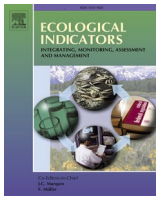
Ecological Indicators 171 (2025) 113202

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



Plant species richness increases the relationship between soil microbial and extracellular enzyme activities and enhances soil fertility

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ARTICLE INFO

Keywords:
Plant species richness
Microbial functional
PICRUSt2
Extracellular enzyme
Soil fertilizer

ABSTRACT

Diverse plant species are crucial for the sustenance of soil health and the facilitation of nutrient cycling by reconstructing the soil microbial communities and improving extracellular enzyme activities (EEAs). However, it is unclear whether the effect of plant species combination on the interaction between soil microbial communities and EEAs contributes to the improvement of soil quality. Therefore, we selected three dominant and seven subdominant plant species from the northern Yanchi desert steppe of Ningxia to assess plant species richness (monoculture and 4-, 6-, 8-, and 10-species mixtures). We found the following: (1) The number of ASVs of soil bacteria and fungi in monoculture was generally higher than that in mixed communities. Although the microbial abundance varied among plant species richness levels, the core microbial communities were the same. EEAs in monoculture were higher than species mixtures, but EEAs did not show a consistent trend with the increase of species richness. (2) Phylogenetic Investigation of Communities by Reconstruction of Unobserved States predicted 5 primary functions, encompassing 25 secondary functions dominated by bacterial metabolism, and 5 primary functions, encompassing 29 secondary functions dominated by fungal biosynthesis. (3) The Mantel test results demonstrated a strong correlation between soil carbon (C), nitrogen (N), and phosphorus (P) acquisition enzyme activities and the functional cellular processes of bacteria. (4) Structural equation modeling revealed that plant species richness directly negatively affected soil C, N, and P-acquiring enzyme activities. However, the functional activities of bacterial and fungal communities positively influenced soil organic carbon (SOC), total nitrogen (TN), and total phosphorus (TP) by indirectly regulating EEAs. The final model explained 69% of the SOC, 61% of the soil TN, and 25% of the soil TP. This study aimed to provide valuable data to support theoretical frameworks for conserving grassland biodiversity and maintaining soil health.

1. Introduction

Since 1500, approximately 30 % of the global species have faced the threat of extinction or have been driven to extinction (Isbell et al., 2023). In recent years, global climate change and intensified agricultural practices have emerged as primary drivers of the global biodiversity crisis (Li et al., 2024). Should this trajectory persist without intervention, the proportion of species at risk or endangered may escalate to 37 % by 2100 (Isbell et al., 2023). Research indicates that the reduction of plant diversity has been recognized as a major threat to ecosystem functions and services (Cardinale et al., 2012; Liang et al., 2016). Plant diversity influences soil functionality through the reconfiguration of soil microbial communities and modifications to extracellular enzyme activities (EEAs) (Kardol et al., 2010). However, whether the loss of plant diversity will reduce soil functionality remains unclear. Therefore, studying the interactions between plant species richness and soil microbial communities and EEAs is critical to maintaining soil health.

Within the context of biodiversity decline, both biotic and abiotic elements significantly impact soil microbial functional groups and EEAs (Xiao et al., 2018; Hutengs et al., 2021), which play a key role in the maintenance and restoration of soil ecosystem functions (Kardol et al., 2010). Soil microorganisms can decompose organic matter and release

Abbreviations: EEAs, Extracellular enzyme activities; ASVs, Amplicon sequencing variants; PICRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; SEM, Structural Equation Modeling; SOC, Soil organic carbon; TN, total nitrogen; TP, total phosphorus; KEGG, Kyoto Encyclopedia of Genes and Genomes; MetaCyc, MetaCyc Metabolic Pathway Database.

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https://doi.org/10.1016/j.ecolind.2025.113202
Received 13 October 2024; Received in revised form 12 January 2025; Accepted 4 February 2025

nutrients such as carbon(C), nitrogen(N), and phosphorus(P). Moreover, certain soil microorganisms are directly involved in processes like nitrogen fixation, phosphorus enhancement, as well as nutrient and water acquisition (Pedrinho et al., 2024). Microbial communities that are found in natural ecosystems are characterised by the presence of a small number of highly abundant taxa, alongside large number of rare taxa (Nemergut et al., 2011). Traditionally, microbial taxa with higher relative abundances have been considered more important within the community (Saunders et al., 2016). However, rare microbial communities play a pivotal role in global nutrient cycles and the functioning of ecosystems (Jousset et al., 2017). It is widely believed that the structure, composition, and functional groups of microbial communities change in response to specific microenvironment (Li et al., 2025). Since different plants have different physiological characteristics, changes in plant species and biomass may alter the activity and abundance of microbial functional groups (Wang et al., 2021). Soil EEAs can reflect the activity of soil function mediated by microorganisms (Chodak and Niklińska, 2010; Liu et al., 2021). For instance, soil microorganisms regulate β-1,4-glucosidase and β-1,4-N-acetylglucosaminidase activities, influencing soil nutrient cycling (Song et al., 2019; Chuan et al., 2020). Therefore, the interaction between microbial functional groups and EEAs is an important indicator for studying soil function (Burns et al., 2013; Liu et al., 2021). In addition, soil physical and chemical factors, including soil temperature (Steinweg et al., 2013), pH, moisture (Baldrian et al., 2010), and organic matter content (Sinsabaugh and Shah, 2012) are important drivers of changes in soil EEAs. These effects can maintain or enhance soil function and provide a beneficial environment for subsequent plant growth (Li et al., 2022). However, due to the differences in soil physicochemical properties and soil microbial functional groups of different plant communities, there may be significant differences in soil nutrient cycling processes among plant communities. Therefore, understanding the factors affecting soil microbial composition and EEAs in different plant communities is crucial for the nutrient cycling.

To sum up, most studies conducted have concentrated on examining changes in soil microbial community or EEAs concerning ecosystem functioning. Nevertheless, these studies have often overlooked the

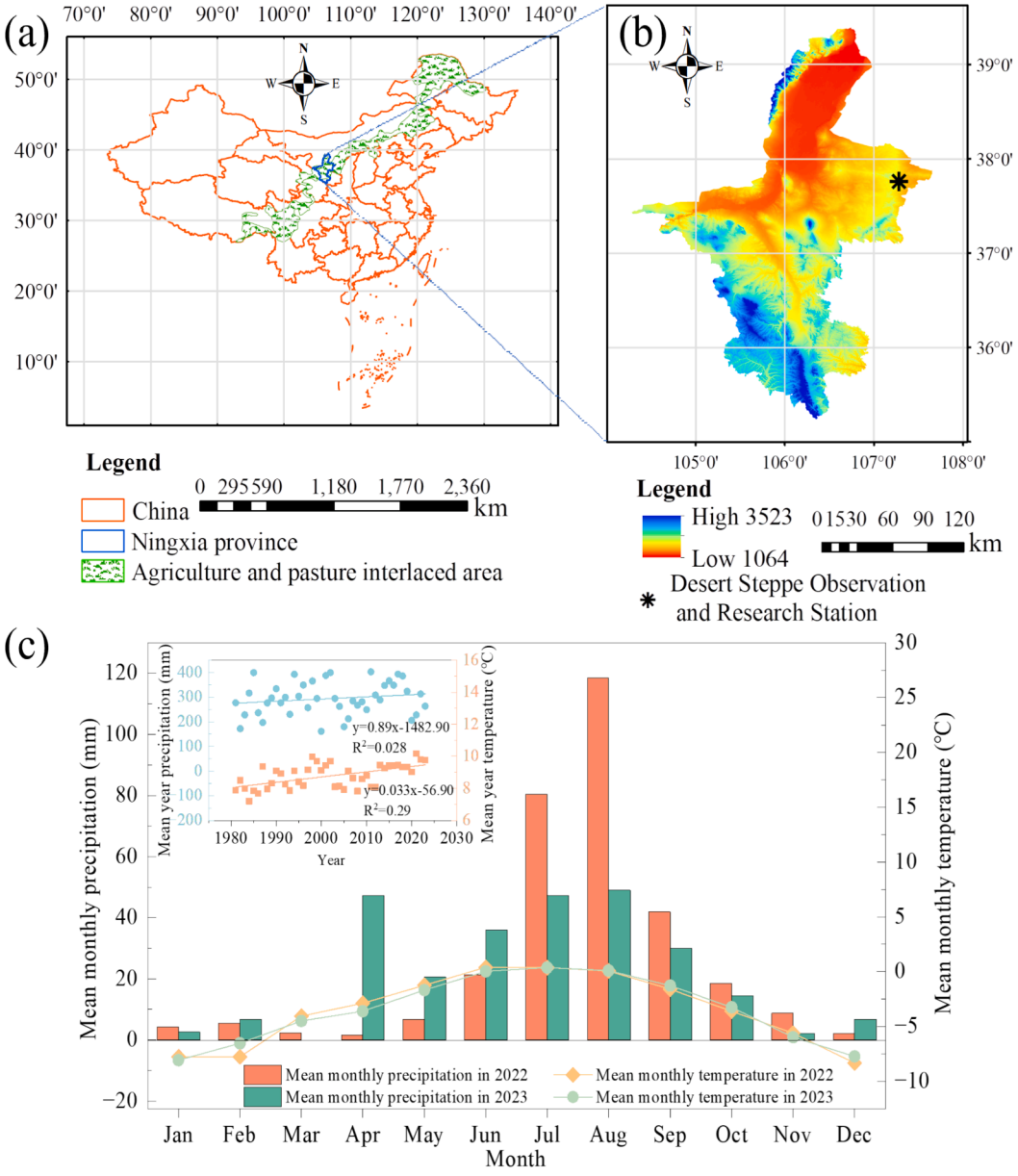

Fig. 1. Profile of the study area. (a) Intersection of agricultural and pastoral areas and location of Ningxia province in China; (b) Northern Yanchi Desert Steppe Observation and Research Station located in Yanchi County, Ningxia province; (c) Temperature and precipitation in Yanchi County from 1981 to 2023.

critical synergistic interactions between the two, which significantly influence soil functioning. By examining the specific and combined impacts of plant species richness on microbial communities and EEAs, we can gain valuable insights into how plant species diversity affects soil functionality. Therefore, we selected three dominant and seven subdominant species from the northern Yanchi desert steppe of Ningxia for monoculture and mixed-species to determine plant species richness (monoculture and 4-, 6-, 8-, and 10-species mixtures). We investigated the aboveground productivity, soil properties, microbial community composition, functional potential, and soil EEAs for the different species combinations, linking EEAs, microbial community and potential function, and abiotic factors with soil nutrient. The following two issues were addressed: (1) Plant species richness increased the relationship between soil microbial functional potential and EEAs. (2) Soil microbial function mixed with different species had a positive effect on soil nutrients by regulating EEAs. The results will deepen the understanding of the relationships among plant species richness levels, soil microbial functional groups, and EEAs and provide evidence for a theoretical framework for conserving grassland biodiversity and maintaining grassland soil health.

2. Materials and methods

2.1. Study sites

This study was conducted in the northern region of the Yanchi desert steppe (37°76′ N, 107°28′ E). It represents an intersection of agricultural and pastoral areas within northern China (Fig. 1a). The average altitude is estimated to be around 1600 m, and the climate is believed to be temperate continental, with a mean annual temperature of approximately 8.7°C and annual precipitation of around 294 mm (1981–2023), with over 60 % of this occurring between July and September (Fig. 1b and c). The annual evaporation is high at 2132 mm. The main soil types are calcareous, sandy, and silty, with relatively low fertility. The typical zonal vegetation includes desert steppes, with three dominant species, *Astragalus melilotoides*, *Agropyron mongolicum,* and *Lespedeza potaninii*; seven subdominant species, *Glycyrrhiza uralensis, Sophora alopecuroides, Agropyron cristatum, Cleistogenes squarrosa, Artemisia desertorum, Astragalus laxmannii,* and *Elymus dahuricus*; and other companion species, for example, *Achnatherum splendens, Leymus secalinus, Pennisetum centrasiaticum, Setaria viridis, Salsola collina, Polygala tenuifolia,* and *Allium mongolicum*.

2.2. Manipulated experiment

The experiment was conducted from May 16 to May 18, 2022, across approximately 6500 m^2 of a homogeneous habitat previously cultivated for agricultural production at the Northern Yanchi Desert Steppe Observatory and Research Station of Ningxia, China. Before the start of the experiment, the land was used to produce a single crop (mainly silage maize). After the harvest in the autumn of 2020, the land was deeply cultivated and fallowed in 2021. In order to reduce the pressure of weeds, a total of 4 times of weeding were carried out in the field. Before the start of the experiment in 2022. Deep plowing was conducted using a rotary tiller and plant roots, apoplastic materials, and plant residues were manually collected. Three dominant and seven subdominant species were selected from the northern Yanchi desert steppe of Ningxia for monoculture and mixtures to establish five plant species richness levels (monoculture and 4-, 6-, 8-, and 10-species mixtures). Species combinations included monocultures of 10 species, three groups of 4-species mixtures, three groups of 6-species mixtures, three groups of 8-species mixtures, and one group of 10-species mixtures, resulting in was 20 species combinations. Among them, there were at least one dominant species in 4-species mixtures, at least two dominant species in 6-species mixtures, and three dominant species in 8- and 10- species mixtures. subdominant species were filled according to species richness and functional groups. Monculture has 1 functional group, 4-, 6- and 8-species mixtures have 2–3 functional group, 10-species mixtures have 3 functional group (Table S1). Each treatment was replicated seven times. Additionally, there were 12 control plots without plants, resulting in 152 plots. A completely randomized experimental design was adopted, with plots measuring 5 m × 5 m and an aisle width of 1.5 m (see Fig. S1 for the sample plot schematic and aerial images).

Seeds in the monoculture sowing was sown at the maximum environmental capacity, and the proportion of mixed sowing was calculated as the actual price of the seeds used (i.e., the product of seed purity and germination) as a proportion of the weight of the monoculture sowing (sowing seed quantities in Table S2). All sample plots were weeded regularly to ensure that plant species richness was maintained at planned levels or slightly below planned levels where communities were not established. The stubble was mowed eight weeks after sowing, leaving approximately 10 cm of stubble, and the biomass was removed to support the establishment of the sown species. Unestablished species were replanted in mid-August 2022 using the following criteria: (1) species richness below 50 seedlings per m^2 and (2) monoculture species coverage of less than 5 %. No fertilizer was used in the experiment. Water was added occasionally after planting based on the soil moisture to ensure proper seed germination and emergence. No watering of the sample plots was performed since 2023.

2.3. Sample collection and measurement

Aboveground productivity measurements were carried out from August 15 to August 20, 2023, avoiding the edge of the plot (1.5 m), by randomly selecting a 1 m × 1 m sample plot and harvesting plant aboveground biomass by species, leaving a stubble height of 3 cm. Three replicates each of *A. melilotoides*, *A. mongolicum*, *A. cristatum*, *A. desertorum*, and *L. potaninii* were harvested from the monoculture, seven replicates of the 10-species mixtures were harvested, and the remaining species richness was sampled in three replicates. All samples were dried (48 h, 65 °C) to constant weight and weighed.

Three random soil samples (0–10 cm) were collected and thoroughly mixed to create a single representative soil sample using an in-situ soil sampler (Australia Cote VD51) within the sample plots where aboveground plant production was determined. After passing through a 2 mm sieve, one sample was promptly transferred to a 5 mL centrifuge tube, preserved in dry ice, and transported to the laboratory for storage at –80 °C for the determination of microorganisms. The results of soil microbial diversity were obtained based on the GENESCLOUD Platform (https://www.genescloud.cn). One sample is stored in an ice box and then transferred to a refrigerator at 4°C in the laboratory to determine the soil carbon (C), nitrogen (N), and phosphorus (P) acquisition enzyme activities, soil EEAs were ascertained using the 96-micropore enzyme plate method (Qi et al., 2016). One sample was shade-dried and used to determine the physical and chemical properties, and the level of soil organic carbon (SOC) was measured using external heating using potassium dichromate, total nitrogen (TN) was determined using a flow analyzer, and total phosphorus (TP) was determined using the molybdenum-antimony colorimetric method (Wang et al., 2023b). The soil pH was analyzed using a pH-3c meter (Leici, Shanghai, China). Soil temperature and moisture were measured at depths of 0–10 cm using a highly reliable portable temperature and moisture probe connected to an LI-8100 instrument (LI-COR Inc., USA).

2.4. DNA extraction, PCR amplification and Illumina high-throughput sequencing

The soil microbial DNA was extracted by Mobio kit (MOBIO Laboratories, Carlsbad, CA, USA), and the DNA was extracted and purified according to the operation procedure in the product manual. The concentration of the extracted nucleic acid was detected by enzyme-labeled instrument. The obtained DNA samples were used for PCR amplification of bacterial 16S rRNA and fungal ITS gene sequences. Bacterial diversity

analysis used universal primers F (5′-ACTCCTACGGGAGGCAGCA-3′) and R (5′-GGACTACHVGGGTWTCTAAT-3′) to amplify the V3 + V4 region of bacterial 16S rRNA gene. The sequence of ITS gene V1 region was amplified by general primers F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and R (5′-GCTGCGTTCTTCATCGATGC-3′) for fungal diversity analysis. Moreover, 10 bp barcode sequence was added to both ends of the primers to distinguish the samples. The sample DNA was amplified in a reaction system with a total system of 50 μL, including 1 μL Tap polymerase, 1 μL dNTP mixture, 1 μL upstream primer, 1 μL downstream primer, 1 μL template DNA, 25 μL PCR buffer, 20 μL ddH_2O was predenatured at 95℃ for 5 min after preparation, then denatured at 95℃ for 15 s, annealed at 52℃ for 15 s, extended at 72℃ for 45 s, repeated 30 times at 16S, ITS cycle 35 times, and finally extended at 72℃ for 5 min, the amplified product was gelled and recovered. Finally, sequencing libraries were constructed by mixing the purified amplification products in equal quantities, and Illumina MiSeq was used for high-throughput sequencing and the initial bacterial and fungal sequencing data were processed using QIIME 2 (Yang et al., 2023a).

2.5. Statistical analysis

One-way analysis and Tukey's multiple comparison method were used to identify significant variances in aboveground productivity, soil properties, microbial diversity, EEAs, and microbial functional potential among plant species richness levels (Wang et al., 2023b). All analyses were conducted using the SPSS software (IBM Corporation, USA). Soil microbial sequences were analyzed using the GENESCLOUD platform (https://www.genescloud.cn). The abundance of each metabolic pathway in the samples was obtained by referencing the 16S rRNA and ITS gene sequences to the genomic databases Kyoto Encyclopedia of Genes and Genomes (KEGG) and MetaCyc Metabolic Pathway Database (MetaCyc) using PICRUSt2 (Douglas et al., 2020). The analysis process is as follows: (1) First, the 16S rRNA and ITS gene sequences of the known microbial genome are aligned, the evolutionary tree is constructed, and the gene function spectrum of their common ancestor is inferred. (2) Align 16S rRNA and ITS feature sequences with reference sequences to construct a new evolutionary tree. (3) Castor hidden state prediction algorithm was used to predict the nearest sequence species of feature sequences according to the copy number of gene family corresponding to the reference sequence in the evolutionary tree, and then the copy number of its gene family was obtained. (4) The gene family copy number of each sample was calculated according to the abundance of each sample feature sequence. (5) Finally, the gene families were "mapped" to various databases, and the existence of metabolic pathways was inferred by default using MinPath, and then the abundance data of metabolic pathways in each sample was obtained. More details in https://github.com/picrust/picrust2/wiki/. The "LinkET" and "ggplot2" packages were used for a Mantel test to further test the relationships of soil C-, N-, and P-acquisition enzyme activities and bacterial and fungal functions (Peng et al., 2024). Structural equation modeling (SEM) was developed to determine the effects of soil properties, bacterial and fungal functions, and C-, N-, and P-acquisition enzyme activities on soil fertility, as well as their driving influences on SOC, TN, and TP among plant species richness levels, utilizing factors identified by stepwise regression analyses. These analyses were conducted using the "QuantPsyc," "nlme," "lme4," and "piecewise SEM" packages in R 4.4.1 (Yang et al., 2023b).

3. Results

3.1. Effect of plant species richness on soil microbial communities and soil EEAs

Actinobacteria, Acidobacteria, and Proteobacteria were the dominant phyla, there were two dominant phyla in the fungal community: Ascomycota and Basidiomycota (Fig. S2). The abundance of ASVs in bacterial communities was significantly greater than that observed in fungal communities, the number of soil bacteria and fungi in monoculture was higher than mixtures (Fig. 2). The number of bacterial ASVs in 8-species mixtures was higher than 6- and 10-species mixtures, but lower than 4-species mixtures, a comparison of the mixed plant communities showed that the highest number of shared bacterial species was 1541 and 1431 for the 8- and 6-species mixtures, respectively (Fig. 2a). And the highest number of shared species of fungi was found in the 8- and 4-species mixtures, with 143 and 120, respectively (Fig. 2b).

Mixed-species decreased the activity of C-, N- and P-acquiring enzymes activity, especially for C- and P-acquiring enzymes activity (Fig. 3a). The αG of monoculture was significantly higher than 10-species mixtures ($P < 0.05$), βG, CBH, and βxyl in monoculture were significantly higher than mixture ($P < 0.05$), but there was no significant difference between mixtures (Fig. 3b). NAG and LAP of monoculture were significantly higher than 10-species mixtures ($P < 0.05$), there were significant differences in LAP between monoculture and 8-species mixtures ($P < 0.05$), but no significant differences in NAG (Fig. 3c). AKP of monoculture was significantly higher than mixtures ($P < 0.05$) (Fig. 3d).

3.2. Functional potential of soil bacterial and fungal functional community among plant species richness

Five metabolic pathways were predicted for soil bacterial community function at the primary level based on KEGG, with metabolism being the primary function. The secondary level contained 25 functions, with the top two being metabolism-related amino acid and carbohydrate metabolism (Fig. 4, Table S4). The lipid metabolism of the 10-species mixtures was significantly higher than monoculture, but the glycan biosynthesis and metabolism, energy metabolism and nucleotide metabolism of monoculture were significantly higher than the 10-species mixtures (Fig. 4a). The membrane transport of soil bacteria was significantly higher in the 4-species mixtures than in the monocultures (Fig. 4b). Folding, sorting and degradation, and translation were significantly lower in the 8- and 10-species mixtures than in the monocultures. In addition, the genetic information processing of monoculture was significantly higher than 6-, 8-, and 10-mixture species ($P < 0.05$) (Fig. 4c). Cell growth and death and cellular community-prokaryotes of monoculture were significantly higher than 10-species mixtures, and transport and catabolism of 8- and 10-species mixtures were significantly higher than monoculture (Fig. 4d). The endocrine system of the monoculture was significantly higher than that of the mixtures, except for the 10-species mixtures. In addition, the organismal systems of monoculture were significantly higher than 4- and 6-mixture species ($P < 0.01$) (Fig. 4e).

Fungal communities have five metabolic pathways, of which biosynthesis and generation of precursor metabolites and energy are the main functions of MetaCyc. The secondary level contained 29 functions, mainly nucleoside and nucleotide biosynthesis, cofactors, prosthetic groups, electron carriers, vitamin biosynthesis, electron transfer, and respiration (Fig. 5, Table S4). Carbohydrate biosynthesis was significantly lower in the 10-species mixtures than in the other plant species (Fig. 5a). Pyrimidine deoxyribonucleotide de novo biosynthesis I, pyrimidine deoxyribonucleotide biosynthesis from CTP, and pyrimidine deoxyribonucleotide phosphorylation were significantly higher in the 8-species mixtures than in the 10-species mixtures (Fig. 5b). Fermentation and glycolysis were significantly higher in the 8-species mixtures than in the 10-species mixture. In addition, the generation of precursor metabolite and energy of 10-species mixtures was significantly lower than monoculture ($P < 0.05$) (Fig. 5c). Degradation/utilization/assimilation – Other was significantly lower in the 8-species mixtures than in the monocultures (Fig. 5d). There were no significant differences in glycan biosynthesis among plant species richness levels (Fig. 5e).

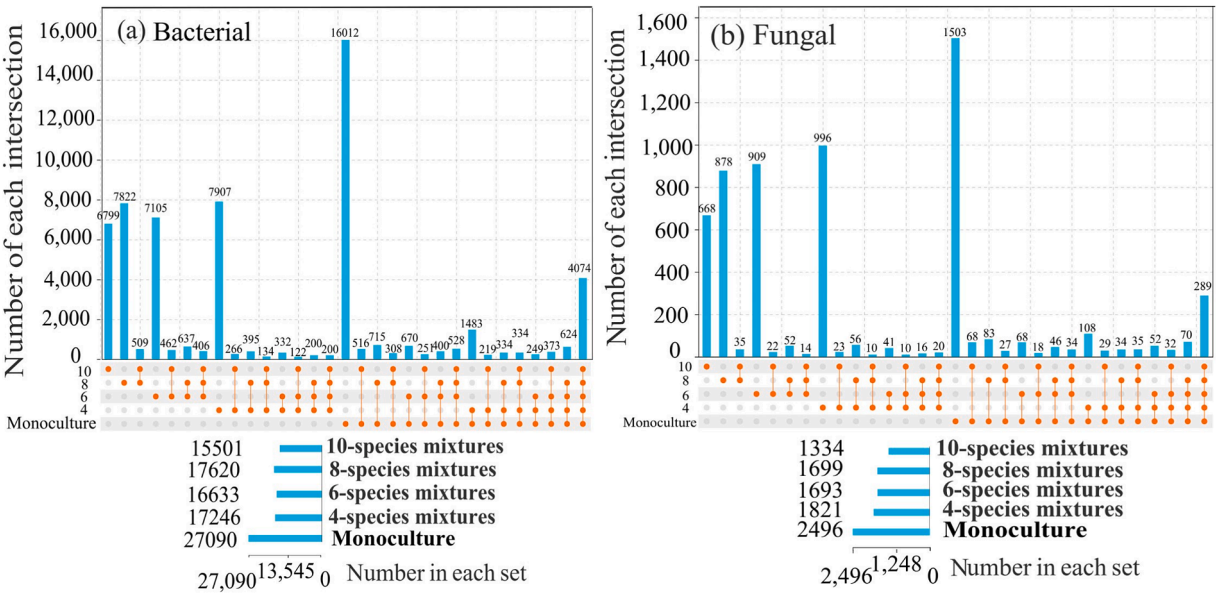

Fig. 2. Venn of the soil bacteria (a) and fungi (b) ASVs among the plant species richness levels (genus level).

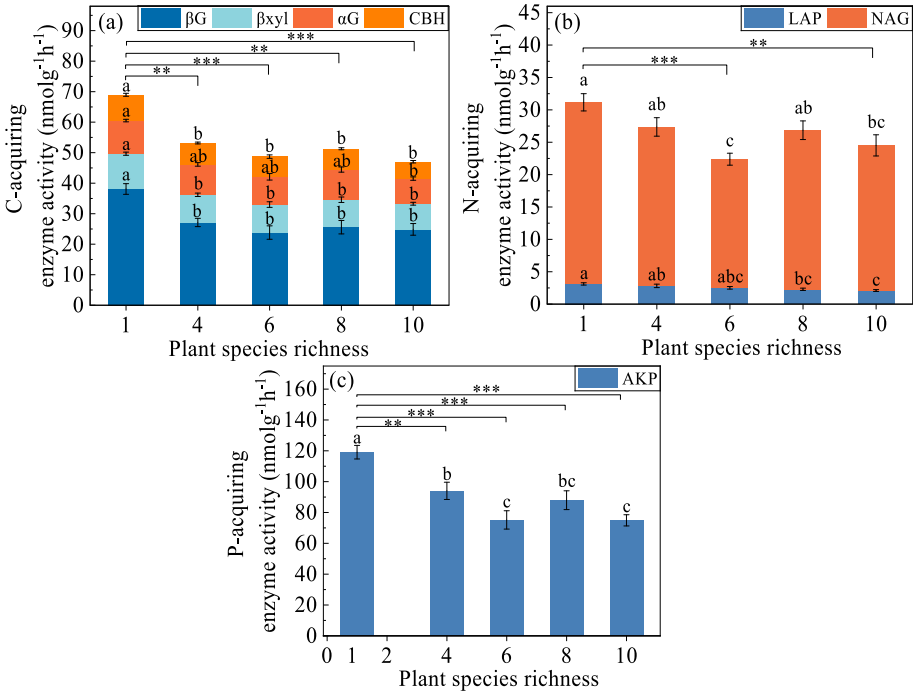

Fig. 3. Changes in the EEAs (a), C-acquiring enzyme activities (b), N-acquiring enzyme activities (c), and P-acquiring enzyme activities (d) among the plant species richness levels. αG denotes α-1,4-glucosidase, βG denotes β-1,4-glucosidase, CBH denotes cellobiose hydrolase, βxyl denotes β-1,4-xyloglucosidase, NAG denotes β-1,4-N-acetylaminoglucosidase, LAP denotes leucine aminopeptidase, AKP denotes alkaline phosphatase. The values represent the mean EEAs with a standard error (± SE). Within the same index, distinct lowercase letters signify statistically significant differences among plant species richness levels, with a significance level set at 0.05. The same below.

3.3. Relationship between the functional potential of soil microorganisms and EEAs among plant species richness levels

The Mantel test results demonstrated that soil C-, N-, and P-acquisition enzyme activities were strongly correlated with cellular processes. There was a significant correlation ($P < 0.05$) between the P-acquiring enzyme activity and soil fungal glycan pathways (Fig. 6a). βG and CBH showed a significant positive correlation with cellular processes, βxyl showed a significant negative correlation with biosynthesis, LAP showed a significant negative correlation with environmental information processing, and AKP showed a significant positive correlation with genetic information processing and a significant negative correlation with environmental information processing and organismal systems (Fig. 6b).

3.4. Direct and indirect pathways of soil EEAs and microbial functional potential to soil nutrients

SEM analysis was used to establish the integrated pathways of

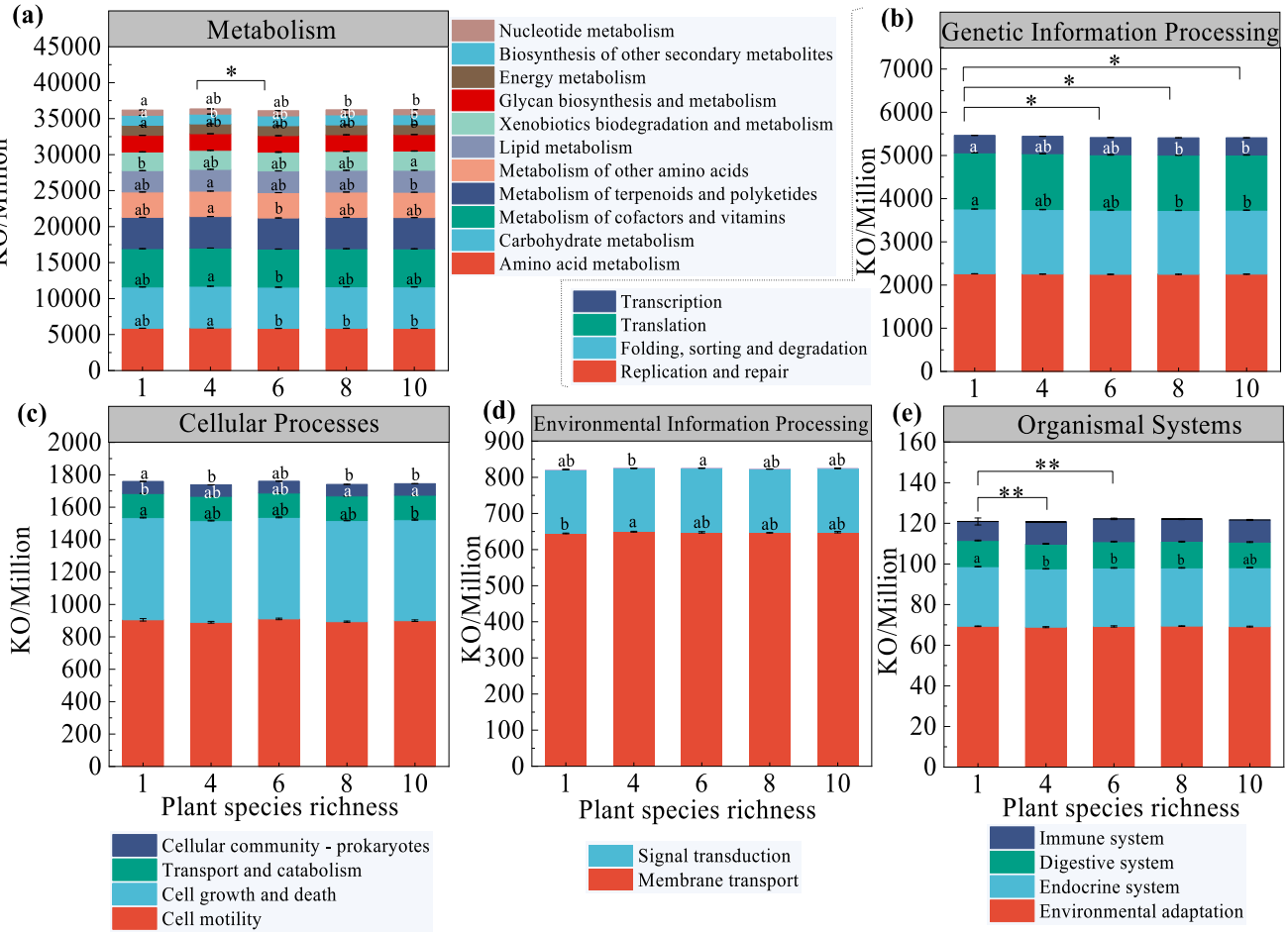

Fig. 4. Changes in the soil bacterial functional potential among the plant species richness levels. (a) Metabolism; (b) Environmental Information Processing; (c) Genetic Information Processing; (d) Cellular Processes; (e) Organismal Systems. The values represent the mean bacterial functional potential with a standard error (± SE). * and ** represent significant differences at $P < 0.05$ and $P < 0.01$ respectively. The same below.

dominant and subdominant plant species richness on SOC, TN, and TP in the northern Yanchi desert steppe of Ningxia. The final model explained 69 % of the SOC, 61 % of the soil TN, and 25 % of the soil TP (Fig. 7). Specifically, SOC was directly positively affected by plant species richness, C-acquisition enzyme activities ($P < 0.001$), and bacterial and fungal community functions, while negatively affected by soil physical properties (Fig. 7a). Soil TN was positively affected by plant species richness, N-acquisition enzyme activities ($P < 0.001$), bacterial community function ($P < 0.01$), and soil physical characteristics ($P < 0.05$) (Fig. 7b). Soil TP was positively affected by plant species richness, P-acquisition enzyme activities ($P < 0.01$), bacterial ($P < 0.05$) and fungal community functions and soil physical characteristics (Fig. 7c). In addition, we found that plant species richness had a direct negative effect on soil C-, N-, and P-acquisition enzyme activities, but bacterial functions positively affected SOC, TN and TP by indirectly regulating soil EEAs, the direct driver of soil TN was bacterial rather than fungal functions (Fig. 7).

4. Discussion

4.1. Effect of plant species richness on soil microbial functional groups and EEAs

A study of monocultures and mixtures of dominant and subdominant species in the northern Yanchi desert steppe of Ningxia revealed that ASVs shared among the five plant species richness levels accounted for 8.62 % of the total. This indicated common characteristics or species compositions among the different plant species mixtures. These shared ASVs likely represented common microbial communities that play significant roles in various plant communities. Actinobacteria, Acidobacteria, and Proteobacteria emerged as the dominant phyla, which is consistent with previous results (Li et al., 2023b). Members of the phylum Actinomycetes, found in environments with varying plant species richness, form hyphae that access water and scarce nutrients. This adaptive mechanism enhances their survival in arid conditions and may improve drought resistance in desert plants (Řeháková et al., 2015). Ascomycota can fix nitrogen and enhance nutrient availability by solubilizing phosphates and degrading residues (Yang et al., 2023a), thereby promoting the growth of desert steppe plants. Ascomycota fungi, which are primarily saprophytic, predominantly utilize easily decomposable carbon components, 8-species mixtures significantly increased the presence of Ascomycetes phyla in the soil compared to other mixtures. Basidiomycota prefer organic substances that are difficult to degrade such as lignin and cellulose (Yang et al., 2023a). It was also found that the core flora was the same among species richness. However, there were differences in community abundance (Fig. S2), further confirming that different plants in the same area share a core microbiota but differ in community abundance owing to the different environments of the sampling sites (Shade and Handelsman, 2012). Soil microbial ASVs exhibited a decreasing trend with increasing plant species richness, except for the 8-species mixture. This indicated that a combination of different species may have an impact on soil microbial communities (Wang et al., 2018). Extracellular enzymes released from plant roots and microbial cells play an important role in biogeochemical cycling in

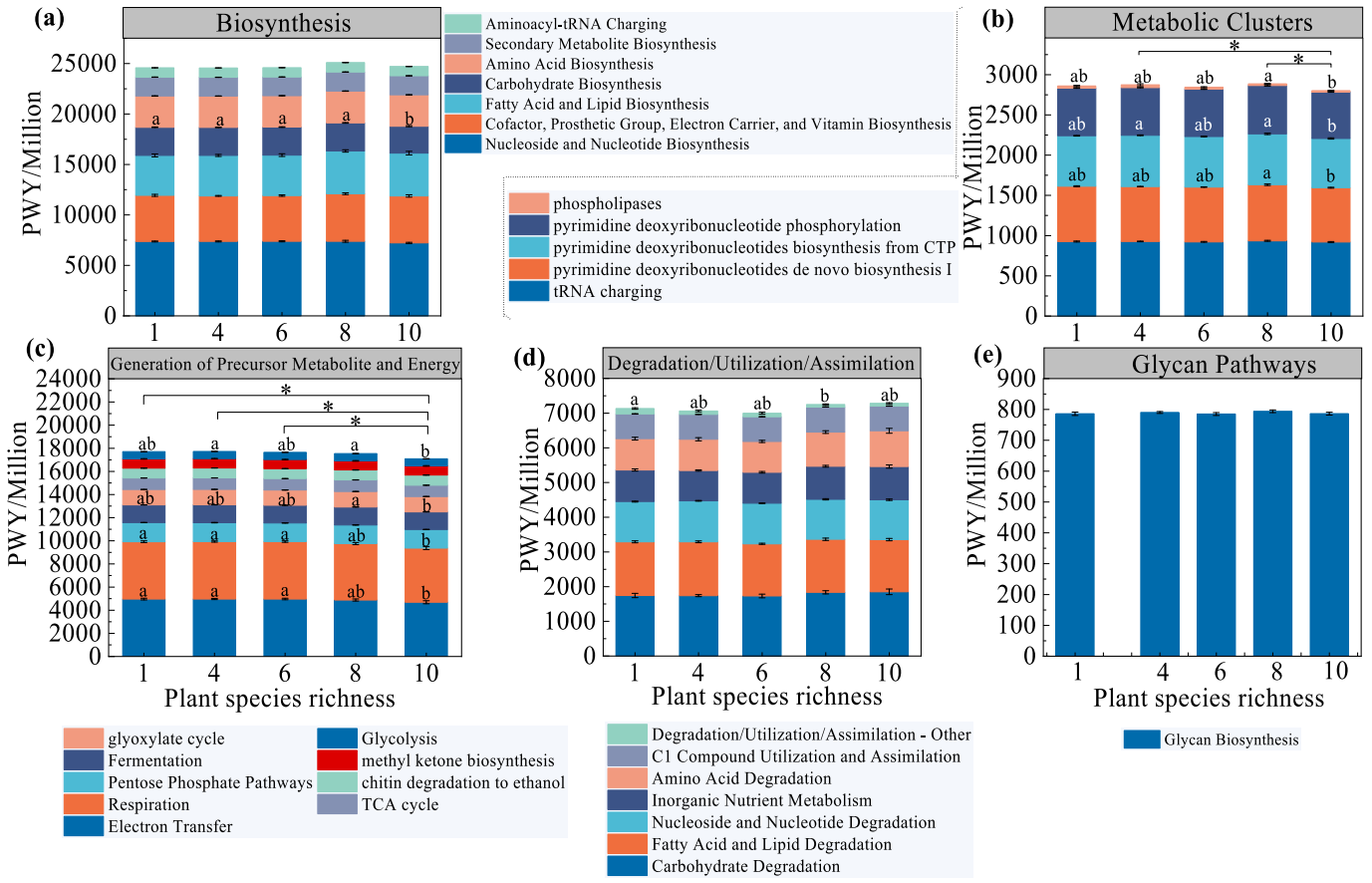

Fig. 5. Changes in the soil fungal functional potential among the plant species richness levels. (a) Biosynthesis; (b) Metabolic Clusters; (c) Generation of Precursor Metabolite and Energy; (d) Degradation/Utilization/Assimilation; (e) Glycan Pathways. The values represent the mean fungal functional potential with a standard error (± SE).

terrestrial ecosystems, by catalysing the mineralisation of organic matter (Chuan et al., 2020). For example, polysaccharides are broken down into dissolved organic carbon by enzymes such as CBH, αG, βG, and NAG (Caldwell, 2005; Wang et al., 2020). Critical enzymes responsible for N acquisition, including LAP and NAG, are essential for degrading proteins and chitin (Uwituze et al., 2022). Microbes secrete various phosphatases that hydrolyze organic phosphorus, releasing it in an available inorganic form, to meet P requirements (Daunoras et al., 2024). However, the microbial community and EEAs did not show a consistent trend with the change of species richness, which may be caused by the difference in the number of legumes in different combinations. Because leguminous plants (such as additional nitrogen input) have been clearly shown to have a disproportionate impact on ecosystem processes (Ledgard and Steele, 1992; Spehn et al., 2002). This finding suggested that higher plant species richness containing more diverse species has a higher potential to improve biological activity, including carbon mineralization, and the associated demand for nutrients, including N and P.

4.2. Effect of plant species richness on the functional potential of soil microbial communities

More than 50,000 ASVs have been identified in the microbial communities of the dominant and subdominant monocultures and mixtures within the northern Yanchi desert steppe of Ningxia. PICRUSt2 predicts the functional potential of microbial communities by identifying 10 primary functions encompassing 54 secondary functions. Metabolism, biosynthesis and generation of precursor metabolites and energy are the most important first-level functional categories, which undertake the vast majority of the basic biological functions and physiological activities of living organisms (Li et al., 2023a). The bacterial community dominated by amino acid metabolism was the most active in the secondary functional layer. Amino acids play multiple roles in plant physiological processes, including acting as protein components, osmotic regulators, regulating ion transport, affecting enzyme synthesis and activity, and acting as precursors for many plants secondary metabolites (Pratelli and Pilot, 2014), and amino acid metabolism was stronger in 4-species mixtures than in other plant species richness levels, suggesting that moderate species richness promotes amino acid metabolism. Amino acids are the main endowment state of organic matter and their degradation promotes bacterial reproduction, whereas metabolism facilitates the utilization of amino acids by bacteria. Carbohydrate metabolism of the 4-species mixtures was significantly higher than that of the other mixed communities. Carbohydrate metabolism can provide energy and metabolites for plant growth and development, and produce intermediates for the synthesis of other substances (Fernie et al., 2004). These findings suggested that a lower plant species richness may enhance the accumulation of these nutrients. The fungal community exhibited the highest abundance of nucleosides and nucleotide biosynthesis, which are essential components of the nucleic acids involved in various metabolic and regulatory activities. However, there were no significant differences in nucleoside and nucleotide biosynthesis with respect to plant species richness. This finding suggested that the storage and expression of genetic information in monoculture and mixed communities were not influenced by plant species richness in this study. Energy metabolism, cofactors, prosthetic groups, electron carriers, and vitamin biosynthesis are all strongly linked to the abundance of bacterial community structures, which directly or indirectly affect plant and soil health (Banerjee and van der Heijden, 2023).

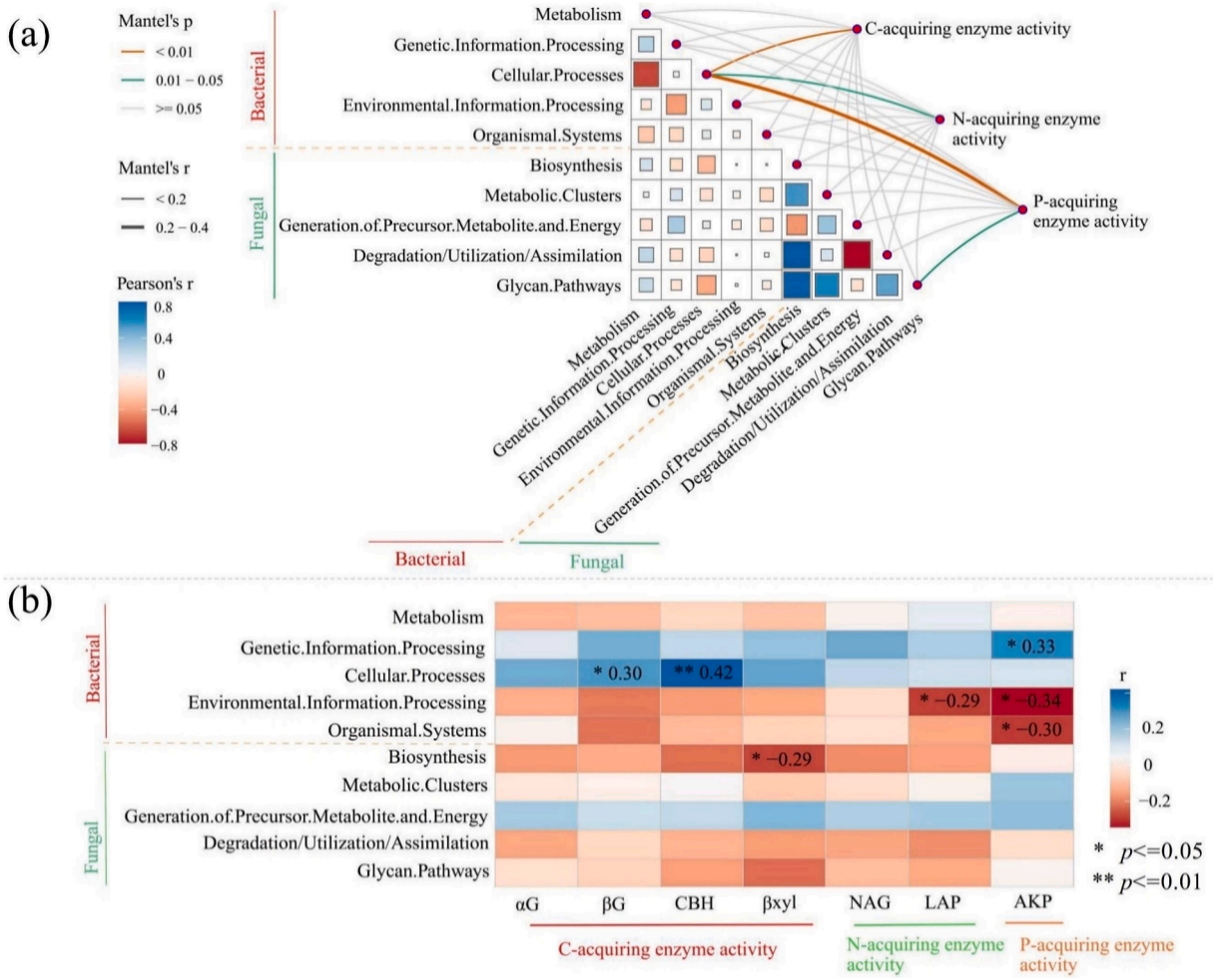

Fig. 6. Mantel test analysis (a) and Pearson correlation analysis (b) between the soil EEAs and the functional potential of soil microorganisms. αG denotes α-1,4-glucosidase, βG denotes β-1,4-glucosidase, βxyl denotes β-1,4-xyloglucosidase, CBH denotes cellobiose hydrolase, LAP denotes leucine aminopeptidase, NAG denotes β-1,4-N-acetylaminoglucosidase, AKP denotes alkaline phosphatase.

4.3. Effect of soil EEAs and microbial functional potential on soil nutrients among plant species richness levels

The aboveground primary productivity of dominant and subdominant species combinations from Ningxia desert steppe showed an increasing trend with the increase of plant species richness (Table S5). The results of the Central European Arrhenatherion grassland species richness experiment showed that the overproduction effect of the community was not related to the species pool or spatial scale, and the positive correlation between dominant species richness and productivity in the Arrhenatherion grassland was more obvious than that of all species richness and productivity (Roscher et al., 2005). Our results are consistent with the experimental results of species richness in Central European Arrhenatherion grassland ('The Jena Experiment' in Germany). There was no significant difference between plant species richness and SOC, TN and TP (Table S5), but the early 'The Jena Experiment' in Germany (Biodiversity experiment) found that the concentration of total dissolved nitrogen decreased with the increase of plant species richness and these relationships were the strongest in the initial stage of the study (Oelmann et al., 2007), the reason may be related to the number of samples collected within a year. One-time sampling, although widely used in field studies, fails to account for temporal fluctuations in soil nutrient and the variation different dominant plant species composition, these dynamics warrant further study in the future.

Soil microbial activity is essential for soil functionality as it significantly influences nutrient cycling and overall ecosystem health. Consequently, nutrient limitation may critically regulate microbial community dynamics, metabolic processes, and their interconnected relationships. In this study, both monocultures and 4-species mixtures with lower plant species richness may have accelerated soil bacterial metabolic rates. These include metabolic pathways, such as amino acids, carbohydrates, energy, and lipid metabolism, which are closely linked to the cycling of soil C, N, and P. Additionally, monocultures and 8-species mixtures with higher plant species richness may enhance EEAs, which are involved in carbohydrate decomposition, thereby supplying more carbon sources for bacteria (Johnson et al., 2020). Notably, EEAs are regulated by resource demand and supply. When microorganisms are limited by a particular resource, they increase the amount of energy and nutrients they invest in to access enzymes preferentially. A notable negative correlation ($P < 0.05$) was observed between plant species richness and C-, N-, and P-acquisition enzyme activities. This suggested that microorganisms can regulate their resource acquisition strategies through differential resource allocation (Wang et al., 2023a). Furthermore, variations in SOC can alter the nutrient balance in the soil and influence the nutrient requirements of microorganisms, subsequently regulating soil EEAs (Song et al., 2019). Extracellular polymers secreted by bacteria significantly influence the adsorption and release of phosphorus, thereby regulating phosphorus cycling in the soil. This regulatory process can increase the effective phosphorus content of the soil, affecting the microbial demand for nutrients and, consequently, regulating soil EEAs. This regulation can positively affect plant growth and

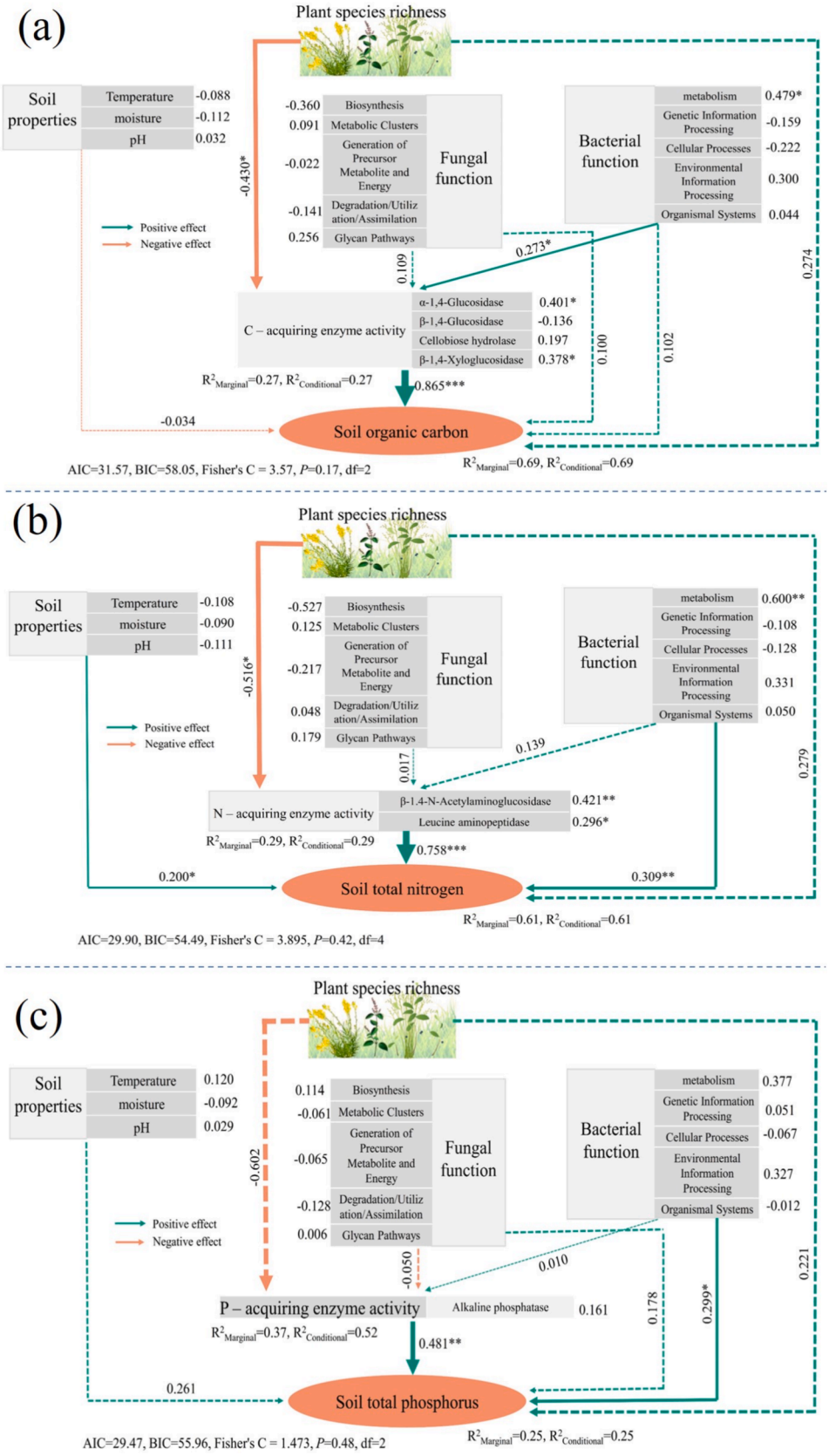

Fig. 7. SEM accounting for the direct and indirect effects of the EEAs and microbial function on SOC (a), TN (b), and TP (c) among the plant species richness levels. Green and orange arrows denote positive and negative effects, respectively, and solid and dotted lines indicate significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) and nonsignificant ($P > 0.05$) coefficients. Arrows with numbers between composite variables represent direct paths and standardized path coefficients, and the width of the arrow is proportional to the strength of the path coefficient.

development (Burns et al., 2013). TP did not exhibit significant differences in plant species richness (Table S5). This consistency can be attributed to the fact that P is one of the most limited elements in the agricultural and pastoral regions of northern China (Ma et al., 2020). In this study, the higher activity of extracellular enzymes observed in 8-species mixtures may result from plant species richness inducing shifts in the microbial community (Banerjee et al., 2018), as well as changes in soil physical conditions (e.g., temperature, moisture, and pH) (Sinsabaugh et al., 2008). Additionally, plant species can indirectly affect soil enzyme activity by controlling the secretion of enzymes from microorganisms and modifying the soil microenvironment (Liu et al., 2021). High soil pH reduces the activity of fungal communities but increases the activity of bacterial communities (Rousk et al., 2010), thereby increasing the response of bacterial communities to plant growth and accelerating rapid bacterial-mediated nutrient turnover in soil (de Vries et al., 2012). In contrast, lower N and P levels may facilitate the acquisition of soil nutrients by plant-regulating microorganisms. Furthermore, the overall differences in soil environments between the monocultures and mixtures were small (Table S5), which may be because the differences in species composition between the selected plant communities were smaller than those in previous studies (Cui et al., 2019). Moreover, the growth of dominant and subdominant species may mitigate the negative effects of the soil environment on soil microorganisms, which may be the reason for the increase of microbial α diversity after mixing (Fig. S3, Table S6).

In summary, microbial communities in monocultures and mixtures of dominant and subdominant species in the northern Yanchi desert steppe of Ningxia perform various metabolic functions, including amino acid, carbohydrate, nucleoside, and nucleotide metabolism. Microbial metabolism in communities with varying plant species richness highlights diverse metabolic pathways used by microorganisms in response to environmental changes. Cofactors are vital to microbial metabolic systems and participate in numerous biosynthetic processes. The presence and activity of these functions maintain the effectiveness of nutrients essential for plant growth, particularly in arid and semiarid regions with nutrient-poor soils. If the soil does not receive organic matter, decomposition and metabolism within the humus layer are accelerated. This resulted in an increased abundance of genes associated with microbial metabolic functions (Zhang et al., 2017). In addition to their robust metabolic functions, mixed microbial communities exhibit rich capabilities related to environmental adaptation. These include the biosynthesis of secondary metabolites and versatile metabolic pathways suited to diverse environments. Such traits bestow these communities with exceptional adaptability and play a significant role in carbon sequestration. Consequently, soils harboring mixed microbial communities represent a valuable resource for populations of carbon-sequestering microorganisms that are resistant to environmental stressors. The mixing of dominant and subdominant plant species and the changes they induce could alter the conditions under which microbial communities develop and consequently regulate soil nutrient cycling.

5. Conclusions

This study is one of the few to examine the combined effects of plant species richness on soil EEAs and microbial community characteristics, and their impact on the soil nutrient drivers of dominant and subdominant plant communities. Our findings provide clear empirical evidence that variations in plant species richness drive soil nutrient cycling through microbial metabolism and biosynthetic functions that indirectly regulate C-, N-, and P-acquisition enzyme activities. The importance of microbial carbon metabolism functions in mixed communities makes mixed community soils a tremendous trove for mining carbon-sequestering microbes and environmentally stress-resistant microbial communities. The results of this study emphasize the importance of changes in plant community composition as a key control on biogeochemical cycling in the agricultural and pastoral areas of northern China. Although PICRUSt2 function prediction can analyze the microbial functional potential, its prediction range is based on the size of the database and has some limitations. The study provides an important reference for subsequent metagenomic research, and it is expected to improve grassland productivity by optimizing the microbial environment through the combination of species functional groups in agricultural production.

CRediT authorship contribution statement

Xu Luo: Methodology, Data curation, Software, Visualization, Writing – original draft. **Yingzhong Xie:** Methodology, Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Cui Han:** Software, Investigation. **Yaxin Zhao:** Investigation, Data curation. **Ying Zhao:** Investigation, Data curation. **Jianping Li:** Methodology, Funding acquisition, Supervision, Writing – review & editing.

Funding

This work was supported by the Natural Science Foundation of Ningxia (grant numbers 2023AAC02029 and 2023AAC03092), the National Natural Science Foundation of China [grant number 32160336], and the Top Discipline Construction Project of Pratacultural Science of Ningxia University [grant number NXYLXK2017A01].

Declaration of competing interest

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

Acknowledgements

Yingzhong Xie and Jianping Li provided the financial support for the research and preparation of this study. Yingzhong Xie directed the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2025.113202.

Data availability

Data will be made available on request.

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