. (2022). The total DNA extraction was performed using an E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, United States). The quality of extracted soil DNA was assessed with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, United States).

Metagenome sequence analysis was conducted at the Beijing Genomics Institute (BGI, Hong Kong, China). The DNA from each sample was fragmented, end-repaired, and adenylated. Adaptors were then ligated to the ends of the fragments, and a polymerase chain reaction (PCR) was carried out to amplify the product. The PCR product was then subjected to circularization, and single-stranded circular DNA molecules were produced via rolling cycle amplification. These were loaded onto patterned nanoarrays and sequenced using combinatorial Probe-Anchor Synthesis. All the raw data were trimmed using SOAPnuke v.1.5.2, and the trimmed reads were mapped to the host genome to remove host-originated reads (only for samples of host origin). High-quality reads were assembled using MEGAHIT software, and contigs with lengths less than 200 bp were discarded. Genes were predicted using MetaGeneMark, and redundant genes were removed using CD-HIT with identity and coverage cutoffs of 95 and 90%, respectively. Salmon software was used for quantification. Moreover, annotation information was generated by aligning the protein sequences of genes against a functional database (like KEGG) using DIAMOND. Taxonomic annotation was assigned based on the Kraken LCA algorithm, and Bracken software was used to generate the taxonomic and functional abundance profiles. Wilcoxon's rank sum test was used to determine the features with significantly differential abundances across groups, and differentially enriched KEGG pathways were identified using reporter scores. Statistical analysis of the Wilcoxon rank test and the Kruskal-Wallis H test were performed using the R project.

## 2.6 Statistical analysis

The normality of the soil physicochemical properties data and plant-associated data was analyzed and represented as the mean with standard deviation. Statistical analysis was conducted using the software package SPSS version 20.0. Differences between mean values of soil physicochemical properties and plant-associated parameters were assessed via a two-way analysis of variance (ANOVA) followed by *post hoc* comparisons using the least significant difference (LSD) test (Yang et al., 2022). Significance was assessed at the 95% confidence interval ($p < 0.05$). Microbial composition and diversity results were analyzed in the RStudio (Version 2023.06.1). The alpha diversity was quantified by the Shannon, Chao1, and Simpson indices using the relative abundance profiles at the species level with the R package "vegan" (Oksanen et al., 2019). The beta diversity was presented with principal coordinate analysis (PCoA) based on the Bray-Curtis distance. The permutational multivariate analysis of variance, function adonis, transformed data by Bray-Curtis, permutation = 999 (PERMANOVA) was used to analyze the differences in the microbial community profiles with the "vegan" package (Zhang et al., 2023). Mantel test was performed using the

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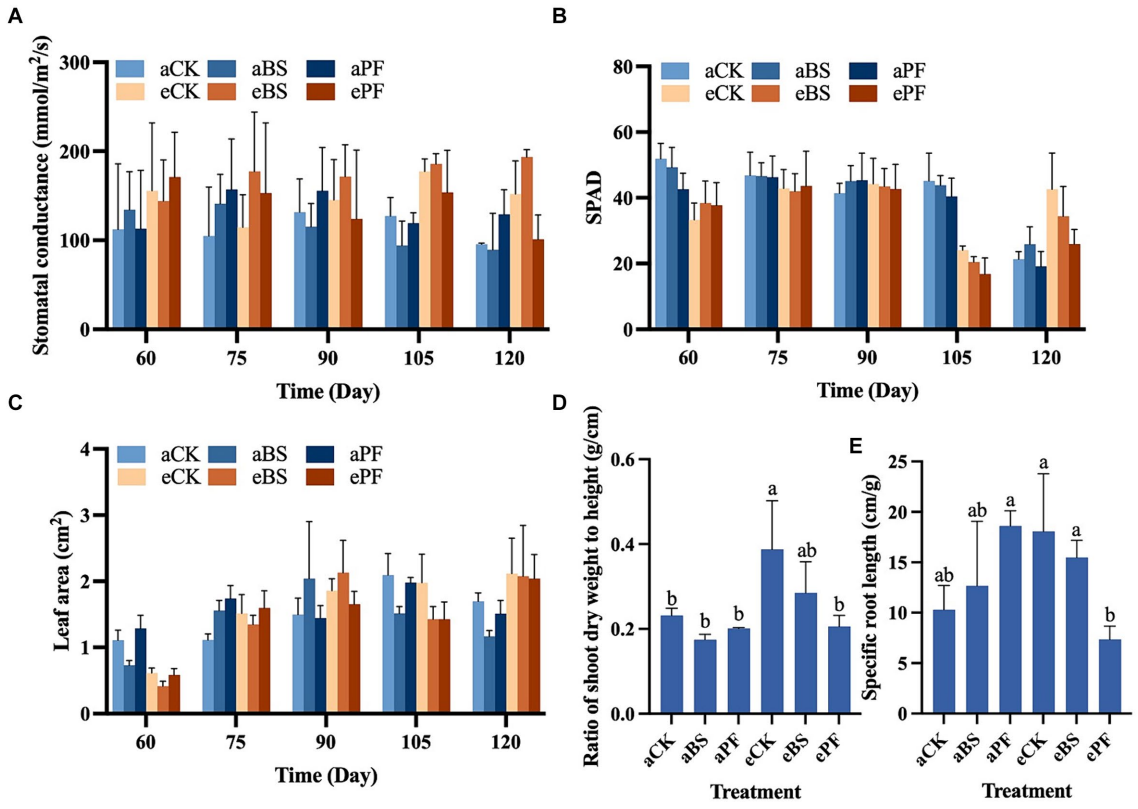


FIGURE 1
Growth traits for *P. heterophylla* individuals cultivated with inoculation of PGPR (*B. subtilis* and *P. fluorescens*) under different atmospheric $CO_2$ levels (ambien: 400 ppm, elevated: 1000 ppm) at 60, 75, 90, 105, and 120 days of development. **(A)** Stomatal conductance. **(B)** Soil plant analysis development (SPAD). **(C)** Leaf area. **(D)** Ratio of shoot dry weight to height. **(E)** Specific root length. Bars represent the mean with standard deviation ($n = 12–19$). Different letters on the top of each bar indicate the significance of differences among treatments ($p < 0.05$).

"vegan" package in R to determine correlations between plant–soil factors and rhizosphere microbial communities. Spearman's correlation analysis of dominant bacterial genera and biological pathways in the rhizosphere was performed using the "psych" package in R (Zhang et al., 2023). Random Forest analysis was used to disentangle the potential main predictors of *Fusarium* pathogen, harvest index, and active compound contents (Ding et al., 2022).

# 3 Results

## 3.1 Plant photosynthesis performance, shoot, and root development

The photosynthesis performance of *P. heterophylla* was significantly affected by $eCO_2$ treatment and $eCO_2$ coupled with BS treatment. With the elevation of $CO_2$, the stomatal conductance ($g_s$) showed a pronounced increase in treatments at $eCO_2$ conditions, especially at 105 and 120 days (Figure 1A). The $g_s$ describes the rate of gas exchange (e.g., $CO_2$ uptake) and functions as the measure of stomatal opening in response to environmental conditions. Greater $g_s$ indicates the photosynthesis rate is higher. Although inoculating PGPR also increased the $g_s$ than control treatments, the differences were not significant. Higher $g_s$ values were observed in the eBS treatments from 75 days to 120 days. Soil plant analysis development (SPAD) measures leaf chlorophyll concentrations and larger values indicating higher leaf photochemical efficiency. The eCK and eBS treatments at 120 days exhibited larger SPAD values than aCK (Figure 1B). Shoot development including leaf area, leaf number, and shoot height was increased by PGPR and/or $eCO_2$ (Figure 1C; Supplementary Figure S3). The leaf area showed a remarkable increase from 60 to 75 days of plant development. It increased by 20–25% at 120 days in eCK, eBS, and ePF treatments, thereby surpassing other treatments during this period (Figure 1C). PGPR coupled with $eCO_2$ enhanced the shoot development, with higher shoot height and larger ratio of shoot dry weight to height ($W/H_{shoot}$) were observed at 75 and 105 days (Figure 1D). Compared to aCK, $W/H_{shoot}$ increased by 70 and 26% at eCK ($p < 0.05$) and eBS, respectively. The root development, especially the specific root length (SRL), was improved under $eCO_2$ (Figure 1E).

## 3.2 Plant productivity and disease incidence

The productivity of *P. heterophylla* was described by yield (tuber biomass and harvest index) and tuber quality (the contents of the active ingredients) (Figure 2). Compared to aCK, the tuber dry biomass significantly increased by 7, 36, and 38% in aBS, eCK, and ePF, respectively. Additionally, the harvest index showed enhancements ranging from 5 to 24% in PGPR and $eCO_2$-treated

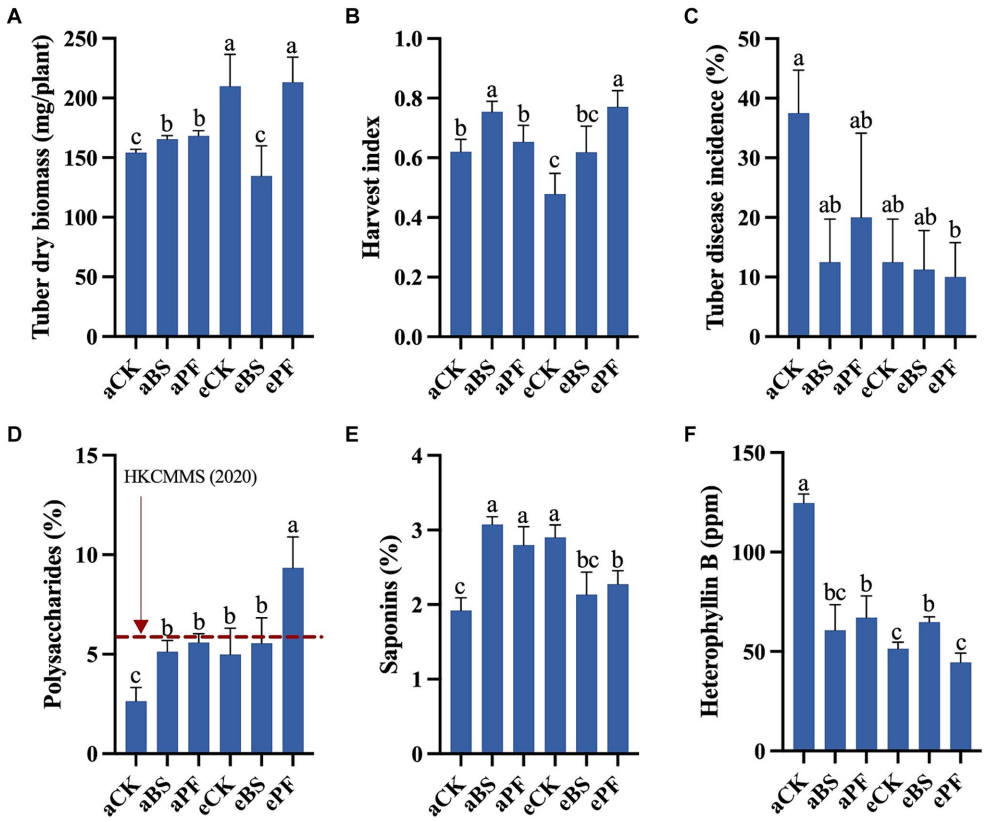


FIGURE 2
Tuber yield, quality, and disease incidence of *P. heterophylla* with different treatments. **(A)** Tuber dry biomass. **(B)** Harvest index. **(C)** Tuber disease incidence. **(D)** Proportion of polysaccharides in tubers. **(E)** Proportion of saponins in tubers. **(F)** Proportion of heterophyllin B in tubers. Bars represent the mean with standard deviation ($n = 12–19$). Different letters on the top of each bar indicate the significance of differences among treatments ($p < 0.05$).

groups. The ePF had plants with the largest tuber dry biomass at 213 mg/plant, as well as the highest harvest index at 0.77 (Figures 2A,B). Taken together, the results showed that the yield of *P. heterophylla* was enhanced the most in the ePF treatment.

Compared to aCK, the proportions of polysaccharides were significantly increased from 2.65 to 9.35%, with a 253% increase (Figure 2D). The highest polysaccharide content was found in ePF, at 9.3% (g/g), which is over 5 times higher than the content found in tubers grown in Anhui Province (1.62%, g/g) (Deng et al., 2018). The percentages of saponins were significantly increased by 60, 46, 51, and 19% in aBS, aPF, eCK, and ePF, respectively, compared to aCK (Figure 2E). The aBS showed the highest saponins content of 3.1% (g/g), which is nearly 7 times higher than the content found in tubers grown in Fujian Province (Ma et al., 2018). As for the heterophyllin B, applying PGPR and $eCO_2$ decreases its percentages by 46–64% relative to aCK (Figure 2F). Overall, the polysaccharides and saponins proportions were positively increased with PGPR and $eCO_2$, while heterophyllin B proportion in tubers was negatively decreased.

Inoculating with PGPR and/or elevation of $CO_2$ reduced tuber disease incidence by 46.7 to 73.3% compared to the control group (Figure 2C). Although the PGPR or $eCO_2$ resulted in a lower tuber disease incidence than aCK, the difference was not significant. The most significant decrease in tuber disease incidence was found in the ePF treatment with a significant 73.3% reduction ($p < 0.05$). The relative abundance of *F. oxysporum* in the rhizosphere showed similar trends as the disease incidence (Figure 3).

## 3.3 Structure and composition of the indigenous microbial community

PCoA results of the beta diversity showed that the untreated group, i.e., aCK, was clearly separated from other treatments (Figure 3A), suggesting PGPR and $eCO_2$ influenced the indigenous bacterial community, but the fungal community change was less evident (Figure 3B). Similar to the Chao 1 index, the elevation of $CO_2$ only increased the alpha diversity of bacterial community (Supplementary Table S2). The fungus *Fusarium* associated with wilt disease showed a marginal decrease in density in response to PGPR and $eCO_2$ application. As depicted in Figure 3C, compared with aCK, the relative abundance of *F. oxysporum* significantly decreased by 57, 57, and 45% in aBS, aPF, and eCK, respectively. Moreover, its abundance was further decreased in eBS and ePF, with a 78 and 72% reduction being observed instead, relative to aCK.

Within the composition of bacterial communities, at the phylum level (Figure 3D), *Proteobacteria* and *Actinobacteria* were the most dominant across all treatments. Compared to aCK, the application of PGPR and $eCO_2$ significantly increased the relative abundance of

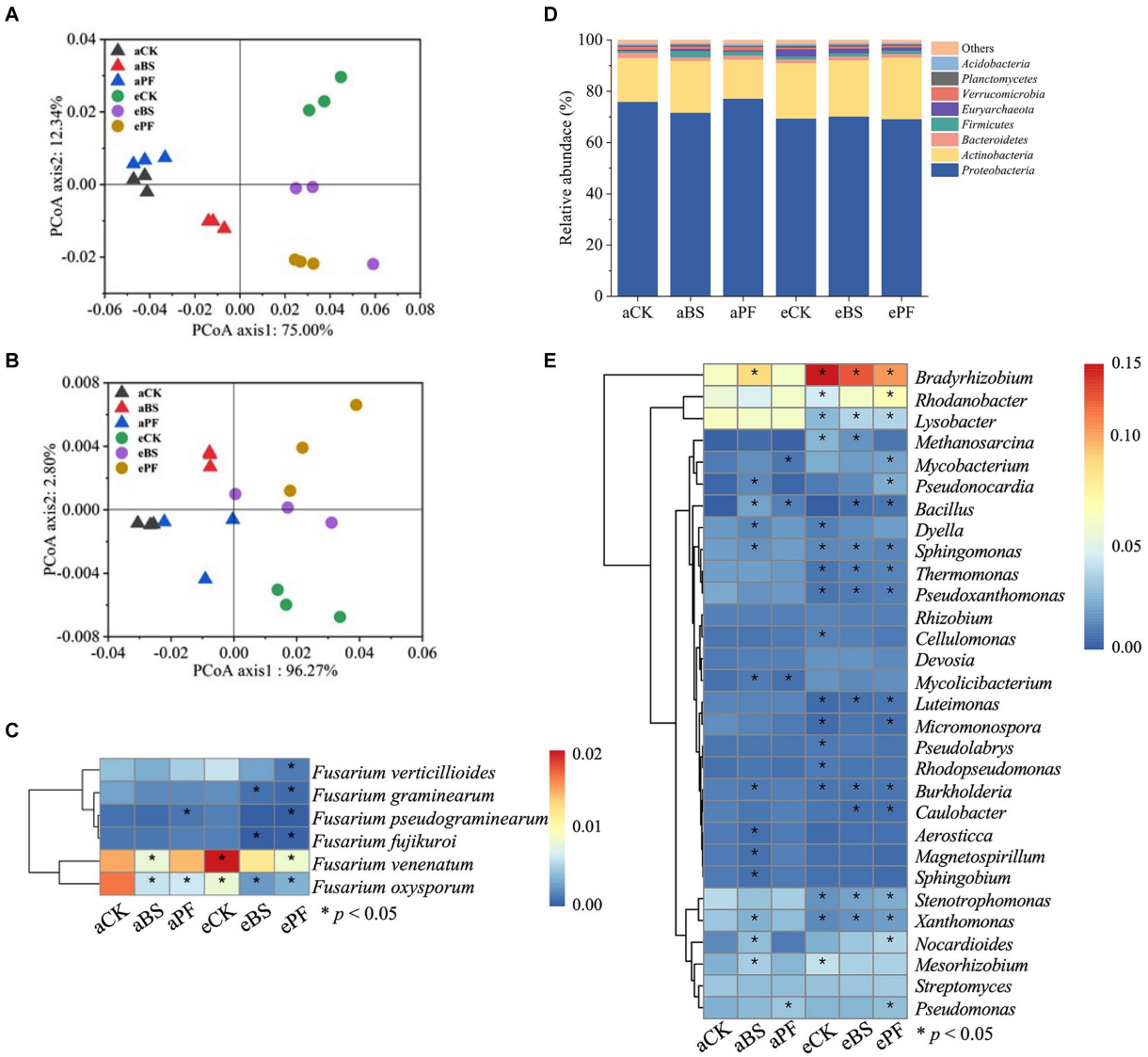


FIGURE 3
The structure and composition of soil microbiome. PCoA was used to visualize the dissimilarity of **(A)** bacterial and **(B)** fungal compositions. **(C)** Relative abundance of fungus *Fusarium*. Relative abundance of bacterial communities at the **(D)** genus level and **(E)** phylum level. The asterisks in the boxes of the heatmaps **(C,E)** indicate the significance of differences among treatments ($p < 0.05$).

*Actinobacteria* by 18–40%, except for the aPF treatment. The dominant bacterial genera were also altered by applying PGPR and $eCO_2$ (Figure 3E). The relative abundances of *Bradyrhizobium*, *Rhodanobacter*, and *Mesorhizobium* were significantly higher in the PGPR coupling with $eCO_2$ treatments. The relative abundances of *Lysobacter* and *Stenotrophomonas* were significantly lower in the PGPR coupling with $eCO_2$ treatments.

## 3.4 Changes in metabolites biosynthesis biological pathways in indigenous microbiome

KEGG biological pathway analysis revealed major transcriptional alterations in different pathways (Figure 4). Spearman correlation analysis showed that soil microbial components were correlated to biological pathways prominence in the rhizosphere (Figure 4A; Supplementary Figure S6). The fungus *Fusarium* and dominant bacterial genera were significantly related to antibiotics biosynthesis pathways and secondary metabolisms pathways ($p < 0.01$). The application of PGPR and $eCO_2$ upregulated the pathways associated with antibiotic biosynthesis, including tetracycline, clavulanic acid, macrolides, and various alkaloids. The normalized abundance of genes in the tetracycline biosynthesis and biosynthesis of various alkaloids were significantly increased by 17–36% in eBS and ePF, compared to aCK (Figure 4B). In contrast to antibiotic biosynthesis, the plant-pathogen interaction and biosynthesis of various other secondary metabolite pathways were downregulated, suggesting relieved pathogen stress and decreased production of some phytohormones.

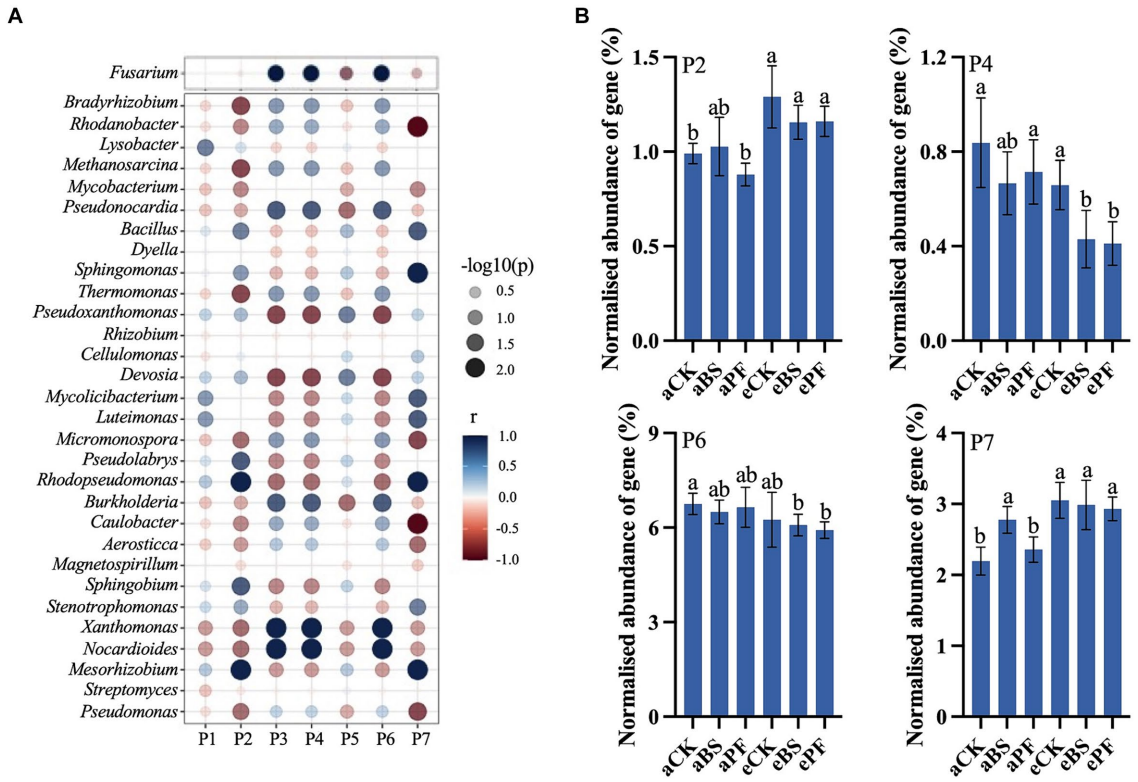


FIGURE 4
**(A)** Spearman correlation between soil microbes and biological pathways. **(B)** KEGG analysis of the normalized abundance of genes in response to PGPR and $eCO_2$. P2: Biosynthesis of various alkaloids. P4: Biosynthesis of various other secondary metabolites. P6: Plant-pathogen interaction. P7: Tetracycline biosynthesis. Bars in **(B)** represent the mean with standard deviation ($n = 3$). Different letters on the top of each bar indicate the significance of differences among treatments ($p < 0.05$).

## 3.5 Relationship between plant and indigenous microbiome

The Mantel test results showed the relationships in the plant–soil network. Plant characteristics were connected to soil microbial components and soil physicochemical properties (Figure 5A). The abundance of *Fusarium* pathogen was negatively related to the harvest index and root development. The abundance of inoculated PGPR was positively related to rhizosphere IAA. Pearson's correlation analyses showed that rhizosphere IAA was a crucial factor affecting root development, harvest index and the proportions of active compounds. Total organic carbon was positively related to root biomass, tuber biomass, and the content of polysaccharides. Additionally, soil bioavailable phosphorus was also related to root growth.

The Random Forest model results showed that rhizosphere microbial components were important predictors for the *Fusarium* pathogen presence, tuber harvest index, and active compound contents (Figure 5B). Two types of PGPR and thirteen recruited bacteria genera as the main microbial predictors of the *Fusarium* pathogen abundance in the rhizosphere. Among nine bacteria genera selected for predicting harvest index, *Bacillus* was the most significant indicator. Regarding the active compound contents, *Fusarium* and eleven bacteria genera were identified as potential microbial predictors.

Pearson correlation analysis revealed that the tuber yield was positively related to root length in all treatments except for aBS and eBS (Supplementary Figure S7). Instead, strong positive correlations among the photosynthetic parameters and tuber yield were found in aBS and eBS treatments. In aCK treatments, tuber quality was positively correlated to the leaf area, $W/H_{shoot}$, and root development. A positive relation was found between the content of polysaccharides and root development in treatments at $aCO_2$ conditions. While polysaccharides contents showed a positive correlation with leaf area in eBS and ePF treatments.

# 4 Discussion

## 4.1 Coupled effects of PGPR and $eCO_2$ on plant growth and productivity

To quantify the coupled effects of two environmental manipulations (PGPR and $eCO_2$) on plant growth and productivity, we used the independent action concept by calculating the combination index (CI) (Table 2). Empirical thresholds of the $CI < 0.9$, $0.9 \leq CI \leq 1.1$, and $CI > 1.1$ are set to indicate synergistic effects (synergism), additive effects, and antagonistic effects (antagonism), respectively (Chou, 2006; Lasch et al., 2020). Applying PGPR and $eCO_2$ together showed an additive effect (CI: 0.98) in the increased tuber yield (dry biomass and harvest index) (Table 2), with the dry biomass of tubers in this study approximately 1.5 times higher than that of a previous study (Ng et al., 2022a). This can be attributed to the

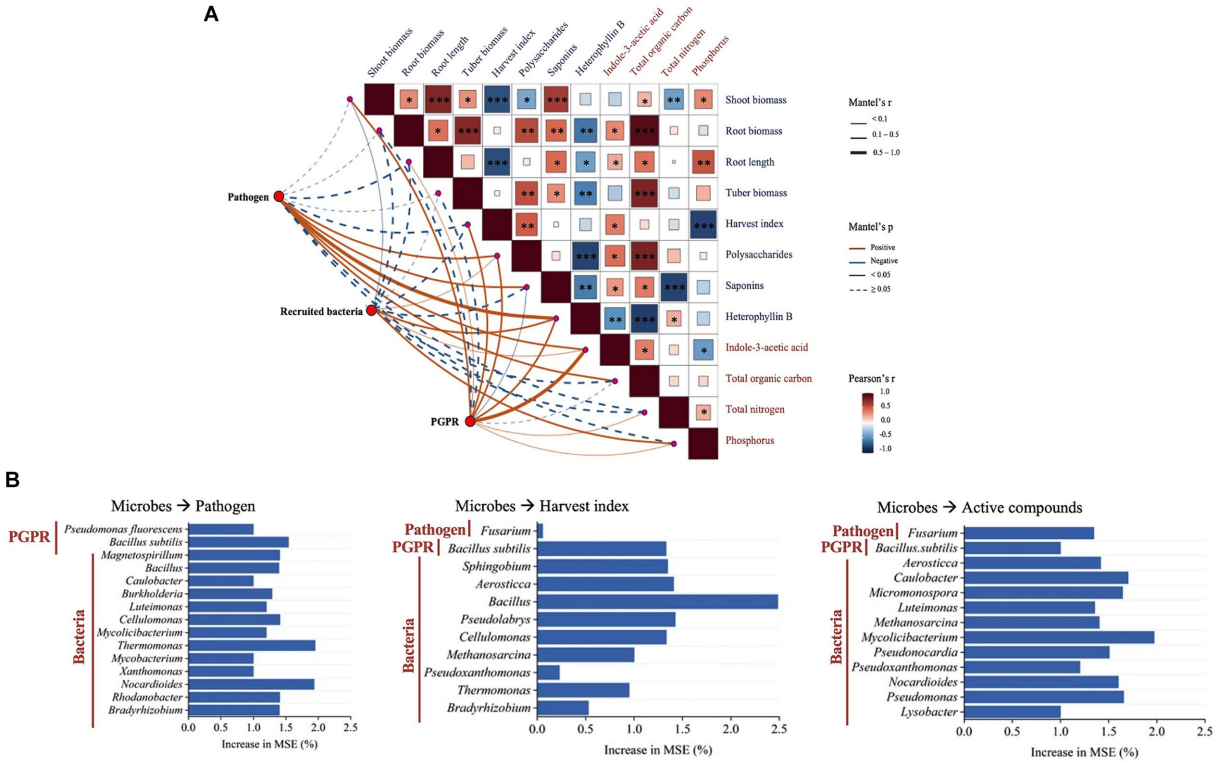


FIGURE 5
**(A)** Mantel test of the relationship between plant characteristics, rhizosphere microbial community components, and soil properties. The asterisks in the box *, **, and *** represent significant differences at the 0.05, 0.01, and 0.001 levels, respectively. **(B)** Random Forest analysis of predictors importance [percentage of increase in mean square error (MSE)] of rhizosphere microbes as drivers for the *Fusarium* pathogen, harvest index, and active compound contents in the tubers of *P. heterophylla*. Higher MSE% values imply more important predictors.

TABLE 2 Summary of combination index of coupled effects of PGPR and $eCO_2$.

| | | Combination index | |
|---|---|---|---|
| | | *B. subtilis* + $eCO_2$ | *P. fluorescens* + $eCO_2$ |
| Tuber morphology | Maximum width | 1.24 (ant.) | 1.05 (add.) |
| | Length | 1.43 (ant.) | 1.65 (ant.) |
| Yield | Tuber dry biomass | 1.55 (ant.) | 0.98 (add.) |
| | Harvest index | 1.24 (ant.) | 0.94 (add.) |
| Active compound contents | Polysaccharides | 1.33 (ant.) | 0.81 (syn.) |
| | Saponins | 1.44 (ant.) | 1.34 (ant.) |
| | Heterophyllin B | 1.34 (ant.) | 2.04 (ant.) |

$eCO_2$, elevated $CO_2$ condition (1,000 ppm); CI, combination index. CI < 0.9, 0.9 ≤ CI ≤ 1.1, and CI > 1.1 were set to indicate synergistic effects (syn.), additive effects (add.), and antagonistic effects (ant.), respectively (Chou, 2006).

promoted root development in ePF, as a strong positive correlation was found between tuber yield and root development (Figure 5; Supplementary Figure S7). The ratio of root length to dry weight (specific root length, SRL) provides a ratio of a standard unit of acquisition (root length) to resource investment (biomass) (Kramer-Walter et al., 2016; Poorter and Ryser, 2015). It characterizes economic aspects of the root system and is indicative of environmental changes, such as elevated $CO_2$ concentrations (Ostonen et al., 2007). In Figure 1E, the results showed the SRL of *P. heterophylla* increased slightly under $eCO_2$, suggesting enhanced nutrient uptake. This observation was different from Ostonen et al. (2017) reported that there was a decrease in the SRL of trees. The reason may be that root development varies from species to species, and it relies on growth conditions, root order, and root locations (Crookshanks et al., 1998). Moreover, PGPR inoculation significantly increased IAA in the rhizosphere, which was significantly correlated with average root length ($p < 0.01$) (Supplementary Figure S4). IAA induces vascular differentiation in the root system and enhances root development, contributing to nutrient and water uptake for plant vigor (Tarnawski et al., 2006). This finding was consistent with the results of another bacteria strain, *P. stutzeri* XL272, which stimulated cucumber growth through IAA production (Sun et al., 2022). The harvest index

illustrates biomass allocation. A higher harvest index in aBS and ePF indicated more efficient resource allocation to the economically valuable part (tuber), potentially increasing profitability. The increased harvest index can be attributed to the improvement in shoot and root development with the application of PGPR and/or $eCO_2$. Higher allocation of resources to root biomass may result in reduced resource allocation to above-ground shoots (Poorter and Ryser, 2015). Changes in the ratio of shoot dry weight to height ($W/H_{shoot}$) describe the response of plant morphology under different growth conditions. For example, *P. heterophylla* showed a larger $W/H_{shoot}$ under higher $CO_2$ conditions (Figure 1D), suggesting elevated $CO_2$ improved plants lateral growth more than vertical growth (Wu et al., 2004).

The content of active compounds in medicinal crops is crucial for their curative properties (Wei et al., 2020). However, little attention has been given to understanding how these qualities are affected by growth conditions and microorganisms. This study revealed that the proportion of three active compounds: polysaccharides, saponins, and heterophyllin B, were significantly influenced (Table 2). The synergistic promotion (CI: 0.81) of polysaccharides content in ePF was significant. The promotion may be due to the increased leaf area (Figure 1C), leading to higher photosynthesis and more carbohydrate accumulation in tubers (Burlak et al., 2013). Additionally, root dry weight and average root length showed increases in PGPR and $eCO_2$-treated groups (Supplementary Figure S3), which may enhance transpiration and partially mitigate the negative effects of $eCO_2$. The transpiration process drives increased carbohydrate transport to the tuber (Sun et al., 2022).

In contrast to polysaccharides, an antagonistic effect (CI: 1.1) of PGPR coupling with $eCO_2$ on the increased saponins and heterophyllin B over aCK was found. Their contents were negatively correlated with total nitrogen and total organic carbon in the soil and were affected by microbial components. This observation was in agreement with a previous study conducted by Xu et al. (2024) that identified the significant role of microbial communities in regulating soil nutrients. Developing a denser network of root hairs and lateral roots in eBS and ePF (Section 3.1) helped improve nutrient absorption ability. However, the higher uptake of microelements, such as Cu and Mn, negatively affected saponin biosynthesis (Wei et al., 2020; Ng et al., 2022a). Heterophyllin B is a cyclic octapeptide whose formation needs nitrogen-contained precursors. The downregulation of the biological pathways, like the biosynthesis of amino acids and various secondary metabolites, may influence its formation (Zheng et al., 2019; Ruan et al., 2023).

Tuberization is a complex process involving interaction between various genetic, biochemical, and environmental factors (Singh et al., 2015). Plants without tuber formation may be due to the varied temperatures during growth, which is one of the most significant factors that affect tuber formation. Besides, the condition factors, such as photoperiod, light (intensity and quality), mineral nutrition, water availability and pathogens can influence tuberization (Abelenda et al., 2014). In addition, at the beginning of the cultivation, some of the seed tubers failed to germinate, which may lead to an increased death rate. Moreover, the survival rate of plants excluding disease ranged from 58 to 75% (Supplementary Figure S9), which probably implied that a high rate of plants died because of disease. Additionally, PGPR inoculants significantly increased the maximum width of tubers under $aCO_2$ conditions ($p < 0.05$) (Supplementary Table S1), suggesting the tuber shape changed from elongated to ellipsoid. This may be due to the changes in the ATP/ADP translocator, which is the only protein dominant in the tuber morphology (Fernie and Willmitzer, 2001).

## 4.2 Suppression of fusarium pathogen by biocontrol bacteria in the rhizosphere

In this study, aCK had the highest abundance of the pathogen fungus *Fusarium*, which has a strong predictive importance for wilt incidence and crop quality (the content of nutrients and active ingredients) reduction (Guo et al., 2022; Tao et al., 2023). As a result, the tubers in aCK show wilting and dry brown rot, and its polysaccharides and saponins contents were lower than tubers of other treatments (Supplementary Figure S5). However, after applying PGPR and $eCO_2$, the *Fusarium* density, in particular *F. oxysporum*, significantly decreased. It has been demonstrated that reductions of *Fusarium* indicated a relieved pathogen threat to plants, leading to a decrease in wilt disease incidence (Deng et al., 2022). The decrease in disease incidences and pathogen densities is associated with the PGPR inoculation and elevation of $CO_2$. The disease-suppressive function of PGPR and $eCO_2$ is related to interactions between pathogenic fungus *F. oxysporum* and bacteria. Previous studies showed that the abundance of dominant bacteria families potentially contributed to the pathogen abundance in the rhizosphere of tomato plants (Deng et al., 2022). Our results enforce the suppression potential of the bacterial communities recruited in soils. They also complement previous findings indicating that bacterial functions related to metabolite biosynthesis pathways can effectively increase plant health and quality by reducing pathogenic fungi in various plant-pathogen systems (Guo et al., 2024; Wei et al., 2015). This suppressed growth of *Fusarium* is in line with research that showed the inhibitory effects of probiotic agents on *F. oxysporum* growth in other herbal plants, e.g., American ginseng (Boulahouat et al., 2023; Dukare et al., 2021; Liu et al., 2020) and tomato plants (Guo et al., 2024). Different from previous research, the decrease in *Fusarium* density was higher under $eCO_2$ conditions. Previous studies revealed the possible mechanism of PGPR against disease pathogens was reshaping the indigenous microbiome composition and function (Deng et al., 2022; Wu et al., 2021). Specifically, the beneficial strains, mostly *Pseudomonas poae*, were recruited into the rhizosphere of *P. heterophylla* to resist *Fusarium* wilt (Yuan et al., 2022). However, our study found no significant variance in the relative abundance of *Pseudomonas poae* among all treatments (Supplementary Figure S9). It is instead identified that the relative abundance of the dominant phylum *Actinobacteria* and the genera, e.g., *Rhodanobacter*, *Bradyrhizobium*, and *Mesorhizobium*, is increased in response to PGPR and $eCO_2$ treatments (Figure 3). As reported by Huo et al. (2018) and Palaniyandi et al. (2013), *Actinobacteria* and *Rhodanobacter* are prolific antibiotic producers in soil and can produce functioning metabolites, like antibiotics, to suppress pathogen growth. As such, it can be said that applying PGPR at $eCO_2$ recruits indigenous biocontrol bacteria, which can contribute to the suppression of *Fusarium* pathogens.

## 4.3 Regulation of metabolites biosynthesis biological pathway to reshape microbial community

The role of PGPR coupling with $eCO_2$ in rhizosphere microbial communities has been investigated using microbial analysis (Yu et al., 2016). To further examine the biocontrol mechanisms at the

molecular level, this study takes advantage of metagenomic analyses to characterize the transcriptional outcomes of microbe interactions in the soil. It was found that distinct gene expression profiles were present in the PGPR and $eCO_2$ treatments compared to the control. The biological pathways related to functioning metabolite biosynthesis significantly correlated with the abundance of *Fusarium* and indigenous dominant bacterial genera (Figure 4). This agrees with studies conducted by Sun et al. (2022) and Deng et al. (2022), which concluded that metabolic interactions play a significant role in reshaping the rhizosphere microbiome. With KEGG analysis, previous studies identified microbial agents against *Fusarium* disease in watermelons and tomatoes by manipulating metabolic pathways related to the synthesis of lignin, acid, and antibiotics (Cai et al., 2021; Zhang et al., 2022). Consistently, this study found that four typical pathways of antibiotic biosynthesis (tetracycline, alkaloids, macrolides, and clavulanic acid) were upregulated, two of which were significant, namely tetracycline and alkaloids. Rhizobacteria *Actinobacteria* has evolved an excellent ability to produce tetracycline, and *Pseudomonas* can produce alkaloids, which has been demonstrated by previous research (Klapper et al., 2016; Lozano et al., 2019; van der Heul et al., 2018). Applying PGPR coupled with $eCO_2$ significantly increased the abundance of *Actinobacteria* and *Pseudomonas*, strengthening our findings that PGPR coupled with $eCO_2$ contributes to the biosynthesis of antibiotics by recruiting biocontrol bacteria. Thus, *Fusarium* pathogen growth can be suppressed by the above biocontrol bacteria via upregulating antibiotic biosynthesis in the PGPR and $eCO_2$ treatments. This was further demonstrated by the decreased abundance of *Fusarium* in Figure 3. Akbar et al. (2022) reported that the plant-pathogen interaction is a significant player in mediating the plant immune response against invading pathogens. The relieved pathogen stress led to a downregulated plant-pathogen interaction, suggesting a decreased pathogen infection in plants following the application of PGPR coupled with $eCO_2$. By creating a favorable and healthy rhizosphere, the growth and productivity of *P. heterophylla*, including yield and quality, in PGPR and $eCO_2$-treated groups were enhanced more effectively.

The structure and composition of rhizosphere microbial communities are crucial for plant growth and health (Wei et al., 2019). The individual effects of PGPR or $eCO_2$ on microbial communities have already been well-studied (Gray and Smith, 2005; Kohler et al., 2009; Šibanc et al., 2014; Yilmaz and Karik, 2022). However, the response patterns of microbial communities to interactions between different manipulations (e.g., $CO_2$ elevation and PGPR inoculation) remain largely unknown. *P. fluorescens* coupled with $eCO_2$ slightly decreased the alpha index, reducing species richness and evenness (Supplementary Table S2). These changes were not observed when PGPR or $eCO_2$ treatments were administered separately, in which bacterial diversity was either unaffected or increased (Berg et al., 2020; Finkel et al., 2020; Jia et al., 2023). This might be due to plants in soil inoculated with PGPR at $eCO_2$ favoring certain bacterial species over others, as observed in Figure 3. This favor was also found in other plant species, such as barley (Zytynska et al., 2020), *Leymus chinensis* (Li et al., 2022), and *Brassica napus* (Mamet et al., 2022). Bacteria that best contributed to plant growth in the rhizosphere were greatly favored in this environment and, as such, were grown in greater abundances, leading to lower bacterial diversity. To bridge links between plant physiology and microbial activities under $eCO_2$, molecular signaling evaluations, including the role of osmolytes and reactive oxygen species, should also be explored. Additionally, further study should incorporate data on plant physiological parameters that would provide valuable insights into the mechanisms of plant-microbe interactions.

## 5 Conclusion

In summary, this study evaluated the coupled effects of different environmental manipulations on plant growth and productivity as well as the rhizosphere microbiome within a medicinal plant cultivation system. We provided evidence that increased root development and indigenous beneficial bacteria were associated with plant productivity (yield and active ingredient accumulation) in response to PGPR under $eCO_2$. Specifically, the recruitment of biocontrol bacteria probably is effective in inhibiting the growth of the pathogenic fungus *Fusarium*. The increase in these probiotics was accompanied by the upregulation of biological pathways related to the biosynthesis of secondary metabolites, which may contribute to the suppression of *Fusarium*. These findings highlighted the importance of PGPR under $eCO_2$ in improving active ingredient accumulation in medicinal plants. The balanced interaction between rhizosphere probiotics and *Fusarium* pathogen could be facilitated by the combined environmental manipulations, demonstrating the potential for sustainable agriculture practices. However, further studies on *in vitro* investigations of *Fusarium* and field applications with long-term cultivation are still needed.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA1083094.

## Author contributions

CN: Funding acquisition, Resources, Supervision, Writing – review & editing. WY: Formal analysis, Investigation, Visualization, Writing – original draft. YX: Methodology, Resources, Writing – review & editing. KT: Methodology, Resources, Supervision, Writing – review & editing. JT: Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1426893/full#supplementary-material

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