

# Comparison of rhizosphere soil microbial diversity of different introduced alfalfa varieties

Chunhui Ma (✉ [chunhuima@126.com](mailto:chunhuima@126.com))

The College of Animal Science and Technology

Jiangjiao Qi

The college of Animal Science and Technology

Xue Yu

the college of animal science and technology

Lihe Su

the college of animal science and technology

Tingting He

the college of animal science and technology

Xuzhe Wang

the college of animal science and technology

Fanfan Zhang

the college of animal science and technology

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# Abstract

Alfalfa (*Medicago sativa* L.) is an important forage legume in farming and animal husbandry systems. In this study, MiSeq high-throughput sequencing was applied to assess the relationship between bacterial and fungal community structures and alfalfa growth characteristics and soil physical and chemical properties induced by different cultivars alfalfa (*Victoria*, *Kangsai*, *Aohan*) in the grey desert soil. The results showed that the diversity of bacterial and fungal in *Victoria* was higher, and the bacterial diversity was significantly lower for alfalfa with *Aohan* than for the others, and the fungal diversity was lower for alfalfa with *Kangsai* than for the others. Heatmap showed that total nitrogen, fresh weight, pH and organic have significantly affect fungal community structure, whereas pH and organic carbon also significant effects on bacterial community structure. LefSe analysis showed that the growth adaptability of introduced alfalfa is mainly related to fungal and bacterial species, and the beneficial microorganisms with significant differences and relative high abundance are significantly enriched in *Victoria*. Pathogens with high relative abundance are mainly concentrated in *Aohan* alfalfa soil. Based on our findings, *Victoria* is the high-yield alfalfa suitable for planting in gray desert soil, while planting *Kangsai* and *Aohan* alfalfa needs probiotic for adjuvant.

## Introduction

Alfalfa is a kind of legume forage with high protein content, which plays an important role in the development of livestock breeding (Wang et al. 2017). Different types of soil has significant differences on the growth and development of introduced alfalfa, which seriously affects the land utilization rate and alfalfa yield (He et al. 2017). In particular, after autumn, the growth difference of alfalfa is more obvious. It can be caused by issues such as the decrease of outside temperature and light (Fiutak et al. 2019). Therefore, in recent years, introduction has become a focus of improving alfalfa yield, although the relevant research remains lacking.

The soil microbial community is highly diverse (Berendsen et al. 2012) and plays a vital role in increasing nutrient availability to plant roots and improving soil structure (Xia et al. 2019). However, compared to non-rooted bulk soil, the rhizosphere, which is the important place where microorganisms interact with plants (Berg et al. 2014), and is considered as one of the most complex ecosystems (Bakker et al. 2013). The increase microbial abundance and activities in rhizosphere environment are due to the release of organic carbon by plant root exudation (Tkacz et al. 2015). Soil microorganisms are chemotactically attracted to root exudates, which allow them to proliferate in this carbon rich environment (Raaijmakers et al., 2009). Similarly, rhizosphere microbial communities can also directly and / or indirectly affect the composition and biomass of plant communities in natural and agricultural ecosystems (Philippot et al., 2013). The study of plant-micro interaction reveals the profound influence on plant growth, development, nutrition, disease and productivity. Plants and microorganisms are adapted to use their close ties to achieve mutual benefit. This is best exemplified by the symbiosis between rhizobia and legumes (Ji et al. 2018). The rhizosphere microorganisms can release carbon dioxide and metabolize acids, which dissolve insoluble minerals like phosphorous and increase their absorption via plant roots (Bakker et al. 2013).

They also secrete plant growth-stimulating substances, such as indoleacetic acid (Abd et al. 2020). Accordingly, the rhizosphere soil microbiota is crucial for the growth and development of its host. When the normal microbiota is affected by change of external environment, its self-balance will be broken, which may threaten the health of soil or even induce disease and insect pests of the host. Recent studies have shown that the types of microbes in the soil are abundant and can be affected by the external environment. Besides the physical and chemical characteristics of soil (Wang et al. 2019), ages and physiological conditions of plants also affect the structure of rhizosphere microbial community (Mendes et al. 2013). However, the characteristics of rhizosphere soil microbiota in alfalfa with different cultivars remain unclear.

This study aim to utilize high-throughput 16S rRNA and fungal ITS genes sequencing to comprehensively analyze and compare the bacterial and fungal community structures in soil of alfalfa with different cultivars. A better understanding of the biotic and abiotic components of planting different cultivars alfalfa, with the goal of providing a scientific basis for planting alfalfa, managing soil and reasonable application of bacterial fertilizer.

## Materials And Methods

### Sample collection and processing

Samples were collected from the experimental herbage station of Shihezi University in Shihezi City, Xinjiang Uygur Autonomous Region, China (N44 ° 20 ', E88 ° 30', altitude 420 m). The site is characterized by a temperate continental arid climate, mean annual precipitation of 225 mm, and mean annual evaporation of 1250 mm. In addition, the region experiences short and hot summers, and long and cold winter, with an average annual temperature of 8.1°C. The site was divided into 3 variety zones in May 2018, and the *Victoria* (VT, Fall Dormancy (FDC)=6.0, *Medicago sativa* L, Canada), *Kangsai* (KS, FDC=4.0, *Medicago sativa* L, America) and *Aohan* (AH, *Medicago sativa* L, Inner Mongolia of China) were planted, respectively. Each experimental zone measured 8 m × 6 m, and the plant and row spacing were 0.2 m and 0.6 m respectively. Rhizosphere samples were collected in mid-September 2019 from 3 random quadrats (1 m × 1 m) per zone by the "shaking root method" (Chen et al. 2020; Bungonsiri et al. 2019). Then six rhizosphere soil samples were collected from each quadrat and immediately pooled into a single soil sample. Finally, three soil samples were obtained for each variety zone. The rhizosphere soil samples were passed through a 2 mm sieve to remove any organic contaminants and snap frozen in liquid nitrogen. One part was stored at -80°C for high-throughput sequencing and another for physicochemical analysis. In addition, alfalfa plants from each quadrat were collected to determine plant height (PHT), number of branches (NOB), number of internodes (NOI), internode length (ILH) and fresh weight (FW).

### Analysis of soil physicochemical properties

pH was measured using the PHS-3C pH meter (Model PHS-3C pH Meter, Shanghai INESA Scientific Instrument Co.Ltd., Shanghai, China). The content of organic carbon (OC) and available nitrogen (AN) were respectively measured with the potassium dichromate-external heating method and alkali solution

diffusion method. The content of available phosphorus (AP) in the sodium bicarbonate-extracted soil samples was determined by molybdenum–antimony colorimetry. Available potassium (AK) was extracted with  $1\text{ mol L}^{-1}$   $\text{NH}_4\text{OAc}$ , and then determined by flame absorption spectroscopy. The samples were digested in  $\text{HClO}_4$  and  $\text{H}_2\text{SO}_4/\text{HCl}$  respectively to measure total nitrogen (TN) and total phosphorus (TP). The content of total potassium (TK) was determined in the molten sodium hydroxide soil extract by flame photometry (Bungonsiri et al. 2019).

### **DNA Extraction, Amplification and Sequencing**

Total DNA was extracted using the PowerSoil DNA isolated Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocol. The quality and concentration of the extracted DNA were measured using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, United States). The 16S rRNA and ITS genes were amplified using primers 338F-806R (Xu et al. 2016) and ITS1F-ITS2R respectively. The PCR products were mixed with the same volume  $2\times$  loading buffer and were subject to 1.8% agarose gel electrophoresis for detection. Then, the mixture of PCR products was purified using the Axyprep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA). PCR products were quantified using the QuantiFluor™-ST Fluorometer (Promega Biotech, Beijing, China), and the samples were adjusted as required for Sequencing. Sequencing were conducted by Shanghai Majorbio Bio-PharmTechnology (Shanghai, China) on the Illumina Miseq platform.

### **Bioinformatics analyses and functional annotation**

The raw reads were quality filtered under specific filtering conditions to obtain high-quality clean tags on the basis of the QIIME (V1.7.0) quality control process. Sequences that were shorter than 200 bp, ambiguous bases and those with an average mass less than 25 were removed. Chimeric sequences were removed using USEARCH v7.1. The unique sequences with  $\geq 97\%$  similarity were clustered into operational taxonomic units (OTUs) using the UPARSE software (Edgar, 2013). The sequences were then classified and annotated using the SILVA database. They were then taxonomically classified to different levels (phylum, class, order, family, genus, and species) using the Ribosomal Database Program (RDP) classifier.

### **Statistical Analysis**

One-way ANOVA of soil physical and chemical properties was performed using SPSS (version 19.0; SPSS, Chicago, IL, USA). Significance was calculated by Tukey's test ( $p < 0.05$ ). Alpha diversity indices (i.e., ACE, Sob and Shannon) were calculated by QIIME from rarefied samples using for richness and diversity indices of the bacterial community.  $\beta$ -diversity was calculated using unweighted UniFrac and non-metric multidimensional scaling (NMDS), after which Intra-group and Inter-group beta distance boxplot diagrams were generated. The Canoco program for Windows 4.5 (Biometris, Wageningen, the Netherlands) was used for principal component analysis (PCA). Heatmap evaluates the correlation between microbial classification and environmental variables by heatmap. The relationship between soil microbial community structure and each affecting factor was analyzed by Redundancy analysis (RDA).

RDA eliminates redundant variables depending on other measured variables, automatically selecting variables with large effects, and on the variance inflation factor values to gradually remove redundant parameters, and the significance levels are based on 999 Monte Carlo permutations. Linear discriminant analysis (LDA) effect size (LefSe) analysis was performed to reveal the significant ranking of abundant modules in different cultivars samples. A size-effect threshold of 2.0 on the logarithmic LDA score was used for discriminative functional biomarkers.

## Results

### **The alfalfa cultivars differ in terms of growth characteristics and the physicochemical properties of rhizosphere soil**

The VT alfalfa variety showed a significantly higher plant height internode length and fresh weight, along with increased TP, TK and AK levels in the rhizosphere soil samples compared to the KS and AH varieties ( $P < 0.05$ ). In contrast, the number of branches and soil AN were significantly higher for KS compared to that of VT ( $P < 0.01$ ), while AP and AK contents were significantly lower than that of VT and AH ( $P < 0.01$ ). The average number of internodes per plant and soil pH were similar across the different cultivars. Finally, soil TN and OC of AH were significantly higher than that of VT and KS ( $P < 0.05$ ) (Table 1).

### **The diversity of the soil microbiome differs among the alfalfa cultivars**

A total of 515,407 and 577,685 quality-filtered and chimera-checked 16S/ITS rRNA gene sequences were obtained with an average length of 417 and 234 bp across all samples, respectively. The ends of the rarefaction curves tapered off with increasing numbers of sequences per sample, as is commonly observed with sequencing data (Supplement Fig. 1A, B).

Bacterial and fungal community relative abundances (Sob, Ace) and diversity (Shannon;  $\alpha$ -diversity) index values were compared for different alfalfa soil (Table 2). The Sob and Ace estimator indicated that bacterial community abundances in KS was significantly higher than those in VT and AH ( $P < 0.05$ ); the fungal community abundance was higher in KS than in the others ( $P > 0.05$ ). The Shannon indices showed that VT and KS bacterial communities was significantly higher than AH ( $P < 0.05$ ), while the fungal community was lower in KS than others.

$\beta$ -diversity is used to analyze the temporal and spatial changes in species composition, reflecting whether there is difference in microbial communities between groups. The PCA clearly grouped the bacterial and fungal communities according to soil of different alfalfa varieties. The first two axes (PC1 and PC2) explained 43.3 and 30.7%, respectively, of the total variance in the bacterial and fungal species in soil of different alfalfa varieties. (Fig. 1A, B). The NMDS plot showing the dissimilarity of community and also revealed a distinct structure between VT, KS and AH (Fig. 1C, D).

### **Relative Abundance and Core Microbiota**

The bacterial phyla with high relative abundance were *Actinobacteria*, *Proteobacteria*, *Chloroflexi* and *Acidobacteria* (Fig. 2A). These bacteria accounted for 83.3, 81.9 and 82.9% of the detectable reads in the VT, KS and AH samples, respectively. The phyla with significant differences in relative abundance between VT and KS was *Actinobacteria* ( $P < 0.05$ ). The phyla with significant differences in relative abundance between VT and AH was *Proteobacteria* ( $P < 0.05$ ). The phyla with significant differences in relative abundance between KS and AH were *Actinobacteria* and *Acidobacteria* ( $P < 0.05$ ) (Supplement Table 1). On genus level, *Arthrobacter*, *JG30-KF-CM45* and *Gemmatimonadaceae* were the dominant genera (Fig. 2B). In the top 10 genera in relative abundance, the genera with significant differences in relative abundance between VT and KS were *Arthrobacter* and *Rokubacteriales* ( $P < 0.05$ ). The genera with significant differences in relative abundance between VT and AH were *Arthrobacter* and *Rokubacteriales* ( $P < 0.05$ ) (Supplement Table 2).

The fungal phyla with relatively higher abundance were *Ascomycota* and *Basidiomycota* (Fig. 2C). These bacteria accounted for 93.6, 89.6 and 94.7% of the detectable reads in the VT, KS and AH samples, respectively. The phyla with significant differences in relative abundance between VT and KS was *Ascomycota* ( $P < 0.05$ ). The phyla with significant differences in relative abundance between VT and AH was *Basidiomycota* ( $P < 0.05$ ). The phyla with significant differences in relative abundance between KS and AH was *Ascomycota* ( $P < 0.05$ ) (Supplement Table 1). In the top 10 genera in relative abundance, the genera with significant differences in relative abundance between VT and KS were *Cladosporium*, *Sordariomycetes*, *Stachybotryaceae*, *Alternaria* and *Penicilium* ( $P < 0.05$ ). The genera with significant differences in relative abundance between VT and AH were *Fusarium*, *Cladosporium*, *Sordariomycetes*, *Talaromyces*, *Mortierella*, *Hypocreales*, *Nectriaceae*, *Alternaria* and *Penicilium* ( $P < 0.05$ ). The genera with significant differences in relative abundance between KS and AH were *Fusarium*, *Sordariomycetes*, *Stachybotryaceae*, *Talaromyces*, *Mortierella*, *Hypocreales* and *Nectriaceae* ( $P < 0.05$ ) (Supplement Table 2).

### **Relationship between the microbial community structure and the soil and plant characteristics**

RDA revealed that the microbial community structure was formed by primary environmental characteristics (TN, AP, OC, pH, NOB, NO, FW). After removal of the redundant variables, seven factors were chosen for RDA. As shown in Supplement Fig. 2, the contribution of soil environmental and plant growth factors to bacterial and fungal communities were 68.16% and 73.67%, respectively. As shown in Fig. 3, on genus level, *Arthrobacter*, *Gaiella*, *Cladosporium* and *Lectera* showed a significant relationship with organic carbon; *Thanatephorus*, *Talaromyces*, *Stachybotryaceae* and *Fusarium* showed a significant relationship with fresh weight; *Sordariomycetes*, *Talaromyces*, *Lectera* and *Fusarium* showed a significant relationship with soil total nitrogen; *Geminicoccaceae*, *Sordariomycetes*, *Cladosporium* and *Mortierella* showed a significant relationship with pH.

### **Microbial communities with statistically significant differences**

Apart from determining  $\alpha$ - and  $\beta$ - diversities, another primary goal of comparing microbial communities is to identify specialized communities in samples. LefSe analysis was performed to reveal the significant

ranking of abundant modules. The cladogram (Fig. 4A, C) showed differences in 18 taxa among VT, KS and AH. The plot from LefSe analysis (Fig. 4B, D) displays LDA scores of microbial taxa with significant differences among VT, KS and AH. On genus level, the bacteria significant differences between the VT group and the other two groups were *Phycisphaeraceae* (genus), *Novosphingobium* (genus), *Dongia* (order to genus) and *AKYG1722* (family to genus); *Lecanicillium* (genus), *Cordycipitaceae* (genus), *Nectria* (genus), *Thielaviopsis* (family to genus) and *Volvariella* (order Agaricales and family Pluteaceae to genus) were the differential fungi. The bacteria significant differences between the KS group and the other two groups were *S085* (class to genus), *Saccharimonadales* (phylum to genus) and *Latescibacteria* (phylum to genus); *Coniochaetaceae* (order to genus) and *Trichoderma* (family Hypocreaceae to genus) were the differential fungi. In contrast, the AH rhizosphere soil did not show significant enrichment of bacteria; *Ascochyta* (family Didymellaceae to genus) and *Didymellaceae* (family to genus) were the only differential fungi (Fig. 4A, B).

## Discussion

In this study, three sample plots of alfalfa with different cultivars were set up in the experimental herbage station of Shihezi University to analyze the relationships between soil bacterial and fungal communities and alfalfa cultivars and the interaction between plant characteristics and soil physicochemical properties resulting from cultivars and bacterial and fungal communities. The predominant flora in the bacterial and fungal communities of rhizosphere soil was generally consistent between the three cultivars regimens, but there were differences in relative abundance, and additionally, each regimen had its own unique microbial populations. Bacterial abundance and diversity were highest in *Kangsai* ( $P < 0.05$ ). Fungal abundance were highest in KS, but the diversity is the lowest. And fungal diversity were highest in *Victoria*. Soil microbial biomass and/or diversity can increase, decrease, or remain the same depending on the type of soil, the management system, ages and physiological conditions of plants (Mendes et al. 2013).

Although bacterial and fungal communities respond to the different alfalfa soil in different ways with regard to relative abundances and diversity, our study showed that the soil microbial community structure changed significantly along the cultivars of alfalfa, which was relative with difference in soil and plant growth characteristics. Although similar findings have been reported, these previous studies often relied on culture plate counting (Wang and Sheng 2012) or denaturing gradient gel electrophoresis (Clegg 2006) to determine the effects of plant cultivars on microbes. As for microbe identification and classification, these methods may not be accurate and/or can analyze only a limited set of microbial species in samples from complex environments. Thus, it's impossible to comprehensive understanding of the effects of planting different cultivars alfalfa on grey desert soil microbial communities. Therefore, this study aimed to provide a more complete analysis of the microbial community in order to achieve a better understanding of the contributions made by the microbial communities.

We found that the different cultivars alfalfa soil physicochemical and plant growth characteristics contribute differently to different microbial groups in the community. And the environmental factors

evaluated explained over 70% of the shift in the microbial communities, which suggests that they are the primary factors influencing microbial community structure (Supplement Fig. 2A, B). The shifts in dominant fungi communities showed a significant relationship with fresh weight, soil total nitrogen, organic carbon and pH; while shifts in dominant bacterial communities showed a significant relationship with soil organic carbon and pH (Fig. 3). The bacterial community structure in our study area was not as significant correlation as the fungal community structure to plant biomass changes. This may be because fungi are more likely to degrade lignocellulose from different plants than bacteria, allowing them to first obtain resources from many of the relevant available substances (Chapin et al. 2011). In addition, we found significant direct relationships of bacterial and fungal community structures with soil organic carbon and pH. The dominant rhizosphere microbial including *Actinobacteria*, *Proteobacteria* and *Acidobacteria* usually survive in acidic soils (Luo et al. 2019), and also correlated with low pH in our study. In addition, the contents of organic carbon, available nitrogen and total nitrogen in alfalfa soil with *Victoria* were significantly lower than *Kangsai* and *Aohan* (Table 1). Nitrogen is one of the most important nutrients for life (Ramara et al. 2016); therefore, plant and microbial activities may gradually reduce the content of nitrogen in soil. However, correlations between microbial communities and environmental factors must be carefully explained because it is often very difficult to firmly establish the relationship between microbial communities and soil nutrient cycling (Bardgett 2005). In addition, this study employed 16S rRNA and ITS sequencing to analyze microbial communities; the gene abundances indicate genetic potential and not necessarily microbial activity. In this study, the productivity of *Victoria* alfalfa is significantly higher than *Kangsai* and *Aohan*.

Compared with soil microbial communities of three introduced alfalfa cultivars, the histogram of relative phylum abundance showed that regardless of what variety, *Actinobacteria*, *Proteobacteria*, and *Chloroflexi* comprised the main dominant bacteria; and *Ascomycota* and *Basidiomycota* comprised the main dominant fungi (Fig. 2A, C). However, the relative abundance of *Actinobacteria* and *Ascomycota* in the soil of *Victoria* were higher than that of *Kangsai* and *Aohan*. Notably, *Actinomycetes* can utilize nitrogenase that catalyzes conversion of nitrogen to nitrate, which is easily absorbed by the plant roots. In addition, *Actinomycetes* play an active role in carbon cycling by decomposing organic matter, promotes the formation of soil aggregates and improves soil structure, all of which are conducive to plant growth and development (Chaurasia et al. 2018). The relative abundance of *Proteobacteria* and *Chloroflexi* in the soil of *Aohan* soil were higher than that of *Kangsai* and *Victoria*. Notably, *Proteobacteria* show rich species and genetic diversity, and promotes nitrogen absorption in addition to controlling plant diseases, and improve the yield of plants (Shang et al. 2016). The phosphate solubilizing function of *Bacillus* can enhance soil phosphate fertilizer and promote plant growth (Ramani 2011). *Chloroflexus* can synthesize amino acids, nitrogen compounds and other substances, and is an important flora in fertile soil (Speirs et al. 2019). In this study, the nitrogen content in *Victoria* soil is lower than that in *Kangsai* and *Aohan*, which may be related to the action of *Actinomycetes*, *Proteobacteria* and *Chloroflexi*.

LefSe analysis showed that on genus level (Fig. 4), most of the significantly enriched microbial clades in *Victoria* showed a significant correlation with total nitrogen (Supplement Table 3). These microbial clades (*Novosphingobium*, *Lecanicillium*, *Cladosporium*, *Cordycipitaceae* and *Nectria*) all have a close



relationship with the growth of plant. Natalia et al. (2020) found that *Novosphingobium* sp. P6W can degradation abscisic acid (ABA) and participates in carbon and nitrogen metabolism. The entomogenous fungi of *Lecanicillium spp.* are important pathogens of insect pests and can maintain the healthy growth of plants (Zhou et al., 2020). Ramy et al. (2020) found that metabolites of *Cladosporium* can effectively inhibit pathogenic microorganisms. *Nectria haematococca* can significantly improve plant growth parameters and promote plant growth under abiotic stress (Prema et al., 2018). Enrichment for *Lecanicillium*, *Novosphingobium*, *Cladosporium*, *Cordycipitaceae* and *Nectria* may be caused by a better growth performance in *Victoria* than in the two other. No significant correlation was detected between Actinobacteria and the main environmental factors measured (Supplement Table 3). This may be because correlations between higher taxonomic ranks and nutrient indicators may conceal relations with environmental parameters at the subfamily level (Cruz-Martínez et al., 2012). In addition, we found that the abundance of *Arthrobacter* in *Victoria* soil was significantly higher than that in *Kangsai* and *Aohan* (Fig. 2B). He et al. (2020) found that *Arthrobacter* are effective nitrogen-removing bacteria that can absorb dissolved nitrogen from the environment and synthesize nitrite. The soil nutrients in *Victoria* may have created the most appropriate growth environment for *Arthrobacter*, which might have accumulated the soil nitrogen in their cells, thereby significantly reducing the soil nitrogen content (Table 1). In Fig. 2D, the abundance of *Cladosporium*, *Nectriaceae* and *Fusarium* in *Aohan* soil was significantly higher than that in *Kangsai* and *Victoria*. Virtually all *Fusarium* species synthesize toxic mycotoxins that threaten the healthy growth of plants (Perincherry et al. 2019). Some species of *Nectriaceae* are harmful to the roots of plants and can threaten their healthy growth (Parkinson et al. 2019). And the abundance of *Talaromyces*, *Hypocreales*, *Penicilium* and *Sordariomycetes* in *Victoria* soil was significantly higher than that in *Kangsai* and *Aohan* (Supplementary Table 2). Sharda et al. (2019) found that *Talaromyces* contains high levels of indoleacetic acid (IAA) and phosphate dissolving activity, which is very important for promoting plant growth. *Hypocreales* are the fungus that controls nematodes and is reported as an insect pathogen (Toledo et al. 2019). As a whole, the abundance of beneficial bacteria in is higher than that of harmful bacteria. This may also be one of the factors for high yield of alfalfa in *Aohan*. Mason et al. (2020) found that *Saccharimonadales* were identified as candidate biological indicators of high phosphorus utilization, and their relative abundance decreased with the increase of P. This is consistent with the results of this study (Table 1). *Trichoderma* can effectively alter soil properties and elevate plant growth and crop productivity. In addition, mowing can greatly accelerate plant shoot regrowth (Zhang et al. 2020). In this study, the number of branches and internodes in *Kangsai* soil were significantly higher than that in *Victoria* and *Aohan*. *Aohan* was primarily enriched for *Didymellaceae*, which can degrade plant litters and promote carbon cycle (Habtewold et al. 2020). This is consistent with the results of this study (Table 1).

In summary, the distribution difference of microbial communities in three introduced alfalfa soils was studied by partial 16S/ITS gene analysis. MiSeq results showed that the diversity of bacterial community in *Victoria* and *Kangsai* are higher, but the dominant microorganisms with significant differences and relative high abundance are significantly enriched in *Victoria*. And most of the beneficial bacteria clades with high abundance were significantly enriched in *Victoria* alfalfa soil. And part of the harmful bacteria

clades with high abundance were mainly enriched in *Aohan* alfalfa soil. Therefore, in order to improve the adaptability of introduced alfalfa varieties in the current study, appropriate use of probiotics should be considered for adjuvant. In addition, changes in microbial communities were primarily attributable to plant fresh weight, pH, and soil total nitrogen and organic carbon. Considering the microbial community structure and environmental factors, we found that *Victoria* is the high-yield alfalfa suitable for planting in gray desert soil, while planting *Kangsai* and *Aohan* alfalfa needs probiotic assistance. Owing to the limited sampling and restricted sampling area, these conclusions cannot be generalized to other gray desert soil. However, our findings provide a theoretical basis for the introduction of alfalfa, and has guiding value for reasonable application of bacterial fertilizer and improving the adaptability of alfalfa.

## Abbreviations

VT	Victoria
KS	Kangsai
AH	Aohan
FDC	Fall Dormancy
PHT	plant height
NOB	number of branches
NOI	number of internodes
ILH	internode length
FW	fresh weight
OC	organic carbon
AN	available nitrogen
AP	available phosphorus
AK	Available potassium
TN	total nitrogen

TP  
total phosphorus  
TK  
total potassium  
OTUs  
operational taxonomic units  
RDP  
Ribosomal Database Program  
PCA  
principal component analysis  
RDA  
Redundancy analysis  
LDA  
Linear discriminant analysis  
ABA  
abscisic acid  
IAA  
indoleacetic acid

## **Declarations**

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### **Authors' contributions**

Chunhui Ma and Jiangjiao Qi designed the study. Jiangjiao Qi interpreted the results. Xue Yu, Lihe Su, and Tingting He performed the experiments. Jiangjiao Qi participated in writing the manuscript. Xuzhe Wang, Fanfan Zhang and Chunhui Ma supervised the study. All authors read and approved the final manuscript for publication.

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### **Availability of data and materials**

Please contact the authors for all requests.

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Conflict of interest**

None declared.

### **Author details**

College of Animal Science & Technology, Shihezi University, Shihezi, 832003, Xinjiang, China

† Contributed equally to this work and are co-first authors

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## Tables

**Table 1. Physicochemical properties and growth characteristics of alfalfa with different varieties**

Index	VT☐Canada☐	KS☐America☐	AH☐China☐	P-Values	SEM
(TN☐mg/Kg)	108.5±0.8718 <sup>c</sup>	122.9±0.9165 <sup>b</sup>	167.6±0.9539 <sup>a</sup>	0.001	0.7469
(AN☐mg/Kg)	83.4±0.2646 <sup>c</sup>	105.1±0.4359 <sup>a</sup>	98.2±0.6245 <sup>b</sup>	0.005	0.3801
(TP☐mg/Kg)	759.2±0.1732 <sup>a</sup>	632.5±0.9292 <sup>b</sup>	583.7±0.0577 <sup>c</sup>	0.003	0.4464
(AP☐mg/Kg)	34.5±0.0577 <sup>a</sup>	29.1±0.6028 <sup>c</sup>	31.8±0.5033 <sup>b</sup>	0.001	0.3712
(TK☐g/Kg)	22.3±0.7201 <sup>a</sup>	18.5±0.9198 <sup>b</sup>	19.7±0.9042 <sup>b</sup>	0.002	0.6964
(AK☐mg/Kg)	209.5±0.9019 <sup>a</sup>	176.3±0.5774 <sup>c</sup>	185.6±0.1528 <sup>b</sup>	0.001	0.5099
(OC☐g/Kg)	34.9±0.7937 <sup>c</sup>	37.4±0.6083 <sup>a</sup>	38.2±0.5320 <sup>a</sup>	0.002	0.5340
pH	6.73±0.2879 <sup>a</sup>	6.49±0.3993 <sup>a</sup>	6.52±0.1967 <sup>a</sup>	0.610	0.2499
PHT (cm)	60.7±2.2517 <sup>a</sup>	49.8±1.0817 <sup>b</sup>	41.3±0.9849 <sup>c</sup>	0.002	1.2658
NOB (Pieces)	48.0±2.6458 <sup>c</sup>	73.0±1.5275 <sup>a</sup>	56.0±0.5774 <sup>b</sup>	0.001	1.4657
NOI (Pieces)	9.0±0.5774 <sup>a</sup>	10.0±1.5374 <sup>a</sup>	9.0±1.1547 <sup>a</sup>	0.921	0.9428
ILH (cm)	6.3±0.2646 <sup>a</sup>	4.4±0.1002 <sup>c</sup>	4.9±0.0451 <sup>b</sup>	0.003	0.1350
FW (g/ plant)	278.4±4.5133 <sup>a</sup>	213.1±3.7723 <sup>b</sup>	236.9±4.2771 <sup>b</sup>	0.002	3.4284

PHT= Plant Height; NOB= Number Of Branches; NOI= Number Of Internodes; ILH= Internode Length; FW= Fresh Weight. According to Duncan's new multiple range test, different letters in the same row indicate statistical significance among different fall dormancy grades of alfalfa. The effective values were expressed as: adjacent letters, P < 0.05; interphase letters, P < 0.01. Data were represented as mean ± standard error

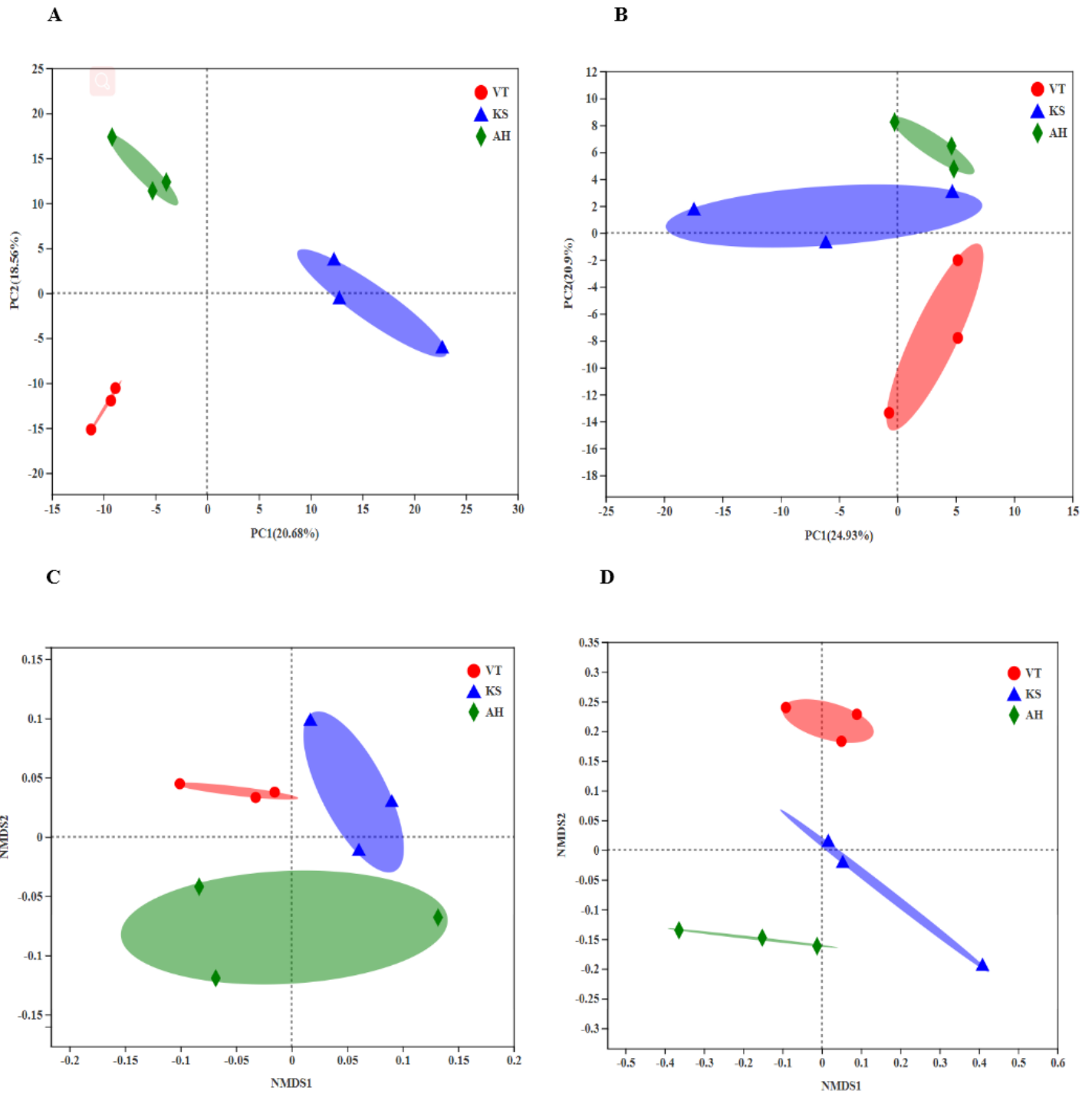
**Table 2. Microbial diversity index of alfalfa rhizosphere with different varieties**



	Sample	sobs	shannon	ace	coverage
Bacterial	VT (Canada)	2516±11.15 <sup>b</sup>	6.47±0.03 <sup>a</sup>	3224±10.82 <sup>b</sup>	0.9826±0.0011 <sup>a</sup>
	KS (America)	2747±19.31 <sup>a</sup>	6.53±0.05 <sup>a</sup>	3546±31.39 <sup>a</sup>	0.9823±0.0009 <sup>a</sup>
	AH (China)	2449±6.56 <sup>b</sup>	6.39±0.08 <sup>b</sup>	3265±20.53 <sup>b</sup>	0.9800±0.0023 <sup>a</sup>
Fungal	VT (Canada)	409±13.53 <sup>a</sup>	3.90±0.31 <sup>a</sup>	473±23.92 <sup>a</sup>	0.9985±0.0005 <sup>a</sup>
	KS (America)	410±38.70 <sup>a</sup>	3.48±0.58 <sup>a</sup>	491±30.40 <sup>a</sup>	0.9989±0.0006 <sup>a</sup>
	AH (China)	362±17.35 <sup>a</sup>	3.55±0.45 <sup>a</sup>	454±21.38 <sup>a</sup>	0.9988±0.0003 <sup>a</sup>

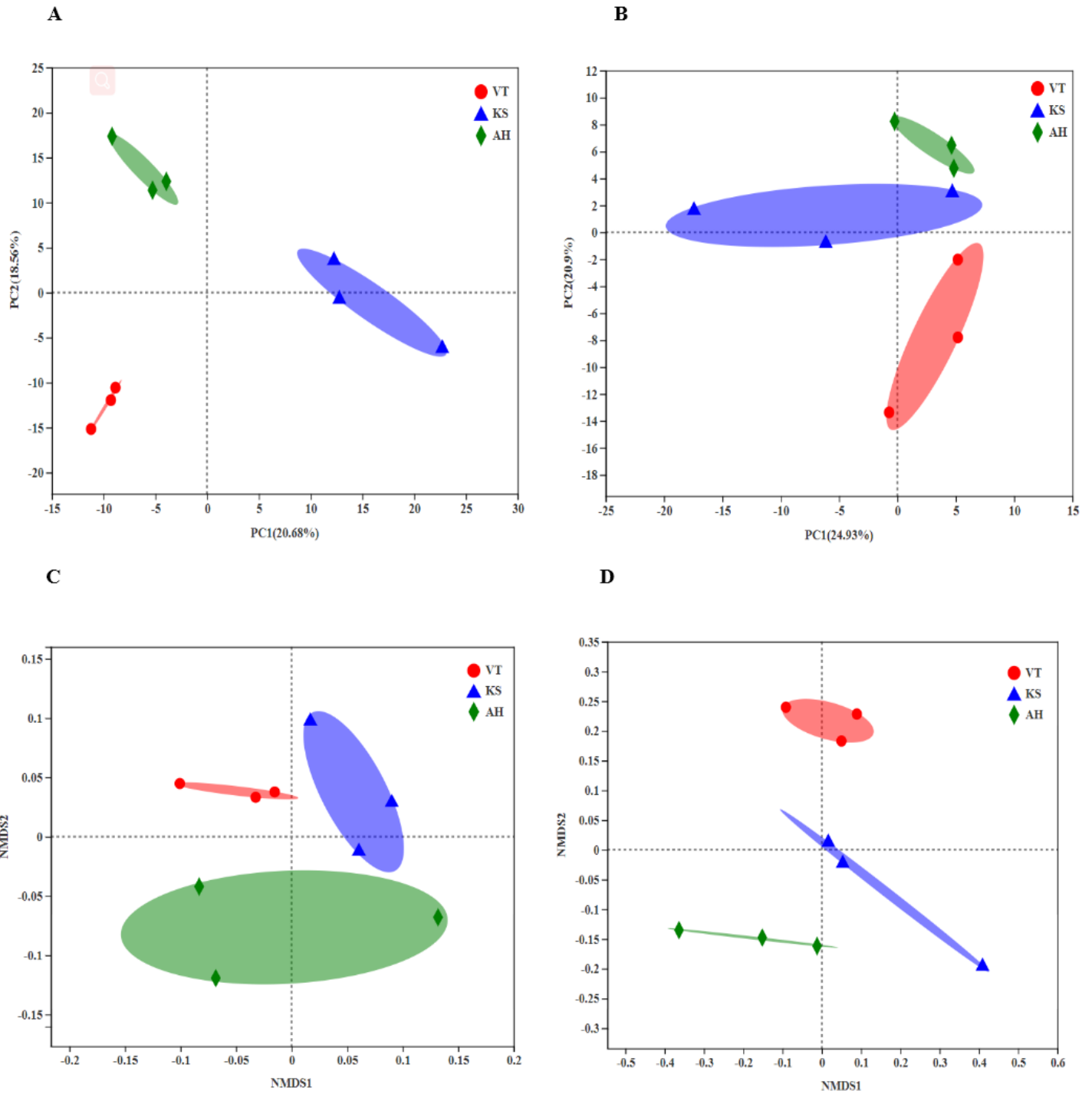
The different letters in the same column indicate the statistical significance among the microbial diversity indexes. The effective values are as follows: the same letter,  $P > 0.05$ ; adjacent letters,  $P < 0.05$ ; interphase letters,  $P < 0.01$ .

## Figures



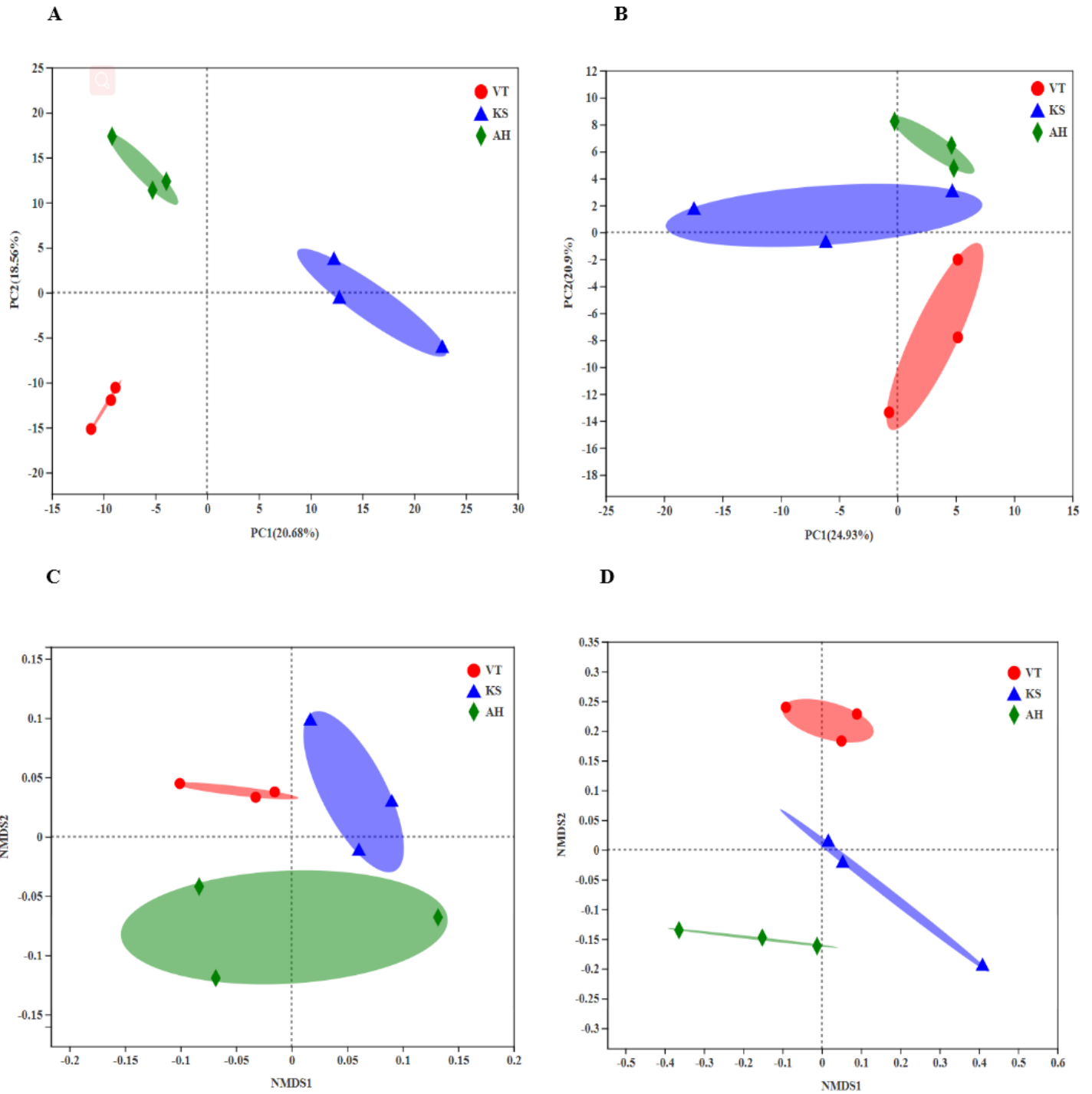
**Figure 1**

Rarefaction curves of alfalfa rhizosphere soil with different fall dormancy. The values of axes 1 and 2 are percentages. Different-colored regions represent different constituents (red, VT; blue, KS; green, AH). The closer the sample points, greater the similarity of species composition.



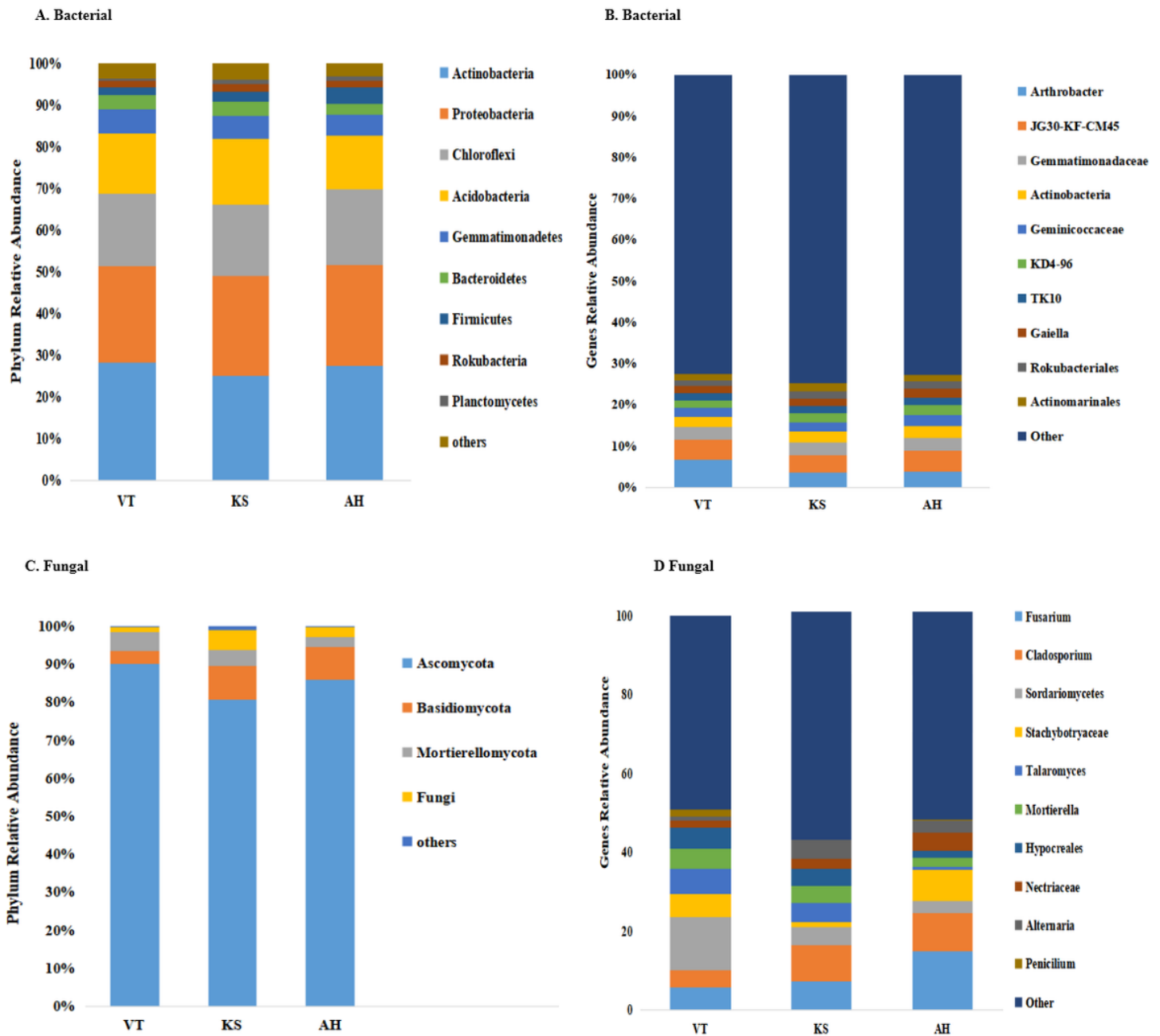
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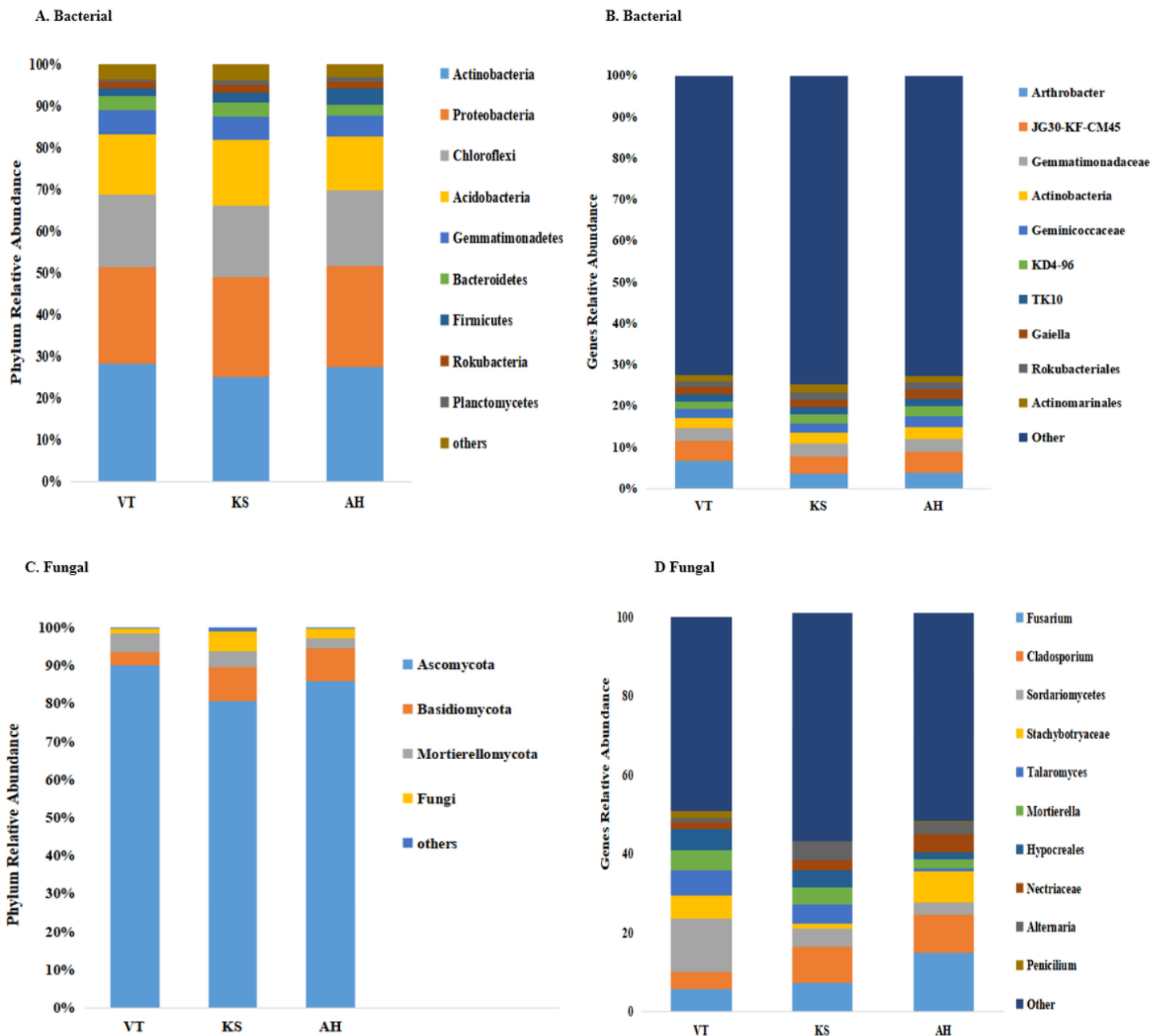
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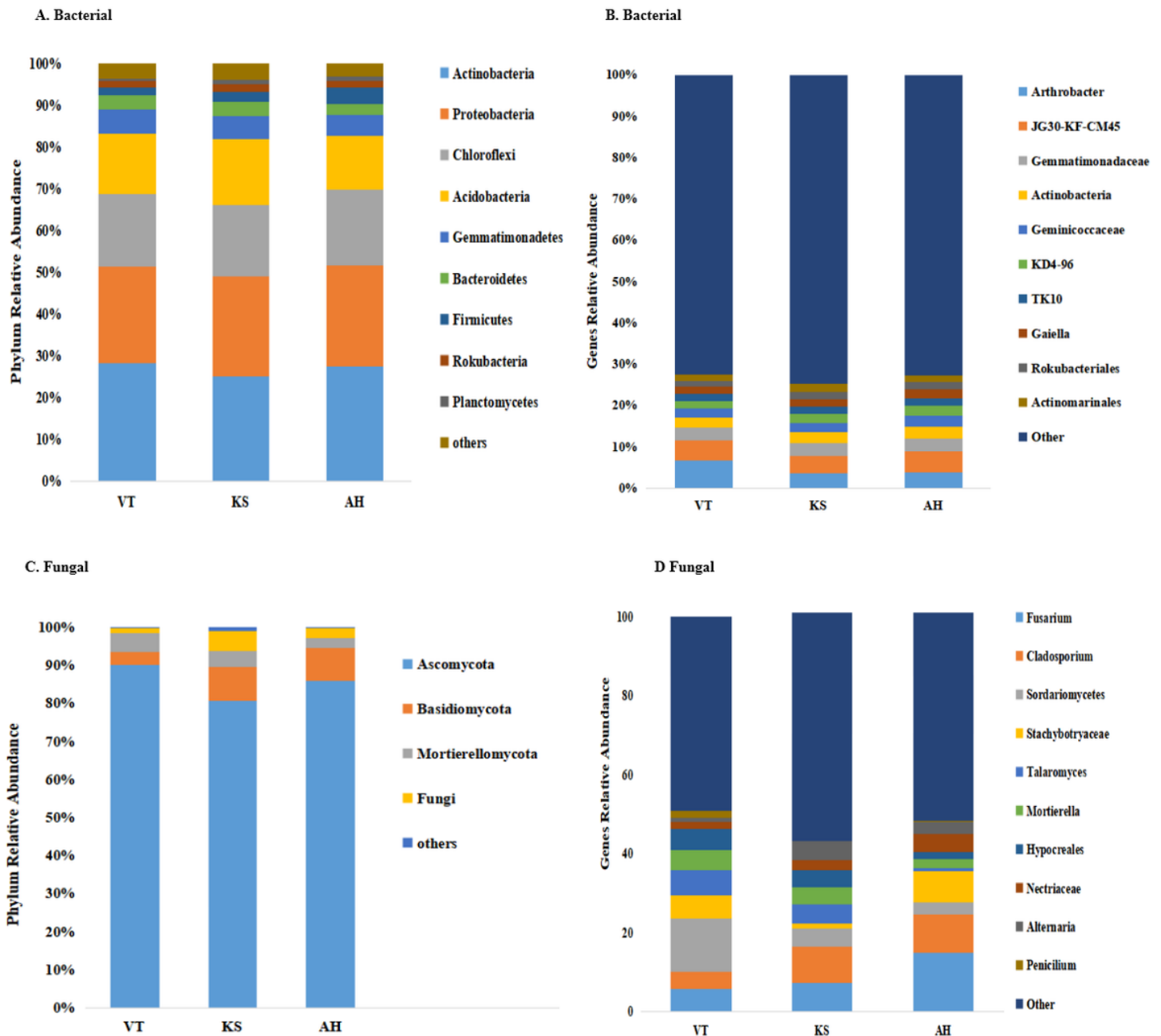
**Figure 2**

Relative abundance of bacteria and fungi in the rhizosphere of alfalfa with different cultivars. (A, B) Relative abundance of bacteria at phylum and genus level in alfalfa cultivars (VT, KS, AH). (C, D) Relative abundance of fungi at phylum and genus level in alfalfa cultivars (VT, KS, AH). Different colors represent different species, and the length of the columns represent their relative proportion.



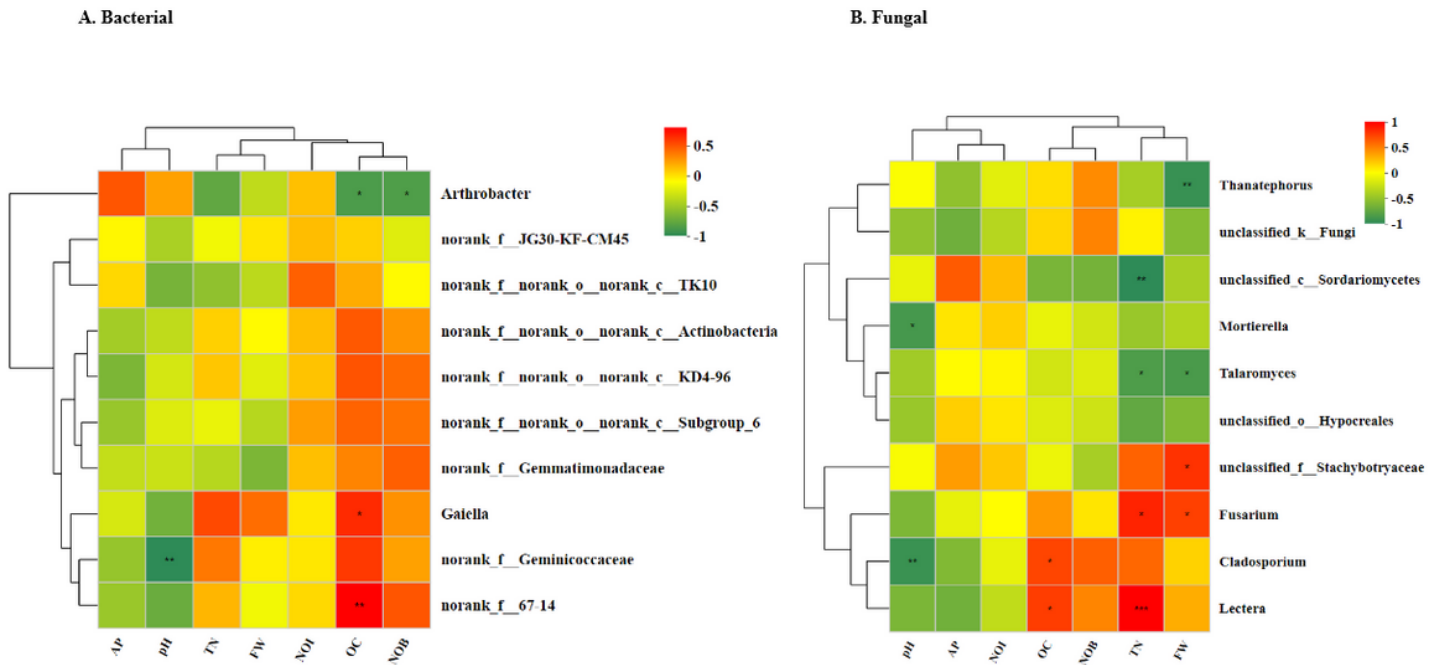
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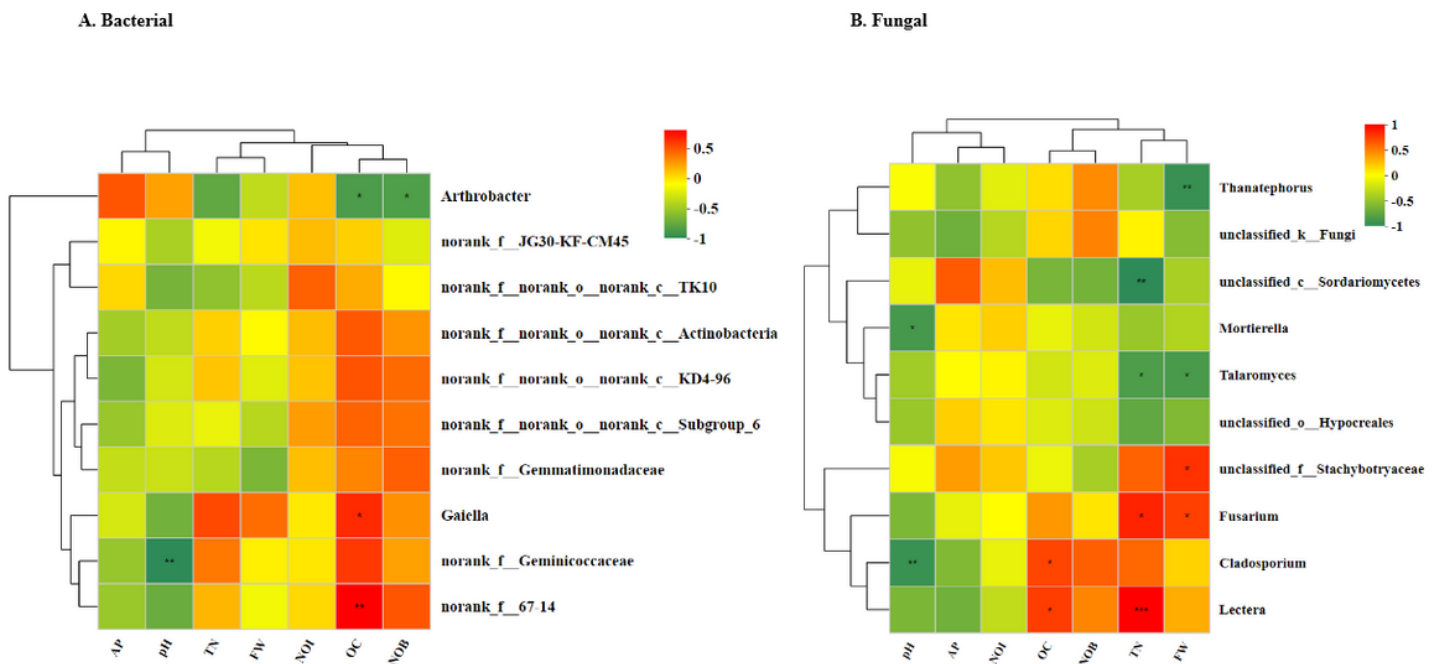
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**Figure 3**

Heatmap analysis of the microorganisms and environmental factors. Note: The X-axis and Y-axis were environmental factors and species, respectively, and the correlation R value and P value were obtained through calculation. The R values are shown in different colors in the figure. If the P value is less than 0.05, it is marked with \*, \* 0.01 < P ≤ 0.05, \*\* 0.001 < P ≤ 0.01, \*\*\* P ≤ 0.001.

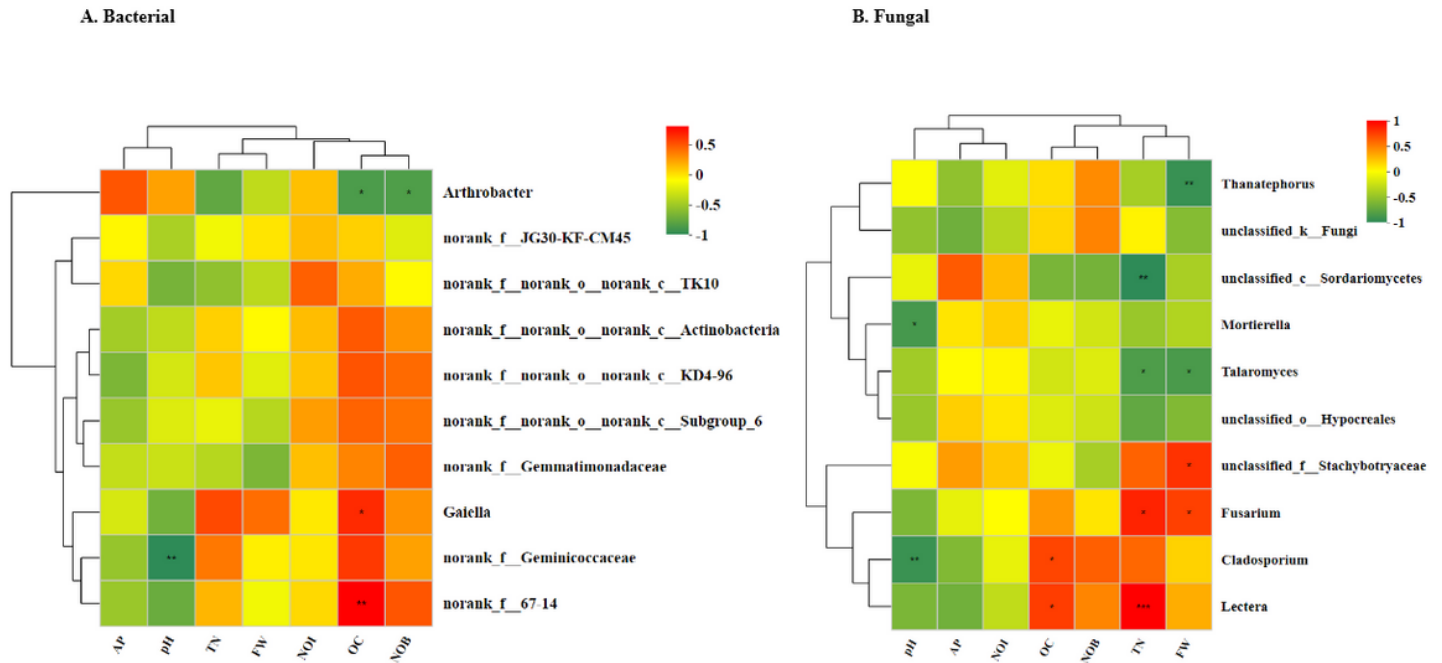


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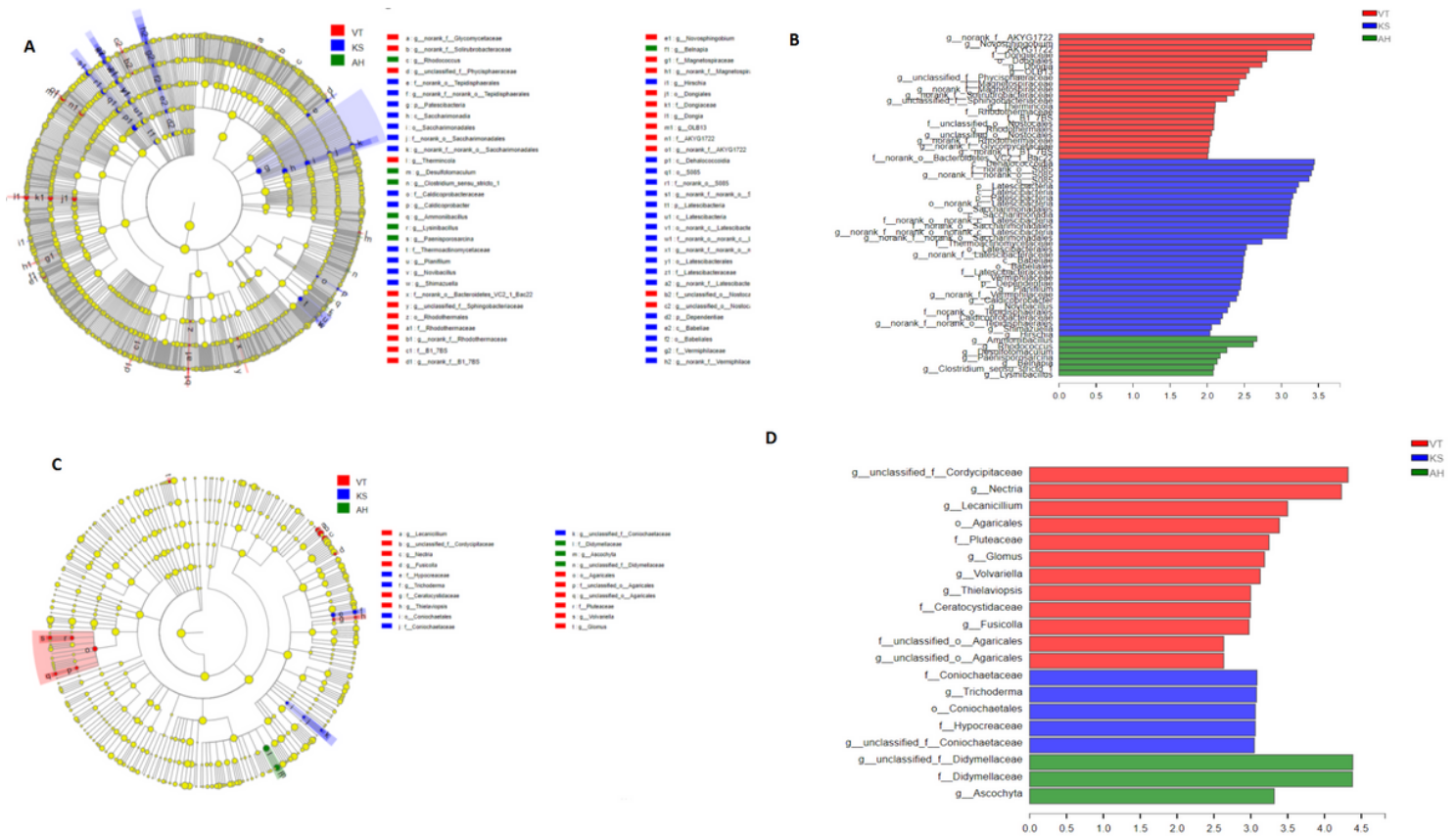


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**Figure 4**

The phylogenetic distribution of the soil microbes from alfalfa with different cultivars. Cladogram showing the phylogenetic distribution of the bacterial (A) and fungal (B) lineages in the soil from alfalfa with different fall dormancy. Circles indicate phylogenetic levels from domain to genus. The diameter of each circle is proportional to the abundance of the group. Nodes with different colors represent significantly enriched microbial groups. Light yellow nodes indicate microbial groups that lack significance. Indicator bacteria with LDA scores of 2 or greater in bacterial (A) and fungal (B) communities associated with soil from the the alfalfa with different fall dormancy classes. Different-colored regions represent different constituents(red, VT; blue, KS;green, AH).

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