THE VARIETY-SPECIFIC IMPROVEMENT EFFECTS OF ALFALFA ON SOIL PROPERTIES AND MICROBIAL COMMUNITIES IN SALINE-ALKALI SOILS

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Abstract. Soil salinity poses a significant threat to global ecological sustainability. Cultivating salt-tolerant crops like alfalfa offers a practical strategy to improve saline-alkali soils. While the effects of alfalfa cultivation on soil properties and microbial ecology have been widely studied, there is limited understanding of how different alfalfa varieties influence these processes. This study used high-throughput sequencing to analyze bacterial, fungal, and archaeal communities in bare soil and rhizospheres of various alfalfa cultivars. Alfalfa cultivation reduced soil pH and electrical conductivity (EC) while increasing nutrient levels, with variations among cultivars. It enhanced microbial diversity, altered microbial community structure, and increased beneficial microbes while reducing pathogens. Different cultivars stimulated key soil functions like nitrification and chitin degradation. Among the varieties, A6 and A4 showed the greatest potential for saline soil restoration. A6 enhanced soil carbon storage and nutrient cycling, while A4 significantly improved microbial diversity. This study highlights the potential of alfalfa cultivation to restore saline-alkali soils and improve ecological functions. By tailoring alfalfa varieties to specific soil conditions, these findings provide valuable insights for sustainable agricultural practices in saline-alkali environments.
Keywords: *alfalfa cultivation, saline-alkali soil restoration, sustainable agriculture, bacterial community, fungal community, archaeal community*

Introduction

The Songnen Plain, one of the world's major regions with saline-alkali soils, is also a crucial grain production area and commercial grain base in China (Shi et al., 2019; Jiang et al., 2022). Saline-alkali soils not only degrade soil and plant health, but also

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have severely impact on the environment and agricultural productivity (Jiang et al., 2024b). Addressing this challenge is imperative to ensure food security and promote sustainable development.

Phytoremediation through the cultivation of salt-tolerant crops represents a key strategy for ameliorating saline-alkali soils (Li et al., 2022a). Alfalfa (*Medicago sativa* L.), a widely used forage crop, plays a vital role in soil and water conservation, ecological restoration, and environmental improvement. It is a preferred pioneer species for grassland restoration and combating desertification (Zhang et al., 2024a; Wick et al., 1998; Gu et al., 2022). Recent studies have highlighted the potential of different alfalfa varieties in addressing stress conditions. Liu et al. (2024) employed transcriptome sequencing and untargeted metabolomics to analyze the responses of two alfalfa varieties to combined cold and saline-alkali stresses, and find that the Zhao Dong alfalfa demonstrated superior resistance compared to Blue Moon alfalfa. Similarly, Jing et al. (2024) evaluated eight alfalfa varieties, including both domestic and international cultivars, by comparing their yield traits, leaf morphological characteristics, and photosynthetic performance. They identified "Gannong No. 9," "WL319HQ," and "SG501" as high-performing varieties, with notable single plant dry weight and leaf biomass (Jing et al., 2024). While significant research has examined alfalfa growth and yield characteristics in saline-alkali soils, few studies have addressed the remediation potential and mechanisms of alfalfa cultivation in severely sodic soils. Identifying optimal alfalfa varieties for soil restoration, mitigating saline-alkali stress on plants, and enhancing land use efficiency are pressing challenges that must be addressed to promote sustainable agricultural development.

Soil microorganisms serve as vital mediators in the cycling and transformation of mineral nutrients, effectively linking the input and output of soil elements (Wu et al., 2024). Among these, bacteria, fungi, and archaea play key roles in shaping soil microbial communities. Bacterial diversity, in particular, plays a pivotal role in driving soil multifunctionality during recovery processes (Wang and Kuzyakov, 2024). Competition for resources between bacteria and fungi significantly influences their adaptation and ecological differentiation in soils (Gong et al., 2024). Lucie et al. (2022) identified soil properties as the primary drivers of diversity and composition in bacterial, archaeal, fungal, and protist communities. Similarly, Kong et al. (2023) highlighted the critical role of microorganisms in regulating soil health, particularly in nutrient cycling. Despite substantial research on bacterial, fungal, and archaeal communities in soils, the interactions among these microorganisms during alfalfa cultivation for saline-alkali soil improvement remain poorly understood. Exploring these interactions is essential to fully grasp the potential of alfalfa in restoring degraded soils.

This study investigated the interaction mechanisms among bacteria, fungi, and archaea within the rhizosphere microbial community of saline-alkali soils improved through alfalfa cultivation. Over an 18-year restoration experiment, the microbial community structures of bacteria, fungi, and archaea were analyzed across eight alfalfa varieties. We hypothesized the following: 1) All eight alfalfa varieties contribute to the improvement of saline-alkali soils; 2) The bacterial, fungal, and archaeal community structures in soils cultivated with alfalfa differ significantly from those in bare land, with these differences primarily influenced by soil properties; 3) The microbial networks of bacteria, fungi, and archaea are shaped by alfalfa cultivation and soil conditions.

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Materials and methods

Experimental area, variety selection, and design

The study was conducted at Sifangshan Farm, located in Zhaodong City, Suihua, Heilongjiang Province, China (125°45′E–126°30′E, 46°12′N–46°22′N). The site features carbonate meadow soil and carbonate meadow saline-alkali soil, both representative of heavy saline-alkali conditions, with topsoil characterized by white, naturally saline-alkali bare patches. Prior to vegetation restoration, the soil exhibited a pH range of 10.50–11.00, which classifies it as heavy saline soil (pH > 9.5). The site has been under a fully enclosed natural succession process since 2002, making it an ideal location for evaluating alfalfa's impact on soil properties and microbial communities.

Eight alfalfa varieties—LH Wet Tolerant (A1), Longmu 801 (A2), Prairie 1 (A3), WL354HQ (A4), WL319HQ (A5), Gongnong 1 (A6), Challenger (A7), and Marker (A8)—were selected as treatments, with bare soil serving as the control (B). Details of the selected varieties are provided in *Table S1*. The experiment utilized a strip sowing method, with each plot measuring 5 rows × 5 m × 0.3 m. Each treatment was replicated three times, and the alfalfa varieties were randomly assigned to plots of equal size in 2002. In each row, 100 seeds were sown at a spacing of 5 cm and a depth of 2 cm, followed by light compaction and mulching. Manual weeding was conducted during the first year of sowing. From the second year onward, no additional field management was performed until the three-year growing season concluded. Residual alfalfa biomass was returned to the soil as part of the restoration process. This sowing and management cycle was repeated every three years, alongside simultaneous natural remediation processes.

Soil sampling was conducted on July 4, 2020. Samples from bare ground (control) and the rhizosphere soils of eight alfalfa varieties were collected at a depth of 0-20 cm using a five-point sampling method and root-shaking technique (Phillips and Fahey, 2006). One set of samples was air-dried, ground, and sieved for the analysis of soil physicochemical properties in the laboratory. The other set was stored at -80°C for DNA extraction and subsequent analysis.

Soil analysis

Air-dried and sifted soil samples were used for the analysis. Soil pH and electrical conductivity (EC) were measured using a 1:5 ratio of soil to deionized water. Soil organic carbon (SOC) was determined using the ammonium dichromate o-phenanthroline method. Alkali-hydrolyzable nitrogen (AHN) was measured by the alkaline diffusion method, while total phosphorus (TP) and available phosphorus (AP) were determined using molybdenum-antimony colorimetry after concentrated H_2SO_4-$HClO_4$ digestion and $NaHCO_3$ leaching, respectively. Available potassium (AK) was quantified using the flame spectrophotometric method with NH_4OAc leaching (Li et al., 2023).

Sequencing of microbial community

DNA was extracted from 0.5 g of soil using the Mag Pure Soil DNA LQ Kit (Magan) for each mixed sample. The quality of the DNA extraction was assessed using 1% agarose gel electrophoresis. The total DNA was pooled and analyzed using a Nanodrop 2000c. For bacterial analysis, the V3-V4 region of the 16S rRNA gene was amplified by PCR using primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-

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GGACTACHVGGGTWTCTAAT-3'). To amplify the ITS1 region of fungal ITS rDNA, the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') were used. For archaeal analysis, the V3-V5 region of the 16S rRNA gene was amplified using primers 344F (5'-ACGGGGGYGCAGCAGGCGCGA-3') and 915R (5'-GTGCTCCCCCGCCAATTCCT-3'). The PCR reaction mixture (20 μL) contained 4 μL of 5x FastPf buffer, 2 μL of 2.5 mmol dNTPs, 0.8 μL of each forward and reverse primer (at a concentration of 5 μmol), 0.4 μL of FastPf DNA Polymerase, 10 ng of DNA template, and ddH_2O to a final volume of 20 μL.

PCR amplification for bacterial and fungal samples was conducted separately using the following thermocycling conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. A final extension step was performed at 72°C for 10 min. For archaeal amplification, the conditions included an initial denaturation at 95°C for 3 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 10 min. PCR products from three replicates per sample were pooled and analyzed by electrophoresis on a 2% agarose gel. The desired PCR products were excised from the gel, purified using a DNA Gel Recovery Kit, and eluted with Tris-HCl. The PCR products were quantified using a blue fluorescence quantification system and combined according to the sequencing volume for each sample. The amplicons from the 16S rDNA PCR amplification were sent to Shanghai Lynn Biologicals for second-generation high-throughput amplicon sequencing, conducted via paired-end sequencing.

Bioinformatic analysis

The Illumina PE250 sequencing data were processed by first barcoding the valid sequences from all samples. The quality of the reads was then assessed and filtered for quality control. Paired-end reads were merged into single sequences based on their overlap. The sequences were subsequently analyzed using the Quantitative Insights Into Microbial Ecology pipeline (QIIME, version 1.8.0; http://qiime.org/) for MiSeq data analysis.

After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) using the UCLUST method, with a 97% identity threshold (Edgar, 2010). The abundance of each OTU was determined by the number of sequences assigned to it. A representative sequence for each OTU was selected as the longest sequence most similar to others in the cluster (Ge et al., 2012). Low-quality sequences (OTUs) with a relative abundance below 0.001% were removed (Bokulich et al., 2013). The resulting OTU matrix was used for community analysis, and each representative sequence was classified using the SILVA database in QIIME (Quast et al., 2013).

Statistical analyses

The data of this study were statistically analyzed using R 4.3.0. Soil microbial communities were analyzed with the "vegan" and "ggplot2" packages to visualize the effects of treatments on α-diversity and microbial community composition. The β-diversity was visualized using principal component analysis (PCA) based on Bray-Curtis dissimilarity distance (Delgado-Baquerizo et al., 2018). One-way analysis of

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variance (ANOVA) was performed using IBM SPSS Statistics 27 software (Li et al., 2021b). Mante test analysis was conducted using the "linkET" package to assess the relationship between soil chemical properties and microbial community composition (Peng et al., 2024). The microbial co-occurrence network was constructed using the "ggClusterNet" package, filtering out correlations with $r < 0.65$ and $P > 0.01$ (Yao et al., 2025). The functional potential of bacterial and archaeal communities was predicted using PICRUSt2, while fungal function prediction was carried out using FUNGuild (Nguyen et al., 2016).

Results

Effect of different alfalfa varieties on soil chemical properties

Cropping different alfalfa varieties significantly reduced soil pH and EC compared to bare ground, with pH values dropping below 9 ($P < 0.05$) (*Table 1*). The SOC and AHN both increased following alfalfa cultivation (*Table 1*). The A6 treatment showed the greatest increase in SOC content, reaching the highest level compared to other alfalfa varieties, while the A1 treatment exhibited the highest AHN content (*Table 1*). Specifically, SOC in A6 and AHN in A1 increased by 88.64% and 84.09%, respectively, compared to bare ground (*Table 1*). TheA5, A7, and A8 treatments increased AP content in bare ground soil by 93.72%, 82.79%, and 25.42%, respectively ($P < 0.05$) (*Table 1*). TheA2, A3, A6, and A7 treatments increased TP content by 43.85%, 40.13%, 43.37%, and 21.84%, respectively ($P < 0.05$) (*Table 1*). Additionally, A4, A6, and A7 treatments significantly increased AK content by 95.68%, 31.35%, and 3.24%, respectively ($P < 0.05$) (*Table 1*). The highest AP/TP ratio was observed in the A5 treatment.

Table 1. *Soil chemical properties in different treatments*

Treatment	pH	EC	SOC	AHN	AP	TP	AK	AP/TP
B	9.99±0.02 a	683.33±2.08 a	0.44±0.01 c	24.08±2.74 d	50.62±18.76 c	6.18±0.01 c	122.42±1.15 de	0.01±0.00 b
A1	8.32±0.02 f	354.33±0.58 e	0.65±0.00 b	44.33±5.05 a	28.14±0.81 d	1.96±0.85 de	109.19±1.99 f	0.02±0.01 b
A2	8.73±0.02 c	348.00±1.00 e	0.65±0.01 b	30.25±0.50 c	34.53±8.59 d	8.89±0.21 a	121.76±2.29 e	0.00±0.00 b
A3	9.18±0.02 b	376.33±1.53 d	0.66±0.12 b	29.83±1.76 c	33.30±0.88 d	8.66±0.07 a	125.73±2.29 cd	0.00±0.00 b
A4	8.41±0.01 e	420.00±16.46 c	0.51±0.06 c	36.58±1.77 b	48.44±4.98 c	0.91±0.26 e	239.55±2.29 a	0.06±0.02 b
A5	8.60±0.02 d	330.67±1.15 f	0.51±0.06 c	33.42±0.29 bc	98.06±0.22 a	1.75±0.86 de	120.44±2.29 e	0.07±0.05 a
A6	8.64±0.01 d	290.67±0.58 bg	0.83±0.12 a	36.92±1.77 b	7.54±2.67 e	8.86±0.10 a	160.80±1.99 b	0.00±0.00 b
A7	8.38±0.10 ef	444.00±1.00 b	0.62±0.03 b	30.58±0.88 c	92.53±1.77 a	7.53±0.09 b	126.39±1.15 c	0.01±0.00 c
A8	7.97±0.04 g	283.33±1.53 g	0.63±0.02 b	33.83±2.02 bc	63.49±4.47 b	2.38±1.40 d	109.19±1.99 f	0.04±0.03 b

B stands for bare ground; A1-A8 represent alfalfa varieties in the order of LH moisture tolerant, Longmu 801, Grassland 1, WL354HQ, WL319HQ, Gongnong 1, Challenger, and Target, respectively. EC stands for Electrical Conductivity, SOC stands for Soil Organic Carbon, AHN stands for Alkaline Hydrolysed Nitrogen, AP stands for available phosphorous, TP stands for Total Phosphorus, AK stands for available Potassium, and AP/TP stands for available phosphorous/Total Phosphorus. Different lowercase letters represent $P < 0.05$. Same as below

Effects of different alfalfa varieties on the alpha diversity of soil bacterial, fungal and archaeal communities

The Shannon and Chao1 indices of bacterial and fungal communities differed significantly between alfalfa and bare ground, with higher values observed in alfalfa soils ($P < 0.05$) (*Fig. 1A*). Specifically, the Shannon index of bacteria in A3 and A7

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treatments, as well as the Shannon index of fungi in A7, were significantly lower than in other alfalfa varieties. Similarly, the Chao1 index for bacteria in the A2 treatment and for fungi in the A7 treatment were significantly lower than in other alfalfa varieties ($P < 0.05$). The highest bacterial Shannon index was found in the A1 treatment, and the highest fungal Shannon index in the A4 treatment. For Chao1, the highest bacterial index was found in the A6 treatment, while the highest fungal index was observed in the A5 treatment. However, the Shannon and Chao1 indices for archaea did not show significant differences across treatments ($P > 0.05$). Overall, alfalfa cultivation significantly increased the alpha diversity of bacterial and fungal communities compared to bare ground (*Fig. 1A*). Random Forest analysis revealed that AHN, EC, pH, and SOC were the primary drivers of the bacterial Shannon and Chao1 indices, while AHN, EC, pH, TP, and SOC influenced the fungal Shannon and Chao1 indices (*Fig. 1B*).

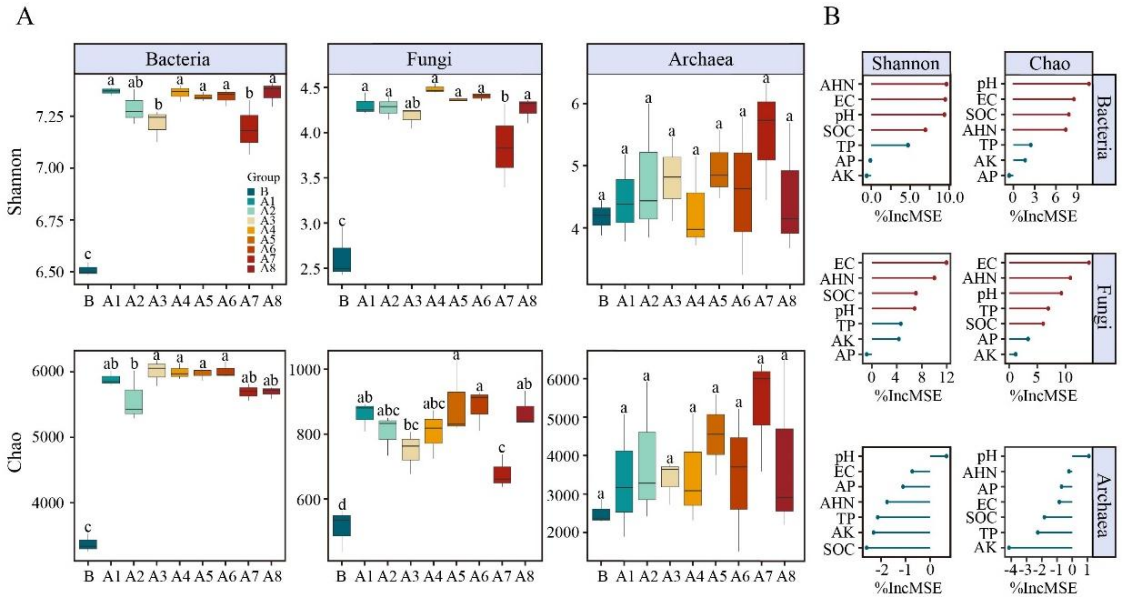

Figure 1. *Analysis of alpha diversity (A) of soil bacteria, fungi and archaea under different treatments and random forest analysis (B) to predict the importance of soil variables to them. B stands for bare ground; A1-A8 represent alfalfa varieties in the order of LH moisture tolerant, Longmu 801, Grassland 1, WL354HQ, WL319HQ, Gongnong 1, Challenger, and Target, respectively. EC stands for Electrical Conductivity, SOC stands for Soil Organic Carbon, AHN stands for Alkaline Hydrolysed Nitrogen, AP stands for available phosphorous, TP stands for Total Phosphorus, AK stands for available Potassium. Different lowercase letters represent $P < 0.05$*

Effect of different alfalfa varieties on beta diversity of bacterial, fungal and archaeal communities

Further analysis of soil microbial communities in both bare ground and different alfalfa varieties revealed significant differences between all alfalfa treatments and bare ground ($P < 0.05$). Notably, differences in the composition of rhizosphere soil bacterial and fungal communities were observed between the A7 treatment and the other alfalfa varieties (*Fig. 2B*). This finding was also supported by beta dispersion analysis (*Fig. 3A*). In contrast, archaeal communities across different alfalfa varieties showed

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significant overlap, indicating that the soil archaeal community structure was similar among the alfalfa treatments (*Fig. 2B*).

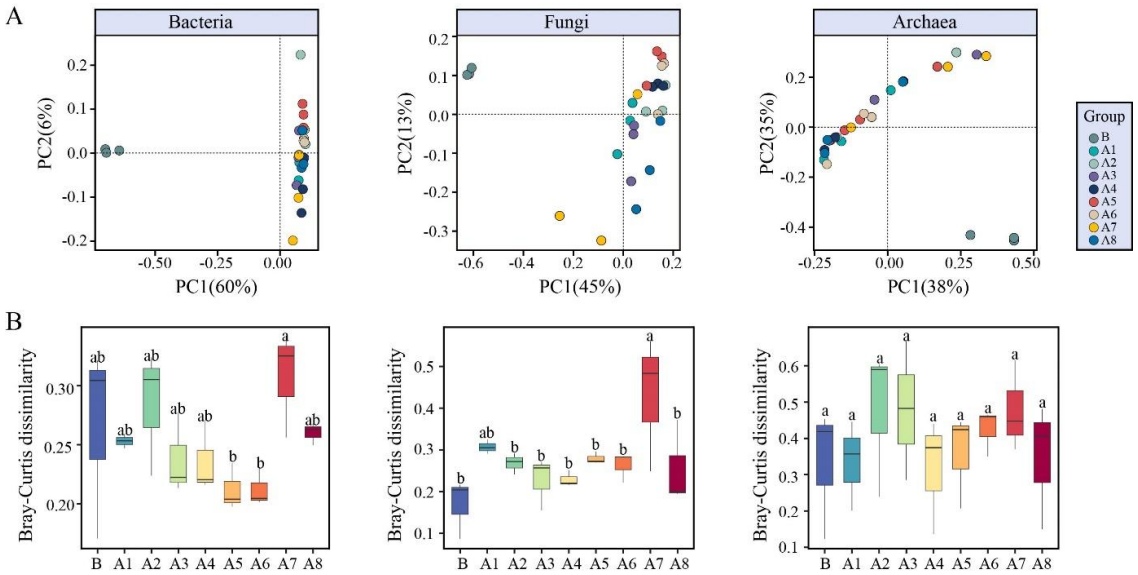

Figure 2. *PCoA analysis (A) and bray-curtis dissimilarity (B) of soil bacteria, fungi and archaea under different treatments. Notes: B stands for bare ground; A1-A8 represent alfalfa varieties in the order of LH moisture tolerant, Longmu 801, Grassland 1, WL354HQ, WL319HQ, Gongnong 1, Challenger, and Target, respectively. Different lowercase letters represent $P < 0.05$*

Effects of different alfalfa varieties on bacterial, fungal and archaeal community composition

In total, eight bacterial phyla in the soil community had a relative abundance greater than 1%, with the following distribution: Proteobacteria (31.49%), Actinobacteria (23.86%), Acidobacteria (19.18%), Chloroflexi (8.55%), Bacteroidetes (5.98%), Gemmatimonadetes (3.26%), Verrucomicrobia (1.98%), and Firmicutes (1.21%) (*Fig. 3B*). Among these, Proteobacteria, Actinobacteria, and Acidobacteria represented the major bacterial phyla, each accounting for more than 10% of the total abundance. Compared to bare ground, alfalfa cultivation increased the relative abundance of Proteobacteria by 16.95%-51.27%, with the highest increase observed in A5; Actinobacteria by 25.91%-46.40%, with the highest increase in A6; and Acidobacteria by 55.02%-117.42%, with the highest increase in A4 treatment (*Fig. 3B*). Together, these three major phyla accounted for 74.54% of the total bacterial community.

The soil fungal community comprised nine genera with a relative abundance greater than 1%, listed as follows: *Fusarium* (36.06%), *Mortierella* (7.46%), *Podospora* (7.09%), *Plectosphaerella* (6.37%), *Archaeorhizomyces* (4.45%), *Latorua* (2.61%), *Trichocladium* (2.53%), *Ascochyta* (2.08%), and *Septorioides* (2.06%) (*Fig. 3B*). Among these, Fusarium, with a relative abundance greater than 10%, was the dominant genus in this study (*Fig. 3B*). Following alfalfa cultivation, the relative abundance of Fusarium decreased by 42.29%-62.27%, with the largest decrease observed in the A8 treatment (*Fig. 3B*). Fusarium accounted for 36.06% of the total fungal community (*Fig. 3B*).

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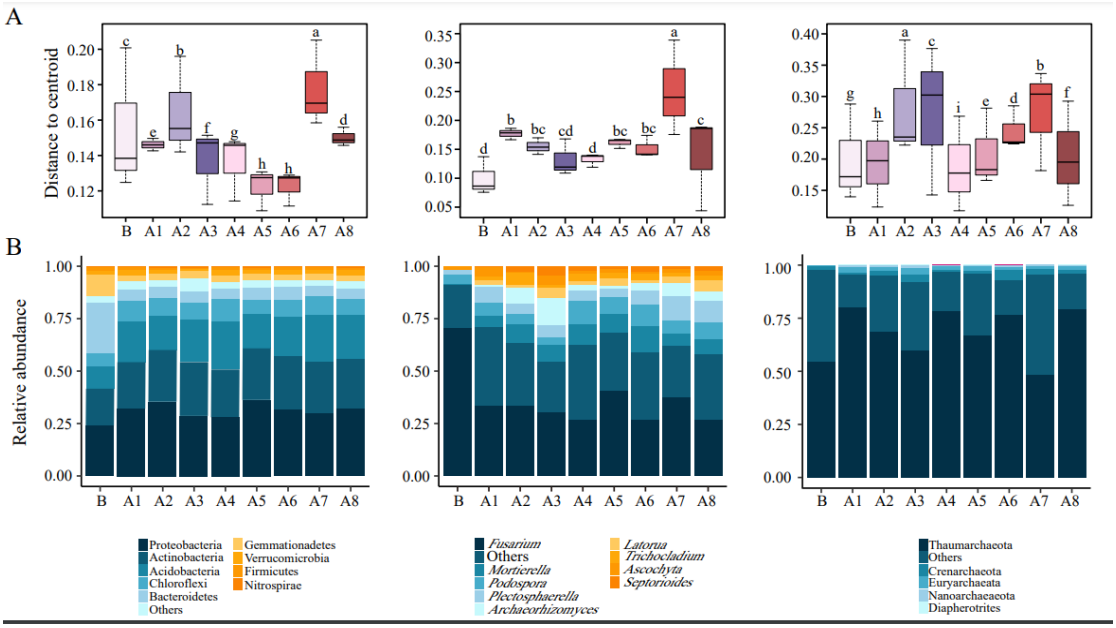

Figure 3. *Beta dispersion (A) analysis of soil bacteria, fungi and archaea, and community composition (B) of species under different treatments. B stands for bare ground; A1-A8 represent alfalfa varieties in the order of LH moisture tolerant, Longmu 801, Grassland 1, WL354HQ, WL319HQ, Gongnong 1, Challenger, and Target, respectively. Different lowercase letters represent $P < 0.05$*

The soil archaeal community consisted of three phyla with relative abundances greater than 1%: Thaumarchaeota (68.03%), Crenarchaeota (2.21%), and Euryarchaeota (1.88%). Thaumarchaeota was the dominant phylum, accounting for over 10% of the archaeal community (*Fig. 3B*). After alfalfa cultivation, the relative abundance of Thaumarchaeota increased by 9.98% to 47.44%, except for the A7 treatment, with the highest increase observed in A1 treatment (*Fig. 3B*). Thaumarchaeota made up 68.03% of the total archaeal community (*Fig. 3B*).

Soil pH significantly affected the composition of bacterial and fungal communities, while AHN influenced the archaeal community composition (*Fig. 4A*). Additionally, pH and EC had a positive effect on Bacteroidetes, Gemmatimonadetes, and Fusarium, whereas EC negatively impacted Nitrospirae (*Fig. 4A*).

Effects of different alfalfa varieties on the assembly of bacterial, fungal and archaeal microbial communities

NST values indicated that deterministic processes played a key role in the assembly of microbial communities in bare land, A5, and A6 treatments for bacteria; in bare land for fungi; and in A1, A2, and A6 treatments for archaea. In contrast, stochastic processes dominated in the other treatments (*Fig. 5A*).

The study also evaluated the influence of stochastic events on microbial community formation using the neutral community model (NCM) (*Fig. 5B*). Results showed that all alfalfa varieties, except A7, exhibited higher dispersal capacity than bare land (*Fig. 5B*). The A5 treatment had the highest bacterial dispersal capacity (Nm = 33,036), while the A8 treatment showed the highest fungal dispersal capacity (Nm = 53,972), both

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exceeding that of bare land (*Fig. 5B*). However, the dispersal capacity of archaea was lower in the alfalfa varieties compared to bare land (*Fig. 5B*).

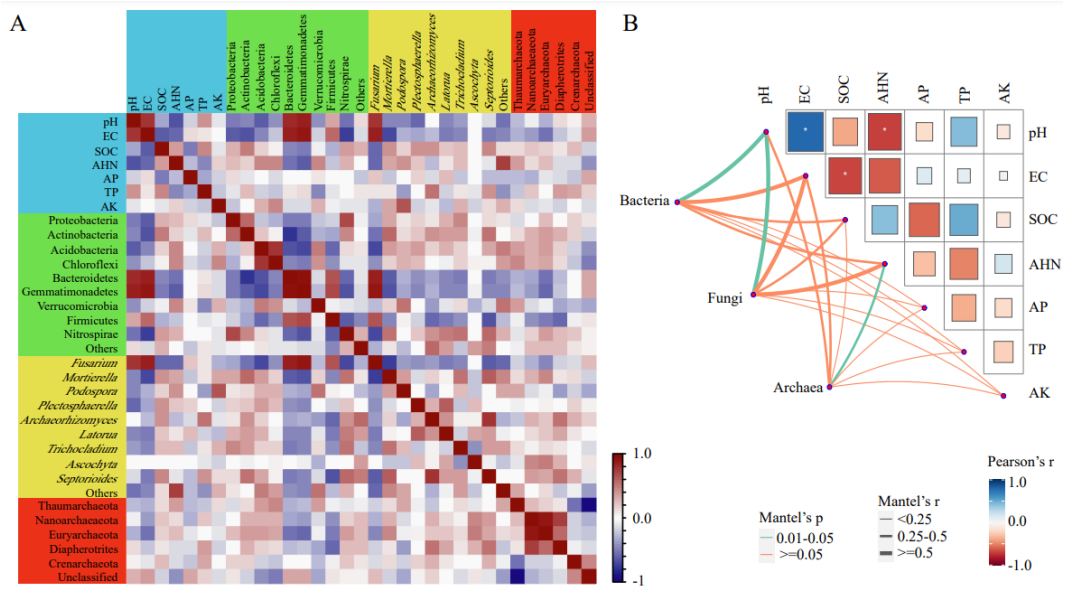

Figure 4. *Correlation analysis of the community composition of soil bacteria, fungi and archaea with soil chemical properties. EC stands for Electrical Conductivity, SOC stands for Soil Organic Carbon, AHN stands for Alkaline Hydrolysed Nitrogen, AP stands for available phosphorous, TP stands for Total Phosphorus, AK stands for available Potassium*

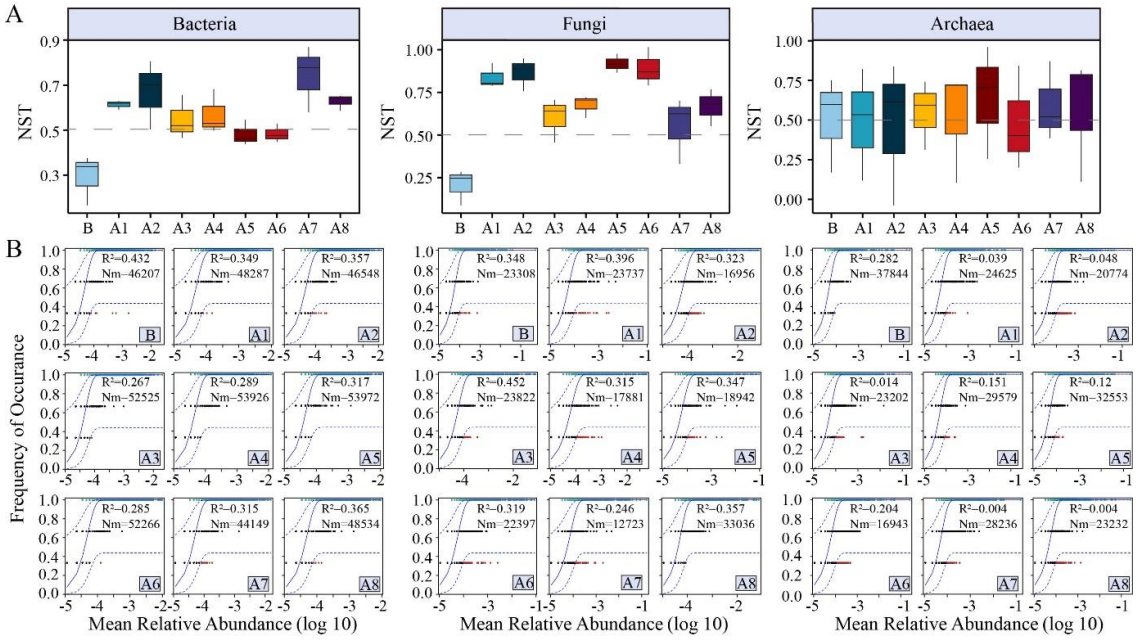

Figure 5. *Stochastic analysis of soil bacterial, fungal, and archaeal communities under different treatments. B stands for bare ground; A1-A8 represent alfalfa varieties in the order of LH moisture tolerant, Longmu 801, Grassland 1, WL354HQ, WL319HQ, Gongnong 1, Challenger, and Target, respectively*

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Effects of different alfalfa varieties on microbial co-occurrence networks

The number of nodes in the bacterial network increased after cropping different alfalfa varieties, with the A3 treatment exhibiting the highest number of bacterial nodes (1133). However, the number of edges decreased. Among all alfalfa varieties, the A3 treatment had the highest values for nodes, edges, graph density, and average degree of the bacterial network (*Fig. 6; Table S2*). In the fungal network, the number of nodes increased after cropping alfalfa for all varieties except A1 and A5, compared to bare land. The A7 treatment had the highest number of nodes and edges, and the network stability and complexity—measured by indicators such as average degree, network diameter, and graph density—were also highest in the A7 treatment (*Fig. 6; Table S3*). For the archaeal network, cropping alfalfa increased both the number of nodes and edges. The A2 treatment had the highest number of nodes, while the A8 treatment had the highest number of edges among all alfalfa varieties (*Fig. 6; Table S4*).

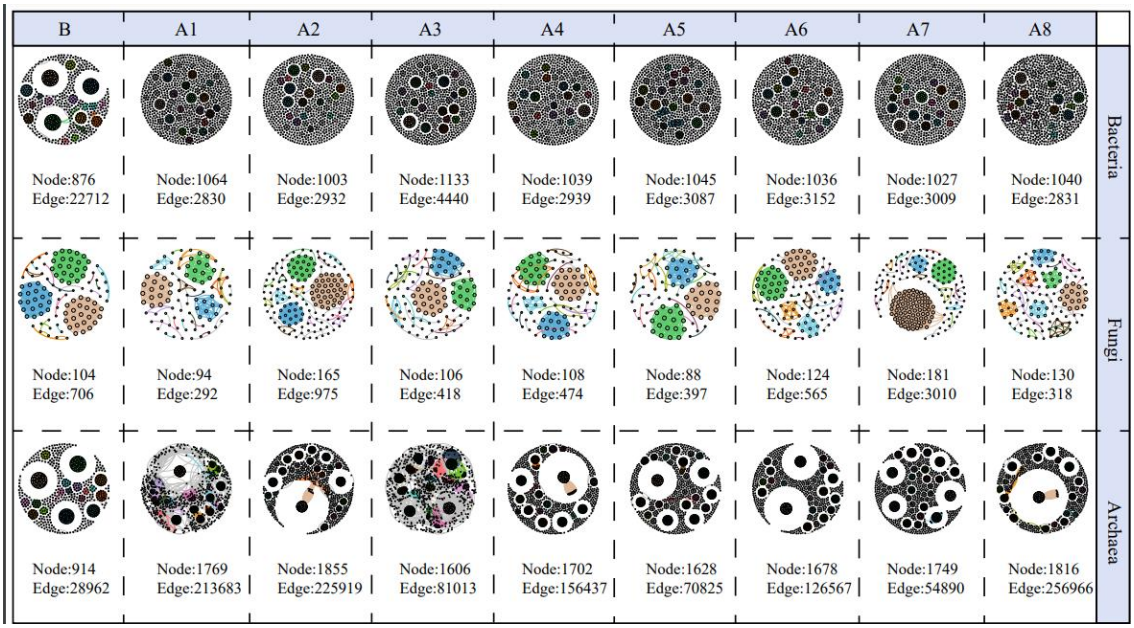

Figure 6. *Co-occurrence network analysis of soil bacteria, fungi and archaea under different treatments. B stands for bare ground; A1-A8 represent alfalfa varieties in the order of LH moisture tolerant, Longmu 801, Grassland 1, WL354HQ, WL319HQ, Gongnong 1, Challenger, and Target, respectively*

Effects of different alfalfa varieties on microbial function prediction

Functional prediction analyses of the soil bacterial community revealed significant differences in most functions, except for nitrate reduction ($P < 0.05$). The relative abundance of functions related to nitrification, chitinolysis, nitrate reduction, and aerobic ammonia oxidation was significantly higher in alfalfa treatments compared to bare land, while the relative abundance of manganese oxidation was lower (*Fig. 7A*). For the soil archaeal community, functional prediction analyses showed significant differences in the relative abundance of methylotrophy, methyl group disproportionation, dark hydrogen oxidation, CO_2 reduction with H_2, hydrogenotrophy, and methanogenesis ($P < 0.05$). These functions were significantly more abundant in

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alfalfa treatments than in bare land, with the A5 treatment exhibiting the highest relative abundance, except for methylotrophy (*Fig. 7A*). In soil fungal communities, functional prediction analyses revealed that the relative abundance of pathotrophs, saprotrophs, and symbiotrophs was lowest in bare land but significantly increased ($P < 0.05$) after cropping alfalfa (*Fig. 7B*). The A8 treatment had the highest relative abundance of pathotrophs, while the A2 treatment had the highest relative abundance of saprotrophs and symbiotrophs (*Fig. 7B*).

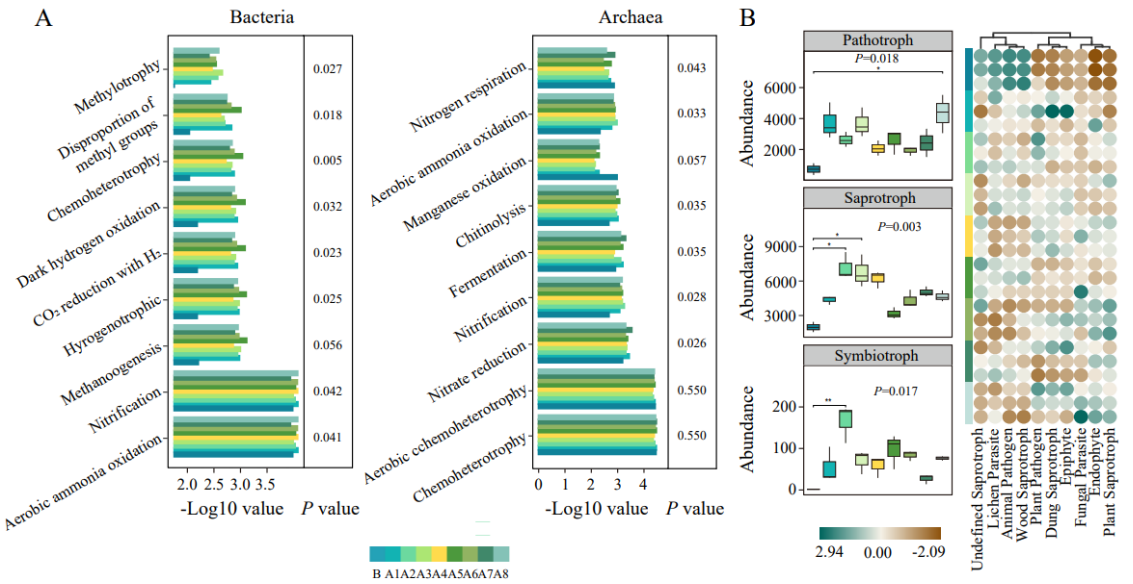

Figure 7. *Functional prediction of soil bacterial and archaeal microorganisms based on the FAPROTAX database. Kruskal-Wallis test was performed between the first 10 functional groups of bacteria and archaea from different treatments and P-values (A) were labelled. Functional prediction of the top 10 functions of soil fungi based on the FUNGuild database, and difference tests were performed between different treatment fungal taxa (B). B stands for bare ground; A1-A8 represent alfalfa varieties in the order of LH moisture tolerant, Longmu 801, Grassland 1, WL354HQ, WL319HQ, Gongnong 1, Challenger, and Target, respectively*

Discussion

Cropping different alfalfa varieties had a positive effect on soil nutrients

Different alfalfa varieties exhibit moderate tolerance to both salt and alkalinity in saline soils, contributing to improvements in soil fertility and ecology (Fan et al., 2023; Shi et al., 2017). Alfalfa roots secrete organic acids in response to abiotic stresses, such as drought and salinity, which helps mitigate these stresses (Wang et al., 2024a; Annicchiarico et al., 2015). This process aids in enhancing the uptake and utilization of soil nutrients by acidifying the soil, which in turn reduces both soil pH and salt content (Ben-Laouane et al., 2021). Furthermore, after 18 years of alfalfa cultivation in soda saline soils, consecutive years of cropping significantly reduce surface water evaporation, thereby preventing soil salinization (Liang and Shi, 2021). In alfalfa management, continuous irrigation can act as a salt leaching mechanism, influencing soil conductivity and balancing ions, ultimately improving soil structure (Su et al., 2024).

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Different alfalfa varieties exhibit distinct adaptations to saline soils, which can lead to variations in their effectiveness in improving soil nutrients. Alfalfa is known for its well-developed root system, and genetic differences between varieties can result in unique root structures. These variations influence plant-soil interactions, including nutrient uptake and inputs to the soil from plant litter and root secretions (Baral et al., 2023; Bucciarelli et al., 2021; Feng et al., 2024). Cropping alfalfa in saline soils increases soil organic carbon, with levels rising significantly over consecutive years of alfalfa cultivation (Wang et al., 2024b). Not only does long-term alfalfa cropping enhance soil organic carbon storage, but alfalfa residues also provide substantial carbon input to the soil. Additionally, alfalfa stimulates microbial activity, leading to effective soil carbon accumulation, particularly in the A6 variety (Kane et al., 2023).

The increase in rhizosphere bacterial richness observed in the A6 alfalfa variety supports this, as the bacterial community may play a role in transforming soil organic carbon. Similarly, the improved performance of the A1 variety in enhancing AHN may be attributed to microbial activity. Among the alfalfa varieties, A1 exhibited the highest Shannon index, with variations in bacterial α-diversity correlating with water-soluble organic nitrogen in the soil (Qiao et al., 2023). The A5 treatment, with its highest AP/TP ratio, suggests that alfalfa may induce nutrient redistribution or phosphorus activation through root secretions. Furthermore, the highest Chao1 index of rhizosphere fungi in the A5 variety indicates a greater fungal abundance, which may include species that solubilize phosphorus. These fungi help release insoluble phosphorus minerals, contributing to the accumulation of available phosphorus (Brazhnikova et al., 2022). This mechanism may explain the significant increase in total phosphorus content in the A2 variety, which, despite having a lower Chao1 index, leads to enhanced phosphorus storage. Additionally, the A4 variety showed the greatest increase in AK, which was linked to changes in fungal diversity. Higher fungal diversity accelerates the decomposition of alfalfa apoplasts, contributing to nutrient cycling (Liu et al., 2023b; Teng et al., 2023). In conclusion, alfalfa varieties influence soil nutrient cycling and accumulation by altering the composition and diversity of soil microbial communities. This, in turn, affects the activity and metabolic processes of functional microorganisms, ultimately improving soil nutrient availability and effectiveness.

Differences among alfalfa varieties shape soil bacterial community structure

This study found a significant difference in the bacterial Shannon index between bare ground and alfalfa-restored soil, with alfalfa-restored soil exhibiting a notably higher Shannon index. The alfalfa rhizosphere, characterized by high nutrient content and low competitive pressure, provides diverse ecological niches for various bacterial types, promoting the richness and diversity of bacterial communities (Yin et al., 2024). Alfalfa root secretions contain a range of organic compounds, such as sugars, amino acids, and organic acids (Annicchiarico et al., 2015; Liu et al., 2024; Jiang et al., 2024a). These compounds serve as carbon and energy sources, nourishing soil bacteria and stimulating bacterial growth and metabolism, which in turn enhances bacterial diversity and abundance. The composition of root secretions may vary among alfalfa varieties, leading to differences in bacterial diversity and community structure. The Shannon index was highest in the A1 alfalfa variety, while the Chao1 index was highest in the A6 variety. These differences may stem from the varying abilities of the alfalfa varieties to improve soil physical and chemical properties. The decomposition of deadfall and root residues in the A6 variety likely contributes organic matter, improving soil fertility and

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indirectly enhancing bacterial richness (Lai et al., 2024; Ridgeway et al., 2024). The A1 variety may form symbiotic relationships with specific rhizobacteria, which could also influence non-symbiotic bacterial communities in the soil (Lagunas et al., 2023). Additionally, the nitrogen-fixing efficiency of A1 alfalfa may alter soil nitrogen levels, impacting the species and abundance of nitrogen-cycling bacteria (Lu et al., 2023). This study further supports the idea that soil nutrients, including AHN and SOC, are key drivers of bacterial diversity, as reflected in the Shannon and Chao1 indices.

The bacterial communities in both bare ground and alfalfa-restored soils were dominated by three phyla: Proteobacteria, Actinomycetes, and Acidobacteria. These phyla are commonly found in soil bacterial communities (Kielak et al., 2016; Vieira et al., 2017). In alfalfa-restored soils, the relative abundance of Proteobacteria, Actinomycetes, and Acidobacteria was significantly higher compared to bare ground. This finding aligns with studies on grasslands, which have shown a positive relationship between plant cover and soil microbial activity (Lange et al., 2015). Such increases may result from enhanced root inputs, changes in root secretions (Bais et al., 2006), or reduced topsoil evapotranspiration due to the higher vegetation density in plant communities (Lange et al., 2014). Proteobacteria are known to play a key role in soil nutrient availability (Xiao et al., 2022), suggesting that the A5 alfalfa variety may be particularly effective in utilizing soil nutrients. Actinomycetes, which are strongly associated with soil disease resistance, showed a higher relative abundance in soils with low disease incidence (Mendes et al., 2014). This suggests that the A6 alfalfa cultivar may enhance soil disease resistance. Additionally, the relative abundance of Acidobacteria, the most significantly altered phylum in alfalfa-restored soils, was notably higher. Acidobacteria are capable of neutralizing and regulating alkaline soils through the production of metabolic acids (Schmalenberger et al., 2013), which can contribute to salt resistance in plants (Xu et al., 2020). Furthermore, Acidobacteria enhance cellulolytic activity and some species may even photosynthesize (Desta et al., 2016; Pankratov and Dedysh, 2010), playing a key role in plant residue decomposition and the carbon cycle. In summary, alfalfa cropping stimulates increased root secretions, which in turn regulate the beneficial Acidobacteria population. As such, A4 alfalfa varieties may be particularly effective in promoting Acidobacteria and enhancing soil health.

Plant rhizosphere bacteria adapt their community structure to specific environmental stresses, co-evolving with their host plants during their adaptation and acclimatization (Wang et al., 2022a). In this study, deterministic processes were found to dominate in the A5 and A6 alfalfa varieties, while stochastic processes were more prevalent in other alfalfa varieties. Jiao et al. explored bacterial community assembly in the oasis-desert transition zone in northwestern China, highlighting the role of soil fungal abundance in regulating microbial community dynamics. They found that under drought stress, fungal abundance influences bacterial community construction, with deterministic processes increasing as fungal abundance rises, while stochastic processes decline (Jiao et al., 2022). This mechanism may similarly apply to alfalfa's role in improving saline soils by modulating soil fungal abundance to reduce the influence of stochastic processes. The increase in deterministic processes in the A6 alfalfa variety may be linked to the enrichment of Actinobacteria, a group of bacteria specialized in microbial colony formation. Changes in ecological niches driven by deterministic processes likely contributed to the growth of Actinobacteria (Wang et al., 2024c). Alfalfa cultivation also improved the structure of bacterial microbial networks, with increased resource and

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nutrient availability being key factors in the greater network complexity compared to bare ground (Dai et al., 2022; Li et al., 2024a). Higher plant densities, particularly in rhizobial symbiotic alfalfa, result in denser root systems, which provide more resources for microorganisms through the decomposition of root fragments and the release of root exudates (Chen et al., 2019). Bacterial networks associated with nitrogen-fixing *nifH* genes were notably enriched, likely due to the increased nutrient availability and the ecological changes in the plant rhizosphere (Li et al., 2024b). Furthermore, alfalfa may stimulate bacterial interactions through the recruitment of nitrogen-fixing bacteria, and the enhanced nutrient availability in saline soils further promotes microbial activity. Alfalfa cultivation significantly increased the relative abundance of microbial functions such as methylotrophy, methyl group disproportionation, and dark hydrogen oxidation. Alfalfa rhizobacteria, capable of biological nitrogen fixation, increase nitrogen availability in the soil (Lagunas et al., 2023; Wang et al., 2020b). This nitrogen boost likely supports the colonization of specific microbial communities involved in dark hydrogen oxidation, which provides energy through hydrogen peroxide, thus enhancing microbial functions (Fan et al., 2022). Additionally, alfalfa's root secretions, which contain low-molecular-weight organic substances such as methanol (Wang et al., 2022b), provide nutrients for microorganisms that utilize methyl as a carbon source, further promoting the enrichment of relevant functional genes and microbial communities.

Soil nutrient dynamics drive soil fungal communities

Soil fungal communities are more sensitive to salinity than bacterial communities, and soil pH has been shown to negatively correlate with fungal diversity (Zhang et al., 2024b; Yang and Sun, 2020). Alfalfa's transpiration helps lower the water table, reducing salt accumulation near the soil surface and inhibiting salt rise. Additionally, organic acids secreted by alfalfa roots, along with those produced by microbial decomposition of plant residues, can help neutralize soil alkalinity. Among the alfalfa varieties studied, A4 had the highest Shannon index, while A5 had the highest Chao1 index, indicating different effects on soil properties that influence fungal diversity. The A4 variety accumulated more available potassium (AK) around its roots, which promotes plant growth and increases rhizosphere secretions, providing resources for a wider range of fungi (Soumare et al., 2023). Potassium ions can also regulate fungal communities by affecting ion exchange between roots and soil, enhancing potassium uptake and supporting the diversity of specific fungal species (Ji et al., 2020; Morgan and Connolly, 2013). Additionally, available phosphorus (AP) explained a significant portion of the variance in fungal community composition, with phosphorus contributing to the increase in fungal richness observed in the A5 variety. Differences in how alfalfa varieties improve saline soils further influence fungal diversity by altering soil nutrient levels and acidity. This was supported by a Mantel test, which identified AHN, EC, pH, TP, and SOC as key factors affecting fungal diversity indices such as Shannon and Chao1. Fusarium, a dominant genus in the fungal community, showed a significant positive correlation with organic nitrogen, which alfalfa contributes through its rhizobacteria and root secretions (Ofosu-Budu et al., 1993). Fusarium enrichment was notably higher in the alfalfa rhizosphere (Qi et al., 2023), and Fusarium oxysporum was more active in surface soils (Li et al., 2023). Alfalfa's root density decreases with depth, with higher secretion and SOC concentrations in the upper layers promoting Fusarium growth (Liu et al., 2023a; Chai and Schachtman, 2022). However, Fusarium is also a

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potential cause of alfalfa root rot, and an increase in pathogenic fungi in the fungal community was observed after alfalfa cropping (Jiang et al., 2021). Salinization can reduce plant immunity, making plants more susceptible to pathogens (Wang et al., 2023), and irrigated field management in alfalfa plots may also elevate plant pathogenic fungi (Deng et al., 2022). Alfalfa cropping increased the relative abundance of Saprotroph and Symbiotroph fungi, with the cover crop providing a greater source of organic carbon and fostering a richer symbiotic fungal community (Schmidt et al., 2019). Symbiotic fungi are crucial for crop health, nutrition, and quality (Igiehon and Babalola, 2017), while saprotrophic fungi play an essential role in nutrient cycling by decomposing organic matter (Maranón-Jiménez et al., 2021). Alfalfa's contribution of organic carbon promotes saprotrophic fungal activity, further enhancing the decomposition of organic matter (Crowther et al., 2012). The introduction of alfalfa led to an increase in deterministic processes during fungal community assembly. With vegetation cover and soil nutrient inputs, deterministic processes favored the development of fungal communities well-adapted to their environment. Conversely, stochastic processes may lead to maladaptive taxa and reduce ecosystem functioning (Ernakovich et al., 2022). Deterministic processes also promote the formation of similar phylogenetic groups, which enhance community resilience to external disturbances (Yu et al., 2021). As with bacterial communities, alfalfa cultivation increased the complexity and stability of fungal microbial networks. Highly connected networks offer greater functional redundancy, making the fungal community more resilient to disturbances (Scheffer et al., 2012). Such networks are also more efficient in carbon utilization and nutrient exchange between species, reducing competition within the microbial community due to improved resource availability (Morriën et al., 2017).

Alfalfa cropping plays a role in regulating archaeal communities

Archaea are a significant component of microbial populations in natural ecosystems (Dubey et al., 2016). These microorganisms are capable of surviving in extreme environments, including areas with high temperature and salinity, which are among the harshest conditions on Earth (Chaban et al., 2006). Yendi et al. identified previously uncharacterized archaeal microorganisms in saline soils (Navarro-Noya et al., 2015). However, our study found that cropping alfalfa in saline soils did not significantly alter the diversity or richness of soil archaea. Similarly, Joseph et al. reported that soil salinity had little effect on the archaeal community, as archaea are genetically suited to thrive in saline environments (Nan et al., 2020; Guevara-Luna et al., 2023). PCA analysis revealed significant differences in the structure of soil archaeal communities between alfalfa-cropped and bare land soils. Soil pH has been identified as a major factor influencing archaeal community composition, with pH playing a crucial role in shaping soil microbial communities. Variations in soil pH contribute to the formation of distinct ecological niches for microorganisms (Tripathi et al., 2013). He et al. also demonstrated through RDA analysis that both electrical conductivity (EC) and pH influence the structure of archaeal communities in soil (He et al., 2019). Furthermore, research has confirmed a strong correlation between soil pH and archaeal populations (Xiao et al., 2017), and alfalfa planting has been shown to effectively reduce soil pH, potentially influencing archaeal diversity. The results of the Mantel test in this study supported the influence of EC, pH, and available nitrogen (AHN) on soil archaeal communities, aligning with previous findings (Zhao et al., 2022). The relative abundance of Thaumarchaeota, the dominant archaeal phylum in soil, was significantly

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higher in alfalfa-cropped rhizospheres compared to bare ground. Thaumarchaeota plays a key role in ammonia oxidation, which is critical for soil nitrogen cycling (Ke et al., 2014). By restoring soil through alfalfa cropping, nutrient inputs from above-ground vegetation enhance soil nitrogen transformation processes and stimulate Thaumarchaeota activity (Li et al., 2021a). Among the alfalfa varieties tested, A1 showed the greatest increase in archaeal abundance, suggesting that this variety may have a superior capacity for nitrogen turnover compared to the others.

Soil archaeal communities play a vital role in biogeochemical cycles and overall soil health (Wei et al., 2020). After planting alfalfa, the number of nodes and edges in the archaeal network increased, with the A2 alfalfa variety showing the highest number of nodes and the A8 variety having the most edges. Soil archaea are known to form stable network structures, which help them resist external environmental disturbances (Wang et al., 2020a). Different microbial taxa respond variably to soil nutrients, with some taxa being more sensitive to nutrient changes, which in turn drives shifts in network complexity (Ren et al., 2019). The A2 and A8 alfalfa varieties significantly boosted soil phosphorus content—total phosphorus (TP) and available phosphorus (AP), respectively—creating favorable conditions for archaea. The A8 variety promoted plant growth by increasing AP, altering root secretion composition, and strengthening interactions among archaeal species (Yang et al., 2020). The higher TP levels in soils cropped with A2 alfalfa likely provided a steady phosphorus supply through microbial mineralization or desorption, supporting a more diverse archaeal community. This long-term phosphorus availability facilitated the coexistence of various archaea and expanded the number of network nodes (Yuan et al., 2024). Moreover, the increase in TP may have alleviated nutrient limitations, promoting ecological niche differentiation and reducing competition between archaea, bacteria, and fungi. The archaeal network, with its increased number of nodes and edges, reflects deterministic processes that foster microbial synergies and enhance functional stability (Li et al., 2024a). Deterministic processes are the primary drivers in constructing the archaeal community in A2 alfalfa soil. By improving the soil microenvironment, alfalfa enriches adaptive archaeal species that are better suited to environmental changes, thus dominating the deterministic processes (Tripathi et al., 2018; Liu et al., 2021). Additionally, we observed a significant increase in the abundance of functions such as methyl group disproportionation and dark hydrogen oxidation in the A5 variety. Alfalfa root secretions and plant residues, which contain large amounts of methylated compounds like lignin and lipids, serve as substrates for methyl group disproportionation (Zhou et al., 2022; Jiang et al., 2018). With alfalfa cropping, organic matter enriches the soil, making the microbial community more metabolically active and increasing hydrogen production. This supports the increase in the relative abundance of dark hydrogen oxidation (Li et al., 2022b). Furthermore, the deep root system of alfalfa enhances soil structure, improves gas diffusion, and further supports archaeal function proliferation. As a nitrogen-fixing crop, alfalfa alters the nitrogen cycle by fixing atmospheric nitrogen through symbiotic rhizobia (Guo et al., 2023; Lagunas et al., 2023). The hydrogen produced during nitrogen fixation provides an energy source for dark hydrogen oxidation, while the associated shifts in the microbial community can enhance methyl group metabolism and hydrogen oxidation processes.

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Conclusions

After 18 years of using different alfalfa varieties to restore saline-alkali soils, changes were observed in the diversity, community composition, and topological properties of the soil microbial co-occurrence networks, including bacteria, fungi, and archaea. Planting alfalfa significantly increased the alpha diversity of both soil bacteria and fungi. Among the treatments, the A7 variety exhibited the highest beta diversity for both bacteria and fungi, while no significant differences in archaea diversity were found across treatments. The planting of A5 alfalfa resulted in the greatest increase in the relative abundance of Proteobacteria, while Thaumarchaeota was most abundant after planting A1 alfalfa, and Fusarium showed the highest relative abundance after planting A8 alfalfa. Additionally, the bacterial network complexity was highest in soils planted with A3 alfalfa, while the fungal network was most complex in the A7 treatment. For archaea, the highest network complexity was observed in soils planted with A2 and A8 alfalfa.

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APPENDIX

Table S1. *Alfalfa variety sources and traits*

Varieties	Source	Genetic background and traits
LH wet tolerant	FGI, USA Waterman-Loomis Research, Inc.	Conventional breeding for resistance to bacterial wilt and yellow wilt, Anthracnose, Phytophthora root rot and other disease resistant varieties
Longmu 801	Heilongjiang Animal Husbandry Research Institute	Heterotetraploid was obtained from wild diploid alfalfa bean (Melissitue ruthenicus C. W. Chang) × tetraploid Zhaodong alfalfa (Medicago sativazhaodong). It is cold-tolerant, salinity-tolerant, drought-tolerant, flood-resistant, high-yielding, resistant to powdery mildew and thrips
Prairie 1	Inner Mongolia Agricultural University	Hybrid alfalfa. Cold and drought tolerant
WL354HQ	FGI, USA Waterman-Loomis Research, Inc.	Clones. Excellent cold resistance, high yield, well-developed root system, good persistence, fast regeneration, good palatability, high resistance to bacterial wilt, Fusarium wilt, anthracnose, Phytophthora root rot, Siluridium root rot, yellow wilt, Siluridium physiological minor 2, aphids, nematodes
WL319HQ	FGI, USA Waterman-Loomis Research, Inc.	Conventional breeding asexual line. Leafy, high yielding, highly resistant to bacterial wilt, yellow wilt, blight, anthracnose, blight root rot, filamentous bursa root rot, and moderately resistant to pea aphid, alfalfa spotted wing aphid, and blue clover aphid
Gongnong 1	Jilin Provincial Academy of Agricultural Sciences, Animal Husbandry Academy Branch	Introduced from the U.S., Grimm's hybrid alfalfa is hardy and productive
Challenger	Dailyland Seed Company, USA, Beeching Corporation, Canada	Resistant to cold, drought, wind and sand, crushing, and highly resistant to bacterial wilt, fusarium wilt, yellow wilt, anthracnose, and root-knot nematode
Marker	Monsanto Corporation, USA	Salt tolerant transgenic, high yielding, high germination, fast emergence, vigorous growth, medium to early maturity, high resistance to bacterial wilt, Fusarium wilt, yellow wilt, anthracnose, root rot of the genus Silphium, pea aphid

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Table S2. *Topology of soil bacterial network under different treatments*

Index \ Alfalfa variety	B	A1	A2	A3	A4	A5	A6	A7	A8
Average degree	51.854	5.32	5.846	7.838	5.657	5.908	6.085	5.86	5.444
Diameter	4	3	2	4	5	4	1	1	3
Density	0.059	0.005	0.006	0.007	0.005	0.006	0.006	0.006	0.005
Modularity	0.716	0.973	0.949	0.935	0.956	0.971	0.952	0.958	0.962
ACC	0.985	0.993	0.998	0.997	0.997	0.989	1	1	0.996
APL	1.079	1.015	1.001	1.009	1.011	1.088	1	1	1.011
PC	99.85%	99.72%	99.11%	98.92%	99.97%	99.71%	99.78%	99.40%	100.00%
NC	0.15%	0.28%	0.89%	1.08%	0.03%	0.29%	0.22%	0.60%	0.00%

APL, Average path length; ACC, Average clustering coefficient; PC, positive correlation; NC, negative correlation

Table S3. *Topology of soil fungal network under different treatments*

Index \ Alfalfa variety	B	A1	A2	A3	A4	A5	A6	A7	A8
Average degree	13.577	6.213	11.818	7.887	8.778	9.023	9.113	33.260	4.892
Diameter	2	2	3	2	1	1	3	4	2
Density	0.132	0.067	0.072	0.075	0.082	0.104	0.074	0.185	0.038
Modularity	0.692	0.733	0.583	0.698	0.736	0.695	0.729	0.164	0.815
ACC	0.988	0.989	0.946	0.986	1	1	0.986	0.983	0.992
APL	1.001	1.003	1.098	1.002	1	1	1.043	1.061	1.006
PC	99.58%	99.66%	77.43%	100%	99.79%	99.50%	93.80%	89.51%	98.76%
NC	0.42%	0.34%	22.57%	0.00%	0.21%	0.50%	6.20%	10.49%	1.24%

APL, Average path length; ACC, Average clustering coefficient; PC, positive correlation; NC, negative correlation

Table S4. *Topology of soil archaeal network under different treatments*

Index \ Alfalfa variety	B	A1	A2	A3	A4	A5	A6	A7	A8
Average degree	63.374	241.586	243.578	100.888	183.827	87.009	150.855	62.767	283.002
Diameter	3	4	12	4	4	5	3	4	5
Density	0.069	0.137	0.131	0.063	0.108	1.053	0.09	0.036	0.156
Modularity	0.704	0.175	0.162	0.678	0.249	0.581	0.507	0.813	0.092
ACC	0.987	0.983	0.977	0.993	0.987	0.944	0.992	0.998	0.983
APL	1.002	1.002	1.611	1.013	1.062	1.011	1	1.006	1.025
PC	99.84%	99.74%	99.86%	99.87%	99.94%	99.92%	99.89%	94.97%	99.88%
NC	0.16%	0.26%	0.14%	0.13%	0.06%	0.08%	0.11%	5.03%	0.12%

APL, Average path length; ACC, Average clustering coefficient; PC, positive correlation; NC, negative correlation

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