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Rhizosphere Microbiome Variations and Soil Amelioration Potential of *Medicago* Species Planted in Saline-alkali Soils

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ABSTRACT

Background: Soil salinization has become a critical threat to global food security and ecosystem sustainability. Conventional physicochemical remediation methods face limitations such as high costs and secondary pollution risks, whereas plant-microbe-synergistic ecological restoration techniques have gained attention due to their sustainability. Alfalfa, a perennial leguminous forage, combines economic, ecological and ornamental values. Investigating rhizosphere microorganisms associated with different alfalfa varieties and their functional roles in saline-alkali soils could provide a theoretical foundation for ecological restoration in salt-affected regions.

Methods: We compared the physicochemical properties of rhizosphere soil from *Medicago sativa* L., *Medicago falcata* L. and *Medicago varia* Martin. planted in saline-alkali land for three years and analyzed differences in their rhizosphere microbial communities using high-throughput 16S rRNA sequencing.

Result: The results showed that planting alfalfa, especially *Medicago varia* Martyn., can significantly reduce soil pH and conductivity and increase the content of available nitrogen, phosphorus, potassium and total nitrogen in the soil, thereby improving the soil. Alfalfa recruited specific microorganisms from the soil to the rhizosphere, with bacteria from *Proteobacteria* and *Bacteroidetes* showing higher relative abundance in the rhizosphere of *Medicago varia* Martyn. These bacteria may serve as key factors in plant growth promotion, stress resistance and soil remediation. Our findings establish a theoretical basis for using alfalfa planting to improve saline-alkali lands.

Key words: *Medicago* genus, Rhizosphere microbiome, Saline-alkali soils, Soil nutrients.

INTRODUCTION

Soil salinization constitutes a critical challenge threatening global agricultural sustainability and ecological security (Purbajanti *et al*., 2024). As a major constraint to agricultural productivity, ecosystem stability and sustainable land use, it drives land degradation, crop failure, vegetation suppression and environmental deterioration (Chen *et al.,* 2021; Zhou *et al*., 2022). Globally, over 1 billion hectares of land are salt-affected (Ivushkin *et al*., 2019), with China accounting for 350 million hectares, of which approximately 13 million hectares hold agricultural potential (Gao *et al*., 2020; Zhao *et al*., 2022). Amid shrinking arable land resources, saline-alkali soil remediation has emerged as a pivotal strategy for ensuring food security and ecological restoration. Traditional physicochemical remediation methods face limitations such as high costs and secondary pollution, whereas plant-microbe synergistic systems offer sustainable alternatives (Ondrašek *et al*., 2021). Studies have demonstrated that salt-tolerant forage species enhance soil structure, reduce evaporation, improve aeration and water retention and directly absorb salts to maintain soil desalination (Li *et al*., 2021; Clay *et al*., 2023). Notably, leguminous forages, integrating biological nitrogen fixation with landscape beautification, exhibit unique potential in saline soil rehabilitation and urban greening.

Leguminous plants enhance soil nitrogen availability and reduce reliance on chemical fertilizers, primarily through rhizobial symbiotic nitrogen fixation (Pourbabaee *et al*., 2021). Alfalfa, a globally cultivated leguminous forage, exhibits exceptional adaptability due to its robust root system, perennial growth habit and strong regenerative capacity (Clément *et al*., 2020). Its multifunctional roles include phytoremediation (heavy metal accumulation), windbreak and sand fixation, as well as soil enrichment *via* nitrogen and carbon sequestration positioning it, as a cornerstone species for soil rehabilitation (Pourbabaee *et al*., 2021; Shi *et al*., 2022). Notably, alfalfa demonstrates salt-alkali tolerance, enabling its establishment in saline-alkali soils. Its root exudates activate microbial communities, driving organic matter mineralization and ion migration (Clay *et al*., 2023), thereby improving soil structure, fertility and regional ecological resilience (Shi *et al*., 2017). Beyond ecological services, alfalfa serves dual aesthetic functions: its prolonged flowering period and vibrant blossoms enhance urban green spaces, while

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its integration into compound vegetation systems synergizes soil remediation, biodiversity conservation and landscape beautification. Crucially, recent research underscores alfalfa's pivotal role as a bioengineering mediator in microbial-driven soil restoration. By modulating rhizosphere microbiomes and nutrient cycling, it bridges soil health restoration and sustainable agroecosystem development (Shi *et al*., 2017).

Soil microbiota serve as the central driver regulating carbon-nitrogen cycling and plant salt tolerance in saline-alkali ecosystems (Manzoni *et al*., 2012). As the most diverse and abundant biological component in soil systems, their community structure and functionality reflect ecological dynamics and serve as critical bioindicators of soil health (Thakur *et al*., 2019). Beyond mediating organic matter decomposition and nutrient cycling, they act as pivotal regulators of climate feedback mechanisms (Zhang *et al*., 2023). Consequently, microbial community stability underpins the integrity of soil ecosystem services, including carbon sequestration and stress resilience (Manzoni *et al*., 2012). Alfalfa rhizosphere microbiomes demonstrate specialized salt-adaptive strategies. Halotolerant taxa (*e.g*., *Pseudomonas*, *Bacillus*) mitigate ethylene stress *via* ACC deaminase secretion while synthesizing osmolytes (proline, glycine betaine) to enhance plant salt tolerance (Qin *et al*., 2016). The *Sinorhizobium meliloti*-alfalfa symbiosis elevates soil nitrogen availability by 30-50% and improves soil aggregation through exopolysaccharide secretion (Guo *et al*., 2022). This mechanism holds particular significance for urban greening: conventional high nitrogen inputs exacerbate soil compaction and eutrophication, whereas legume-microbe symbiosis enables nutrient self-regulation in green spaces. Recent advances in molecular biology have catalyzed microbial-driven remediation strategies, positioning microbial alle*via*tion of salt stress as a frontier approach for enhancing crop resilience (Qin *et al*., 2016).

This study investigated the remediation potential of *Medicago* species in saline-alkali soils, addressing two critical questions: (1) Mechanistic effects of three *Medicago* plant cultivation on soil physicochemical properties (*e.g*., salinity reduction, nutrient cycling, structural stabilization). (2) Rhizosphere microbiome variations underlying differential salt-alkali stress adaptation among three *Medicago* plant. The findings will establish a theoretical foundation for ecological rehabilitation of degraded saline ecosystems and multifunctional green infrastructure development integrating soil restoration with biodiversity conservation.

MATERIALS AND METHODS

Experimental area and plant materials

The experimental site was located in Tumet Left Banner (40°35′18.63″N, 110°34′30.78″E), Hohhot City, Inner Mongolia Autonomous Region, China (Fig 1). The moderately saline-alkali soil had a pH of 8.6, salt content of 4.65 g/kg and electrical conductivity (EC) of 4.29 dS/m. Three *Medicago* species (*Medicago sativa* L., *Medicago falcata* L. and *Medicago varia* Martin.) were cultivated at the site for three years.

Collection of rhizosphere soil samples

In June 2024, during the early flowering stage of all plants, rhizosphere soil samples were collected from root surfaces using the method of Edwards *et al.* (2015). Bulk soil (BS), serving as the control, was collected from adjacent non-vegetated areas. Subsequently, each soil sample was divided into two sub samples. One of them was placed in a 5 mL sterile cryovials and immediately returned to the laboratory with dry ice after rapid freezing with liquid nitrogen. It was stored at -80°C and rhizosphere microbial DNA extraction was completed within 24 hours. The remaining subsample was placed in a plastic bag, transported to the laboratory at 4°C and stored at -20°C for subsequent determination of soil physicochemical properties and enzyme activity.

Analysis of soil physicochemical properties and enzyme activity

Soil pH and EC were measured using a digital pH meter and conductivity meter, respectively. Total nitrogen (TN) was quantified *via* CHNOS elemental analyzer, while total phosphorus (TP) was determined with a continuous flow analytical system. Total potassium (TK) was analyzed by inductively coupled plasma-atomic emission spectrometry. Available nitrogen (AN) was assessed using the alkaline hydrolysis diffusion method, available phosphorus (AP) by molybdenum-antimony spectrophotometry and available potassium (AK) by ammonium acetate extraction-flame photometry. All analyses included three biological replicates. Soil enzymatic activities were determined using commercial assay kits (Solarbio, China) following manufacturer protocols.

DNA extraction, 16S amplification, squencing and data processing

Microbial community total genomic DNA extraction was performed by the DNeasy® PowerSoil® Pro Kit (QIAGEN, U.S.). The V3-V4 variable regions of the bacterial 16S rRNA gene were amplified using the ABI GeneAmp® 9700 PCR system with primers 338F (5′-ACTCCTACGGGAGGCAG CAG-3′)/806R (5′-GGACTACHVGGGTWTCTAAT-3′). Amplicons were sequenced on the Illumina MiSeq PE300 platform. Raw sequencing reads were subjected to quality control using fastp (v.0.19.62). Subsequently, the paired-end sequences were merged to a single sequence with length of ~300 bp using FLASH (v.1.2.11). Then the optimized sequences were clustered into operational taxonomic units (OTUs) using UPARSE 7.1 with 97% sequence similarity level. Representative sequences were classified using RDP Classifier (v.2.2) and annotated against the SILVA reference database (v.138) with a confidence threshold of 0.7.

Statistical and bioinformatics analyses

Statistical analyses were performed using SPSS (v.26.0.0.2). Bar graphs were plotted with Prism 9.5 (GraphPad Software, LLC). Alpha diversity was calculated using QIIME (v.1.9.1). Rarefaction curves based on the Shannon indices were plotted for each sample. Principal Coordinate Analysis (PCoA) based on the Bray-Curtis distance was performed using the R Package "vegan (v.2.5-3)". Species composition bar plots were generated with the ggplot2 (v.3.3.5) package.

RESULTS AND DISCUSSION

The effect of planting alfalfa on soil physicochemical properties

Numerous studies have demonstrated the significant soil amelioration effects of alfalfa cultivation in saline-alkali lands (Li *et al*., 2021; Huang *et al*., 2019). Notably, 3-6 years of cultivation have been shown to significantly reduce soil pH and total soluble salts while improving nutrient availability (Fan *et al*., 2023). Alfalfa's extensive root system enhances soil structure through increased porosity and permeability. In our study, we compared rhizosphere soil properties of three *Medicago* species after three-year cultivation, with bulk soil (BS) as control (Fig 2). The BS exhibited alkaline pH (8.61) and moderate salinity (EC = 4.28 dS/m). All three *Medicago* species showed statistically significant pH reductions compared to BS (p<0.05), with MvM showing the greatest reduction to 8.14. No significant difference was observed between MS and MF (p<0.05). Notably, soil EC of MvM and MS cultivation were significantly reduced (p<0.05), with MvM showing remarkable reductions of 57.71% compared to BS, consistent with previous research findings. Perhaps the salt-alkali tolerance and growth ability of *Medicago varia* Martin. are stronger, therefore its ability to absorb soil acidification and salt ions is stronger.

Soil nitrogen, phosphorus, potassium and organic matter contents serve as crucial indicators of soil fertility (Ram *et al*., 2023). Our results align with reports that alfalfa enhances nutrient availability through symbiotic nitrogen fixation, particularly nitrogen enrichment, thereby rehabilitating degraded soils (Saidi *et al*., 2021). Among the tested species, MvM showed superior performance in elevating available N, P and K levels, followed by MS, while MF exhibited relatively weaker effects (Fig 2). Interestingly, while TN significantly increased only in MvM rhizosphere, all *Medicago spp*. exhibited decreased TP and TK compared to BS. This phenomenon could be attributed to enhanced nitrogen fixation coupled with efficient conversion of insoluble P/K into plant-available forms during vigorous growth. These findings collectively suggest that *Medicago varia* Martin. possesses superior growth vigor and stress tolerance in saline-alkali environments, making it particularly effective for ecological restoration of degraded soils.

The effect of planting alfalfa on soil enzyme activity

Soil enzyme activity serve as a critical bioindicator of biological activity and biochemical processes, reflecting soil fertility, quality and health status (Alkorta *et al*., 2003). Our study revealed significant interspecific differences in rhizosphere enzyme profiles among *Medicago* species (Fig 3), with MvM exhibiting superior performance in overall soil enzyme activity. Both α-glucosidase and β-glucosidase, key drivers of organic matter decomposition and carbon cycling essential for maintaining soil fertility and ecological balance, showed the highest activities in MvM rhizosphere (p<0.05). Notably, MF exhibited significantly higher α-glucosidase activity than MS (p<0.05), while β-glucosidase activities showed no statistical difference between these two species (p>0.05). In nitrogen-related enzymes, MvM displayed the highest alkaline protease activity, followed by MS. Given that protease activity is sensitive to environmental stressors including heavy metal contamination, organic pollutants and suboptimal pH levels (Chae *et al*., 2017), its activity level serve as a valuable indicator of soil environmental quality. Dehydrogenase activity, reflecting microbial metabolic intensity, showed no significant

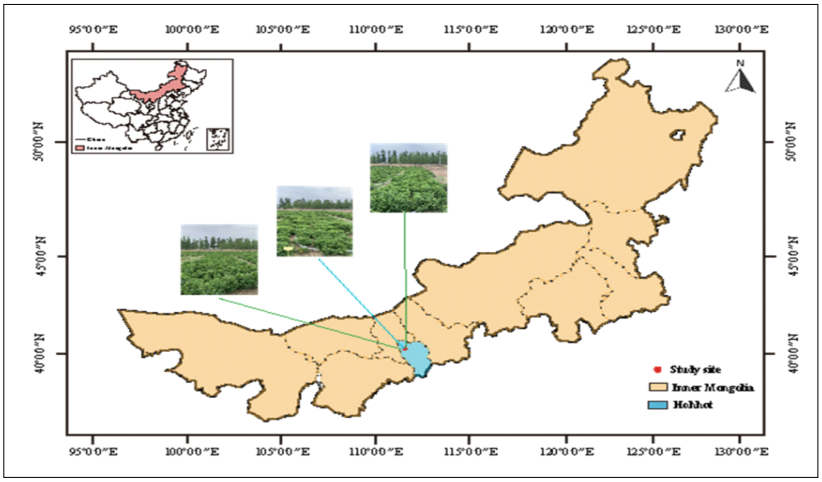
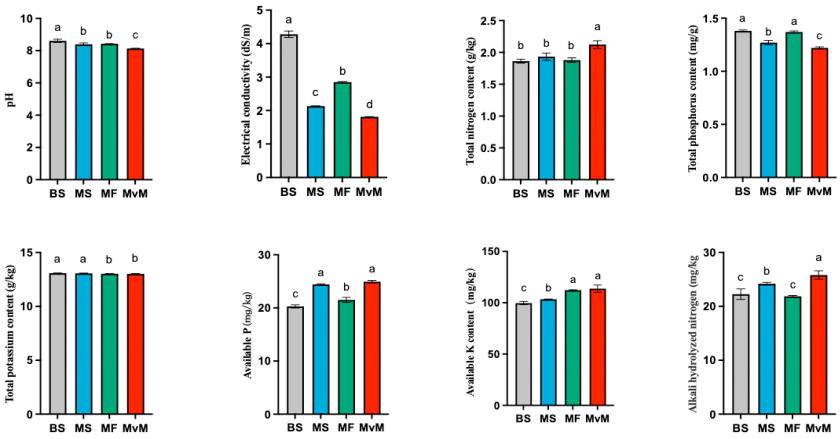


Fig 1: Location map of the alfalfa planting site in this study.

variation among the three alfalfa species (p>0.05), indicating comparable microbial activity enhancement across all treatments. Urease activity, which governs organic nitrogen mineralization and inorganic nitrogen availability (Baheliya *et al*., 2025), along with alkaline phosphatase that catalyzes phosphate hydrolysis for phosphorus mobilization (Hu *et al*., 2015), both reached peak levels in MvM rhizosphere (p<0.05). No significant differences were observed between MS and MF in these enzymatic activities (p>0.05). The comprehensive enzymatic profile analysis confirms that MvM outperforms MS and MF in enhancing soil remediation efficiency, nutrient cycling capacity and microbial ecological functions in saline-alkali environments.

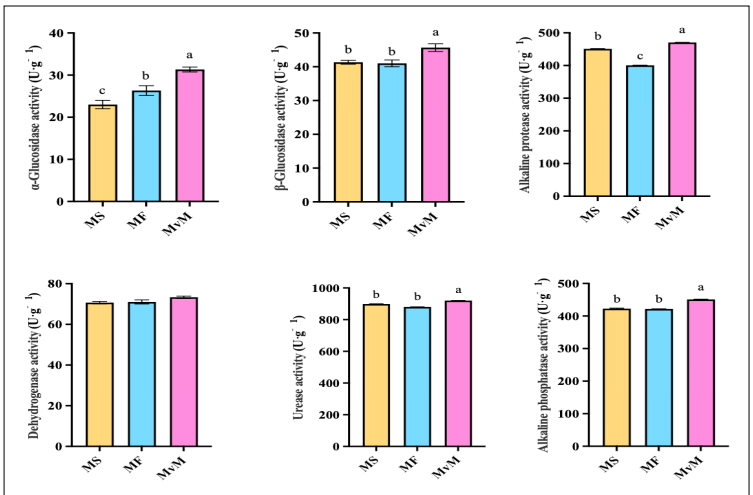
Sequencing reads information, data statistics and otu analysis

Amplicon sequencing of bacterial 16S rRNA V3-V4 regions generated 958,186 high-quality sequences from 12 soil samples. Sequencing depth reached saturation across all four sample types (Fig 4A), confirming adequate coverage for microbial diversity characterization. Cluster analysis at 97% sequence similarity identified 2,290 OTUs, with alfalfa-cultivated soils exhibiting significantly higher OTU richness compared to BS (Fig 4B). This increased bacterial diversity suggests that rhizosphere microbiota play critical roles in alfalfa's adaptation to saline-alkali stress and soil remediation. A Venn diagram (Fig 4C)



One-way ANOVA was used to analyze the effects of different *Medicago spp*. plants on the physical and chemical properties of rhizosphere soil. Error bars represent SD values of three replicates. Different letters above the bars indicate statistically significant differences (P<0.05).

Fig 2: Rhizosphere soil physicochemical profiling across three *Medicago* species.



One-way ANOVA was used to analyze the effects of different *Medicago* plants on the enzyme activity of rhizosphere soil. Error bars represent SD values of three replicates. Different letters above the bars indicate statistically significant differences (P<0.05).

Fig 3: Enzyme activity determined in the rhizosphere soil of three *Medicago* plants.

diisplayed 458 conserved OTUs (20% of total) shared amng all groups, alongside species-specific microbial signatures: MS contained 155 unique OTUs, MF 175 and MvM demonstrated the most abundant exclusive OTUs (231). These interspecific differences indicate that MvM's rhizosphere harbors distinct microbial consortia potentially critical for its superior stress tolerance and growth performance in challenging environments.

Analysis of rhizosphere bacterial diversity

To ensure analytical validity, all samples were rarefied to the minimum sequence count (38,902 high-quality sequences) for standardized comparisons. Alpha diversity metrics (Chao1 richness estimator and Shannon diversity index) were employed to quantify rhizosphere microbial diversity and species richness. All *Medicago* species exhibited significantly higher Chao1 and Shannon indices compared to BS ($p<0.05$). Notably, MvM demonstrated superior diversity metrics versus both BS ($p<0.01$) and MF ($p<0.05$) (Fig 5A, B). The higher bacterial diversity may be due to the higher trend of symbiotic relationships between MvM and bacteria in nitrogen fixation and assimilation, carbon and sulfur cycling and other characteristics (Mendes *et al*., 2014).

The study by Zhao *et al*. (2020) on rhizosphere microbial community diversity in desert leguminous plants reached consistent conclusions. Obviously, as reported by Prudent *et al*. (2019), microbial diversity in rhizosphere soils has been linked to nutrient cycling dynamics, suggesting that high microbial diversity enhances plant growth and stress adaptation. PCoA based on Bray-Curtis distances (ANOSIM, R=0.418, p=0.007) revealed significant separation of microbial communities (PC1=25.63%, PC2=21.07%). All *Medicago spp*.-associated communities

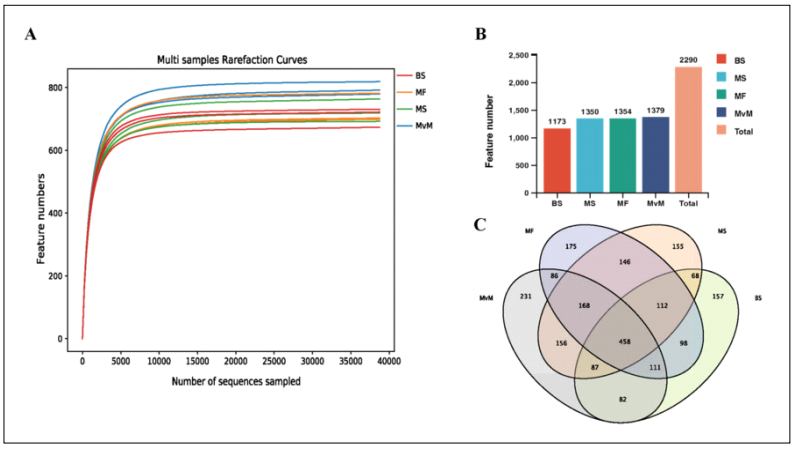
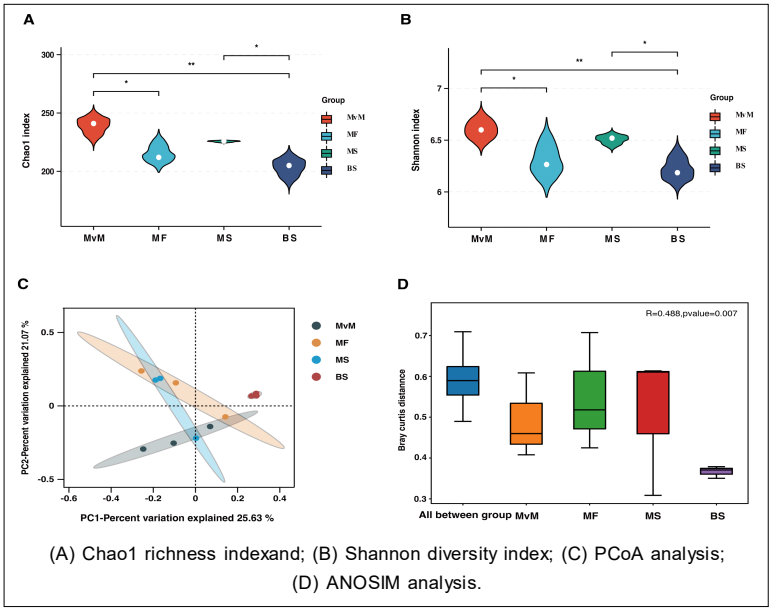


Fig 4: Rarefaction curves (A), OTU number distribution and Venn diagram at the OTU level (C) of the amplicon sequences obtained from the 12 rhizosphere soil samples.



(A) Chao1 richness indexand; (B) Shannon diversity index; (C) PCoA analysis; (D) ANOSIM analysis.

Fig 5: Bacterial community diversity.

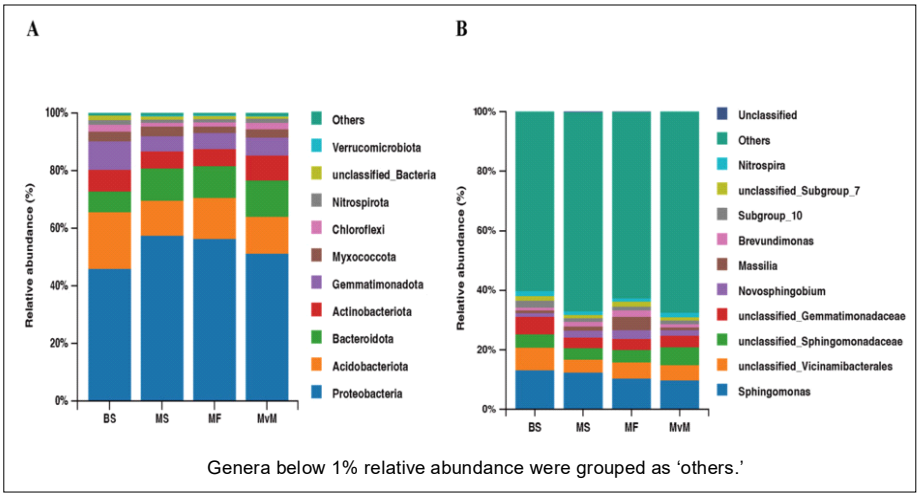


Fig 6: Bacterial community composition at the phylum level (A) and genus level (B).

clustered distantly from BS, indicating that alfalfa recruits bacteria from the soil and forms its unique rhizosphere microbial community (Fig 5C, D). Numerous reports have confirmed this point (Zhao *et al*., 2020; de Vries *et al*., 2020). The similarity in composition among different *Medicago* species indicates a similar community composition structure. This may be because they belong to the same genus of *Medicago* and the *Medicago varia Martin.* is a hybrid of *Medicago sativa* L. and *Medicago falcata* L., so they have many similarities with each other.

Analysis of community structure composition of rhizosphere bacteria

Community composition analysis based on phylum level was conducted on rhizosphere bacteria of different *Medicago* species (Fig 6A). The three phyla with the highest relative abundance in the rhizosphere microbiota of different *Medicago* species were *Proteobacteria*, *Acidobacteriota* and *Bacteroidota*. This finding is similar to the structure and composition of dominant bacterial phyla reported by Sun *et al*. (2017) in the relative abundance statistics of soil microbial community diversity in arid shrublands. Research has shown that salt stress leads to an increase in the relative abundance of *Proteobacteria* and *Bacteroidetes* in the rhizosphere of alfalfa, while the relative abundance of *Actinobacteria* decreases (He *et al*., 2021). In our study, the abundance of *Proteobacterla* and *Bacterodelta* in the rhizosphere of *Medicago spp*. were significantly higher than that in BS, but the abundance of *Acidobacterilota* in BS was significantly higher in the rhizosphere of *Medicago spp*. Notably, MvM exhibited the highest *Bacteroidota* abundance, while MS and MF showed no significant differences in bacterial composition. According to research statistics, about 40% of salt tolerant PGPR belongs to *Proteobacteria* (Zamanzadeh-Nasrabadi *et al*., 2023), making it more effective under salt stress in alfalfa. As Fan *et al*. (2023) found in their study, with increasing salinity, *Proteobacteria* are more likely to accumulate in the rhizosphere of alfalfa varieties with strong salt-alkali tolerance. Our experimental results also confirm this.

At the genus level, the three genera with the highest relative abundance were *Sphingomonas*, unclassified *Vicinamibacterales* and unclassified *Sphingomonasaceae*. Species-specific patterns emerged: *Sphingomonas* dominated MS rhizospheres, *Massilia* was enriched in MF and unclassified *Sphingomonadaceae* prevailed in MvM (Fig 6B). These bacterial genera have been reported to be beneficial growth-promoting bacteria that help plants grow under strss conditions (Fan *et al*., 2023). This interspecific divergence confirms that host genotype shapes rhizosphere microbiome assembly (Fox *et al*., 2020). The main factors contributing to this difference may be related to root exudates and soil physicochemical properties. As reported in current research, root exudates have been recognized as an important medium for plant soil communication (Afridi *et al*., 2024).

CONCLUSION

We conclude that planting *Medicago spp.* can significantly improve the physical and chemical properties and soil enzyme activity of saline-alkali soil, especially for *Medicago varia* Martin., which can significantly reduce soil pH and salt content and increase the content of available nitrogen, phosphorus, potassium and total nitrogen in the soil. We found that alfalfa can specifically recruit relevant bacteria from saline-alkali soil, especially bacteria from the phyla *Proteobacteria* and *Bacteroidetes*. These are potential stress-tolerant and growth-promoting bacteria and compared to *Medicago sativa* L. and *Medicago falcata* L., these bacteria have a higher relative abundance in the rhizosphere of *Medicago varia* Martin. These microbial communities enriched in the rhizosphere of MvM may drive their excellent salt-alkali adaptability and soil remediation ability by enhancing nutrient cycling and osmoregulatory metabolite synthesis.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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