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Article

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The impact of shrub encroachment on the diversity of rhizosphere microbial communities of *Caragana microphylla* in the typical grasslands in Inner Mongolia, China

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Abstract: Soil microorganisms as a pivotal ecological indicator for monitoring shrub encroachment in arid and semi-arid grassland ecosystems. *Caragana microphylla* is a key constructive species in the shrub encroachment of arid and semi-arid grasslands in northern China. It deeply reshapes the structure and function of the rhizosphere microbial community through root exudates, litter input, and root nodule symbiosis. This study focuses on the response characteristics of rhizosphere microorganisms in *Caragana microphylla* under the gradient of shrub encroachment by high-throughput sequencing technology, aiming to reveal the mechanism of microbial mediated shrub encroachment ecological processes and provide microbiological basis for shrub encroachment grassland regulation. The results showed that: (1) The shrub encroachment of *Caragana microphylla* significantly changed the structure of rhizosphere microbial communities, as the degree of shrub encroachment intensifies, the diversity of rhizosphere fungi usually showed a trend of first decreasing and then increasing, the diversity of rhizosphere bacteria usually showed a trend of decreasing; (2) With the aggravation of shrub encroachment, the OUTS numbers of soil fungi and bacteria both showed a decreasing trend, in fungal communities, Basidiomycota decreased first and then increased, Mucoromycota increased first and then decreased, Chytridiomycota decreased; In bacterial communities Actinobacteria decreased first and then increased, Proteobacteria increased first and then decreased, Bacteroidetes

decreased. Research has shown that the shrub encroachment of *Caragana microphylla* changes the rhizosphere soil microenvironment, regulates the structure and functional group distribution of rhizosphere bacterial and fungal communities, and forms a synergistic interaction relationship of "shrub—microorganism—soil", providing theoretical basis for understanding the underground ecological process of typical shrub encroachment grassland in Inner Mongolia and formulating scientific regulation strategies.

Keywords: rhizosphere microorganisms, *Caragana microphylla*, high-throughput sequencing technology, shrub encroachment grassland

Introduction

Woody plant encroachment into grasslands is a global phenomenon that is associated with land degradation via xerification, which replaces grasses with shrubs and bare soil patches¹. The displacement of grasses by woody plants in many arid and semi-arid ecosystems was caused by the changes in climatic, livestock grazing, fire regimes and atmospheric CO₂ enrichment², surface temperature, wind speed, and relative humidity, with elevation, slope, and precipitation³, rather than being driven by a singular factor, the expansion was a result of the combined influence of various factors. Shrub encroachment is often accompanied by large changes in the spatial pattern of soil resources and vegetation, which have been linked with alterations in the structure and functioning of the ecosystem, declines in forage productivity, biodiversity and socioeconomic potential as well as increased erosion, ultimately leading to its desertification^{4, 5}. These changes in vegetation structure are affecting livestock production and terrestrial ecosystem functioning⁶, carbon and nitrogen cycles². Recent researches have found that Seed density of *Caragana stenophylla* decreases and seed proportions inside shrub canopies increase as drought stress intensifies in the Inner Mongolia Grassland⁷, the ongoing Woody plant encroachment contributed to vegetation greening and elevated vegetation productivity by increasing the leaf area index and partitioning more water into transpiration⁸, the shrub encroachment might affect the responses of dominant grass traits to N deposition⁹.

But woody plant encroachment had overarching benefit of carbon sequestration in soil and woody plants in grasslands that help to mitigate climate change and global warming¹⁰. So the benefits and drawbacks of woody plant encroachment should be treated scientifically.

As arid and semi-arid grassland desertification core, the process of grasslands shrub encroachment has become an important field of the study of global changes in terrestrial ecosystems. Shrub encroachment or proliferation of shrubs in grasslands, has become a widespread phenomenon in the Inner Mongolia Grassland, and *Caragana* sp. are the major encroaching shrubs¹¹. There are 15 species belonging to *Caragana* distributed in the Inner Mongolia Grassland¹², among them, *Caragana microphylla* is one of the species that causes shrub encroachment. In this study, we hypothesized that the degree of shrub encroachment caused by *Caragana microphylla* will affect the structure and function of grassland ecosystems. To test our hypotheses, we investigated *Caragana microphylla* communities of Grassland-shrub mosaic plot (GSMP) and Grassland thickening plot (GTP), measured the rhizosphere microbial community of *Caragana microphylla* by high-throughput methods, and analyzed rhizosphere microbial community structure, biodiversity to reveal the mechanism of shrub encroachment in Inner Mongolia grassland, to provide theoretical basis for the restoration and rational utilization of degradation grassland.

Materials and methods

Field experimental site

Overview of field experimental site

West Ujimqin Banner is located at 16°21'—119°03' E, 43°57'—45°23' in the east of Xilingol of Inner Mongolia Autonomous Region in China. West Ujimqin Banner belongs to the temperate semi-arid continental climate, it has long winter and short summer, its spring is windy, and its autumn is cool. The average annual temperature of West Ujimqin Banner is 0-1 °C, the annual precipitation is 345.8mm, the average annual evaporation is 1759.8mm, reduces precipitation in the southeast to the northwest, forming the features of eastern humid, western arid. The main soil type is

Chestnut soil^{13, 14}.

Sampling site

Two experimental sites were selected (Fig.1), and named as Grassland-Shrub Mosaic plot (GSMP): shrubs were embedded in grasslands, with a shrub coverage of 6%, and Grassland Thicketization plot (GTP): Shrubs were widely distributed in grasslands, with a shrub coverage of 50%; Control plot named as the Typical Grassland plot (TGP): shrubs are sporadically distributed in grasslands, with a shrub coverage of 3%, was set in West Ujimqin Banner of Xilingol. In each plot, three duplicate quadrats were set, the basic information of quadrats was shown in Table1. The quadrat size was 5 m × 5 m. The coverage, height for shrubs and herbs were measured in each quadrat. The vegetation cover was calculated by crown diameter, and heights of shrubs and herbs were measured by using a meter, the coverage was calculated by crown diameter. Altogether 40 plant species were recorded in 9 quadrats.

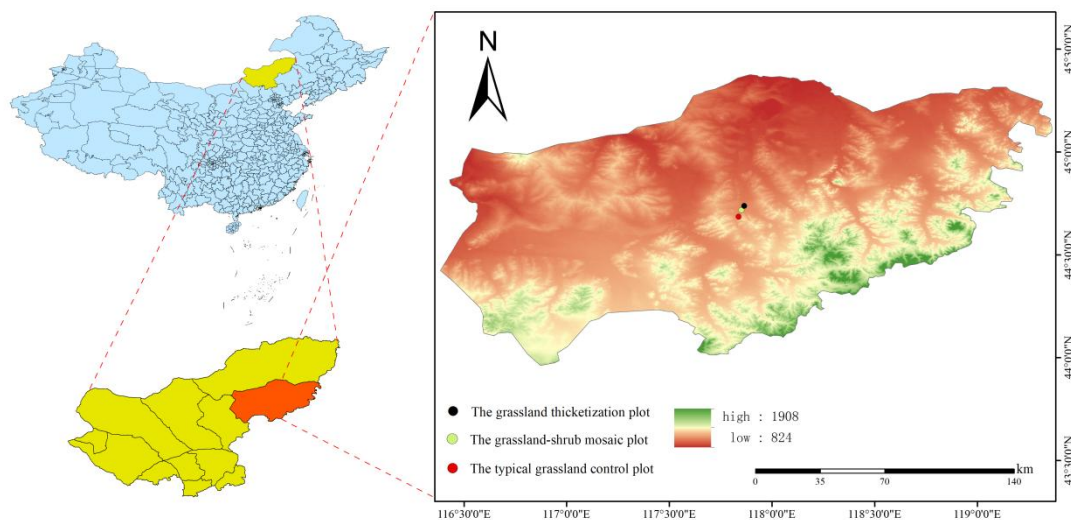


Fig.1 Location of sample plots of *Caragana microphylla* shrub encroachment community in West Ujimqin Banner of Xilingol of Inner Mongolia Autonomous Region in China

Table1 The basic information of *Caragana microphylla* shrub encroachment community in Inner Mongolia Autonomous Region, China

Plot name	Longitude	Latitude	Altitude (m)	Coverage (%)	Slope (°)	Aspect
The grassland thicketization plot 1	117°51'22.6"	44°43'44.0"	1021	80	20	30
The grassland thicketization plot 2	117°51'24.5"	44°43'43.5"	1022	70	20	28
The grassland thicketization plot 3	117°51'20.3"	44°43'44.1"	1016	65	22	35
The grassland-shrub mosaic plot 1	117°51'19.1"	41°43'37.7"	1019	89	15	80
The grassland-shrub mosaic plot 2	117°51'17.2"	44°43'38.4"	1021	90	13	110
The grassland-shrub mosaic 3 plot	117°51'16.8"	44°43'36.9"	1017	90	3	110
The typical grassland plot 1	117°50'18.0"	44°41'05.6"	1050	95	3	110
The typical grassland plot 2	117°50'17.8"	44°41'05.1"	1049	92	3	105
The typical grassland plot 3	117°50'17.8"	44°41'04.8"	1049	90	3	100

Rhizosphere soil collection

The preserved herbarium specimen of *Caragana microphylla* (voucher specimen number: IMNU-Surigua-20230710012) was deposited in the Herbarium of Inner Mongolia Normal University, a public herbarium. Specimen was identified by Suriguga. Selected 10 typical *Caragana microphylla* according to the "S" shape, using Riley and Barber's shaking method¹⁵, excavate soil with complete roots, and then soil on the root surfaces was gently brushed off with a sterile brush and collected as rhizosphere soil samples. Put it into a sterile centrifuge tube, put it into a liquid nitrogen tank, return to the laboratory, and store it in a -80 °C refrigerator for high-throughput sequencing of soil microorganisms, measuring microbial diversity and richness.

Statistical analysis

Excel and SPSS (22.0) software were used to statistically analyze the experimental data. Turkey analyses were used to test the significant differences among different data groups. Differences were either significant ($P < 0.05$) or extremely significant ($P < 0.01$).

Results

Plant community characteristics in three experimental plots

In GSMP and GTP, *Caragana microphylla* formed shrub layer, especially in GTP the important value of *Caragana microphylla* was higher than herbaceous plants' (Fig.2),

and plants of Gramineae no longer dominated. But in TGCP the important value of *Caragana microphylla* was lowest, and the coverage was 3%, but the coverage of the herbaceous plants was 90%. The coverage of herbaceous plants was much larger than the coverage of *Caragana microphylla*, plants of Gramineae dominated in TGCP community, still maintained the species composition characteristics of typical grasslands, and the dominant species were *Stipa grandis*, *Leymus chinensis*, and *Achnatherum sibiricum* (Table2).

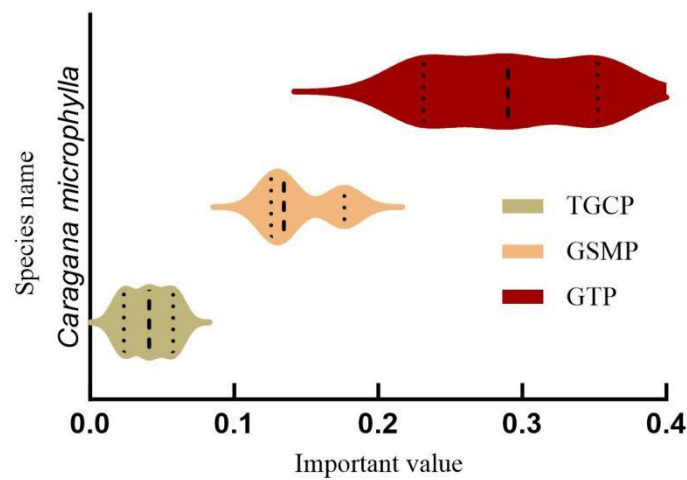


Fig.2 Important value of *Caragana microphylla* in three experimental plots

Table 2. Main species composition and their important values in three experimental plant communities

The dominant species	GTP	GSMP	TGCP
<i>Caragana microphylla</i>	0.2914	0.1456	0.0407
<i>Hedysarum laeve</i>	0.1789	—	—
<i>Calamagrostis pseudophragmites</i>	0.0394	0.1708	—
<i>Leymus secalinus</i>	0.1053	—	—
<i>Stellera chamaejasme</i>	—	0.1517	0.0377
<i>Leymus chinensis</i>	—	0.1748	0.1742
<i>Stipa grandis</i>	—	—	0.3151
<i>Achnatherum sibiricum</i>	—	—	0.1299
<i>Anemarrhena asphodeloides</i>	—	—	0.1488

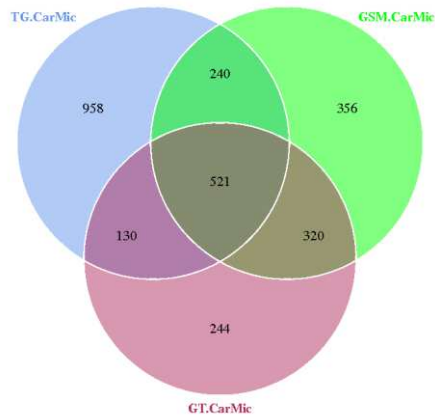
OUTs in Rhizosphere soil of *Caragana microphylla* in three shrub encroachment communities

As shown in Fig.3A, there were 521 fungal OTUs in rhizosphere soil of *Caragana microphylla* shared by the three shrub encroachment communities, 240 OTUs shared by TG.CarMic and GSM.CarMic, 130 OTUs shared by TG.CarMic and GT.CarMic, and 320 OTUs shared by GSM.CarMic and GT.CarMic. Among the three experimental plots, TG.CarMic had the greatest number of unique OTUs (958). The number of unique OTUs in GSM.CarMic was 356. GT.CarMic had the lowest number of unique OTUs (244).

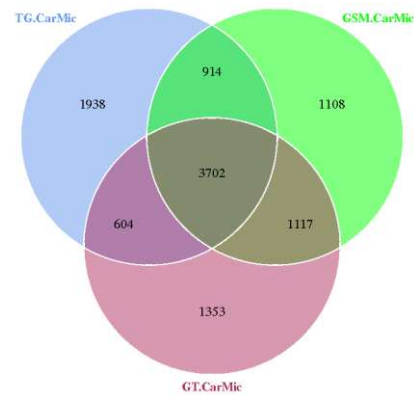
As shown in Fig.3B, there were 3702 bacterial OTUs in rhizosphere soil of *Caragana microphylla* shared by the three shrub encroachment communities, 914 OTUs shared by TG.CarMic and GSM.CarMic, 604 OTUs shared by TG.CarMic and GT.CarMic, and 1117 OTUs shared by GSM.CarMic and GT.CarMic. Among the three experimental plots, TG.CarMic had the greatest number of unique OTUs (1938). The number of unique OTUs in GT.CarMic was 1353. GSM.CarMic had the lowest number of unique OTUs (1108).

In summary, the number of OTUs of the rhizosphere microorganism of *Caragana microphylla* varies between shrub encroachment communities. Among the three shrub encroachment communities, the largest number of fungal OTUs in rhizosphere soil was from TG.CarMic with 1849 OTUs, followed by GSM.CarMic with 1437 OTUs, and the lowest was in GT.CarMic with 1215 OTUs (Fig.3C). TG.CarMic had the most bacterial OTUs in the rhizosphere soil (7157), followed by GSM.CarMic (6841) and GT.CarMic (6776)(Fig.3D).

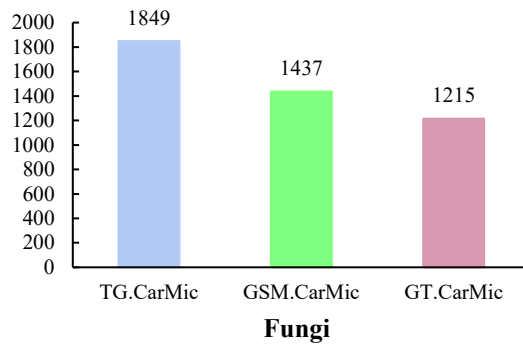
A.



B.



C.



D.

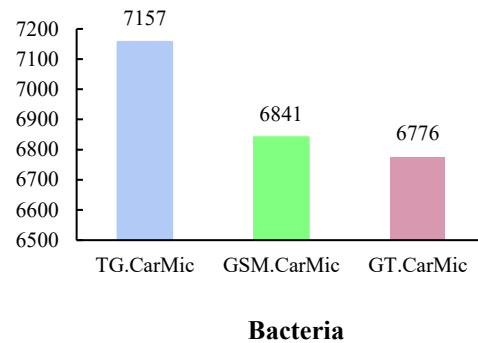


Fig.3 Venn diagram and the number of OTUs in rhizosphere soil of *Caragana microphylla* microorganism

A : Venn diagram of Fungi; B: Venn diagram of Bacteria; C: the number of OTUs of Fungi; D: the number of OTUs of Bacteria

Note: TG.CarMic: *Caragana microphylla* of Typical grassland community; GSM.CarMic: *Caragana microphylla* of Grassland-shrub mosaic community; GT.CarMic: *Caragana microphylla* of Grassland thicketization community;

Differential analysis on microorganism in rhizosphere soil of *Caragana microphylla* in three shrub encroachment communities

From differential analysis and contribution rates at the fungal phylum level (Fig.8A,B), it can be seen that Basidiomycota, Chytridiomycota, Glomeromycota of

TG.CarMic fungal community were significantly higher than GSM.CarMic fungal community; however Mucoromycota of TG.CarMic fungal community was significantly lower than GSM.CarMic fungal community.

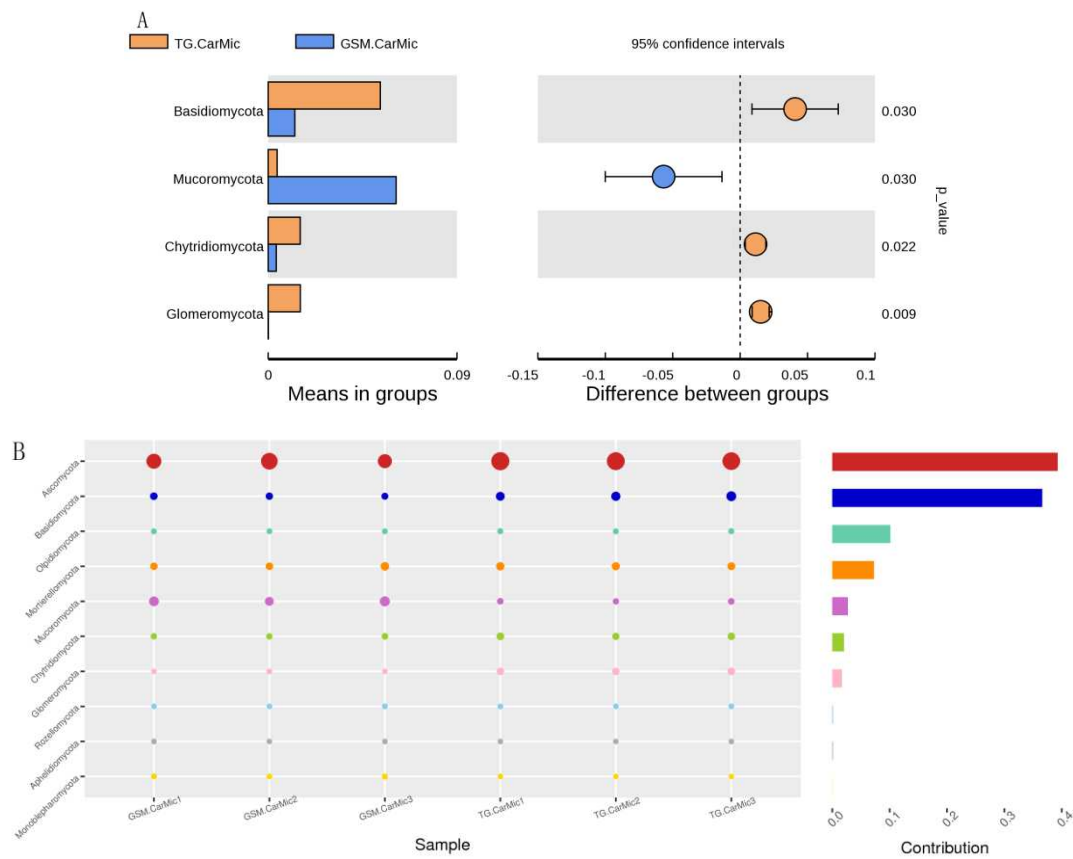


Fig.8 Difference between TG.CarMic and GSM.CarMic fungal community at phylum A:

Differential analysis; B: Contribution rates

From differential analysis and contribution rates at the fungal phylum level (Fig.9A,B), it can be seen that Ascomycota, Chytridiomycota, Glomeromycota of TG.CarMic fungal community were significantly higher than GT.CarMic fungal community; however Basidiomycota of TG.CarMic fungal community was significantly lower than GT.CarMic fungal community.

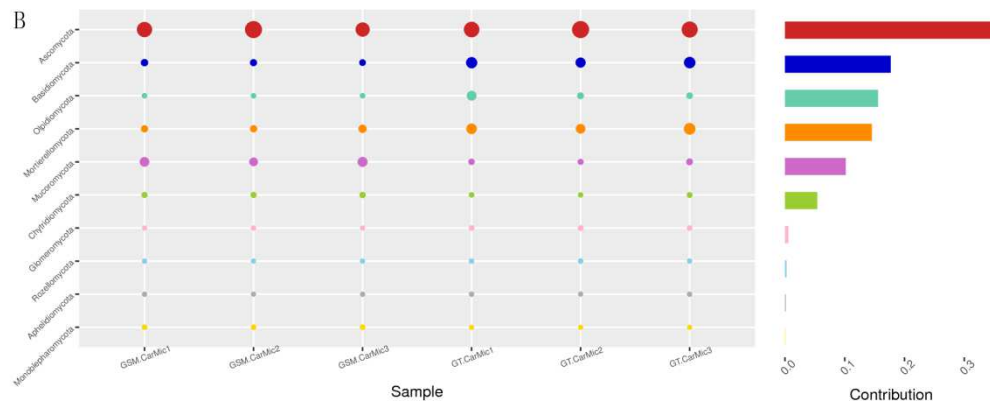
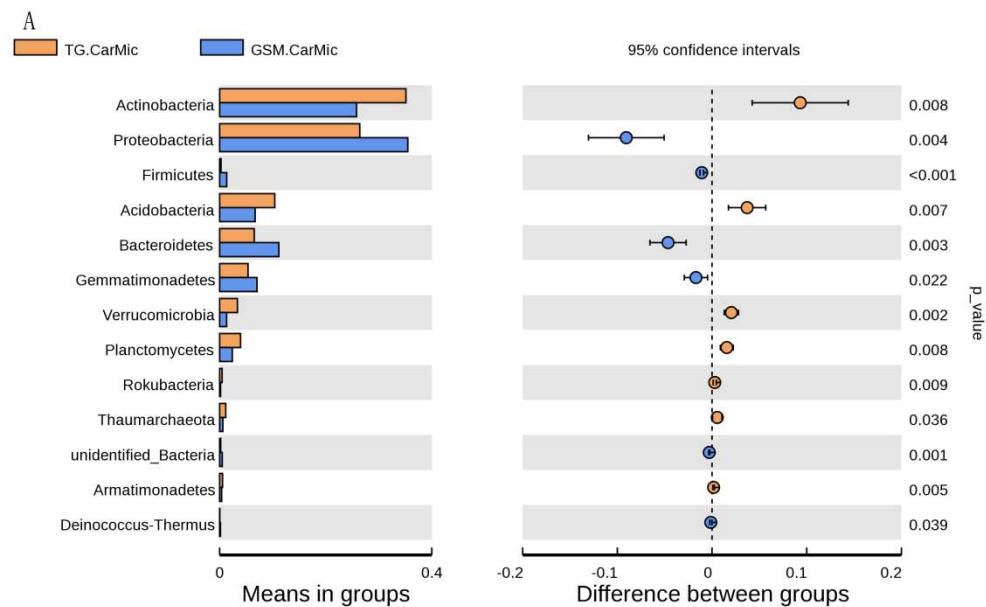


Fig.10 Difference between GT.CarMic and GSM.CarMic fungal community at phylum A:

Differential analysis; B: Contribution rates

From differential analysis and contribution rates at the bacterial phylum level (Fig.11A,B), it can be seen that Actinobacteria, Acidobacteria, Verrucomicrobia, Planctomycetes and Thaumarchaeota of TG.CarMic bacterial community were significantly higher than GSM.CarMic bacterial community; however Proteobacteria, Bacteroidetes and Gemmatimonadetes of TG.CarMic bacterial community was significantly lower than GSM.CarMic bacterial community.



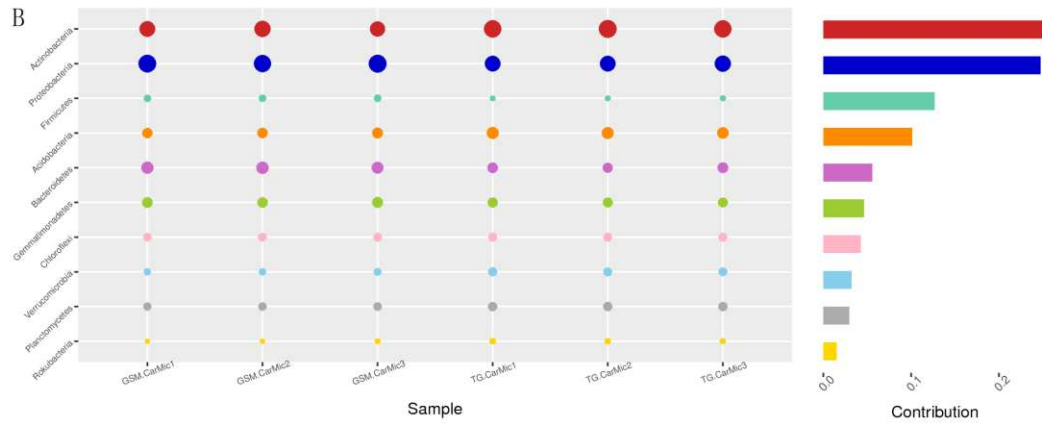
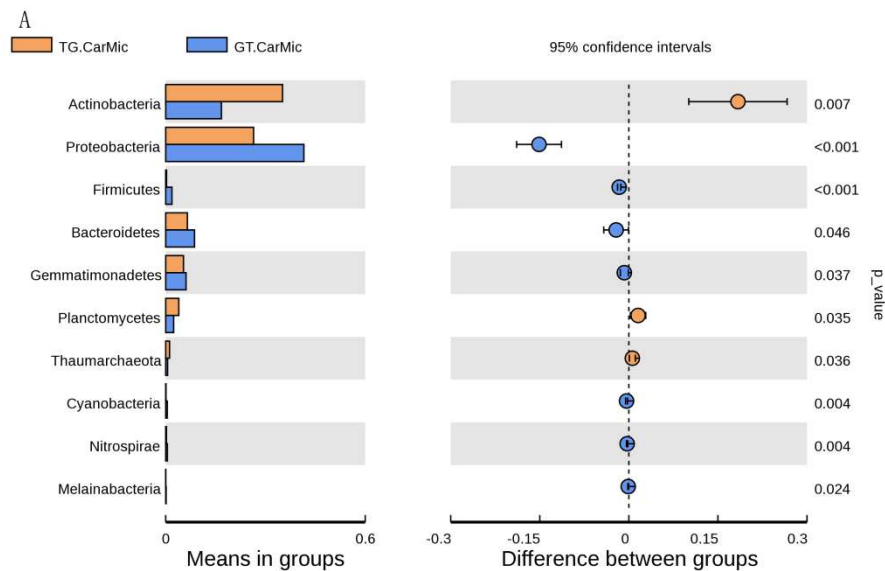


Fig.11 Difference between TG.CarMic and GSM.CarMic bacterial community at phylum A:

Differential analysis; B: Contribution rates

From differential analysis and contribution rates at the bacterial phylum level (Fig.12A,B), it can be seen that Actinobacteria, Planctomycetes and Thaumarchaeota of TG.CarMic bacterial community were significantly higher than GT.CarMic bacterial community; however Proteobacteria, Firmicutes, Bacteroidetes and Gemmatimonadetes of TG.CarMic bacterial community was significantly lower than GT.CarMic bacterial community.



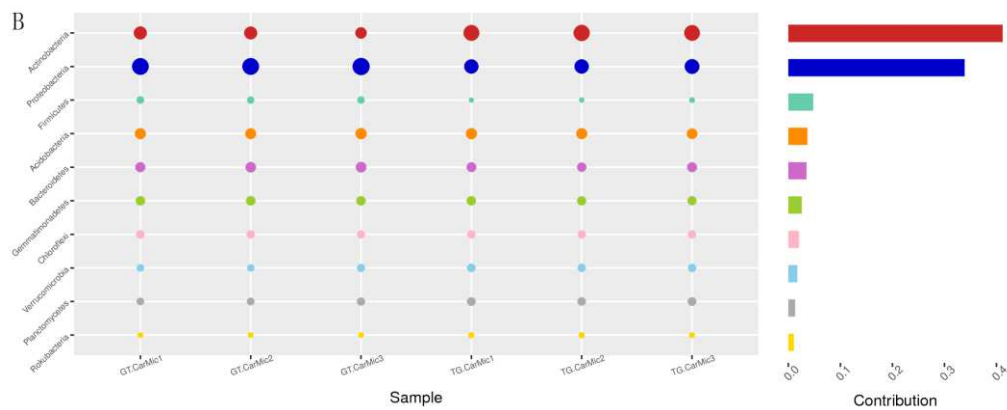


Fig.12 Difference between TG.CarMic and GT.CarMic bacterial community at phylum A:

Differential analysis; B: Contribution rates

From differential analysis and contribution rates at the bacterial phylum level (Fig.13A,B), it can be seen that Actinobacteria and Bacteroidetes of GSM.CarMic bacterial community were significantly higher than GT.CarMic bacterial community; however Proteobacteria, Firmicutes and Acidobacteria of GSM.CarMic fungal community was significantly lower than GT.CarMic bacterial community.

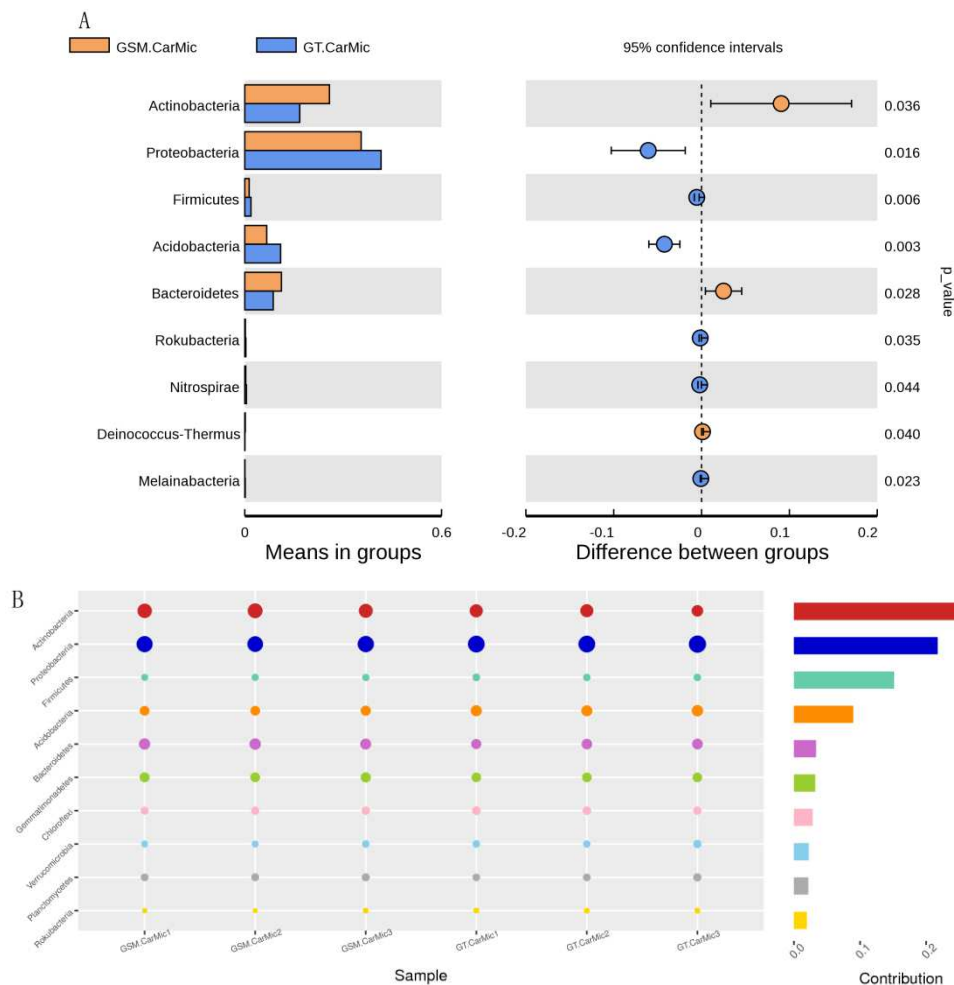
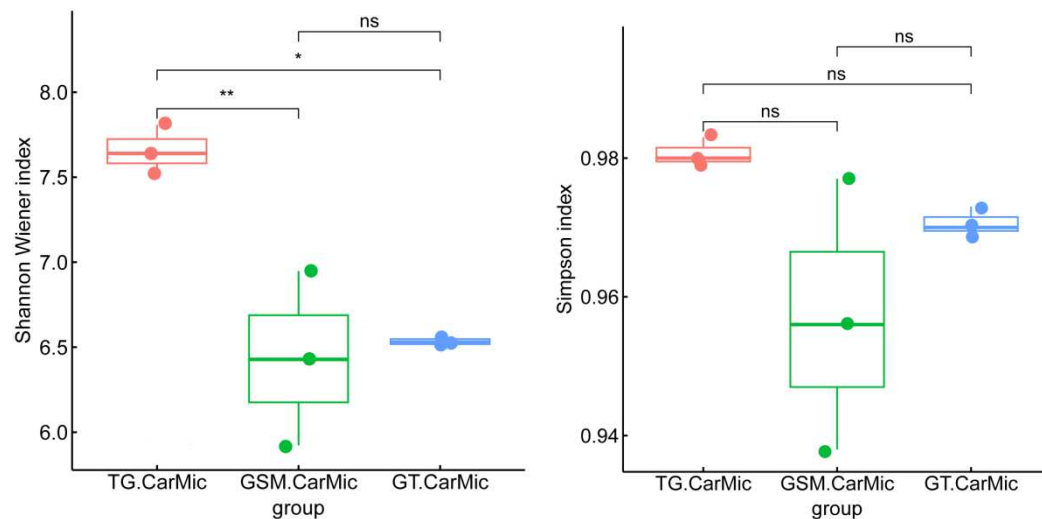


Fig.13 Difference between GT.CarMic and GSM.CarMic bacterial community at phylum A:

Differential analysis; B: Contribution rates

Diversity of Microbial Communities in Rhizosphere Soil of *Caragana microphylla*

Shannon Wiener index of fungal diversity in rhizosphere soil of *Caragana microphylla* of TGCP community was significantly higher than GSMP community ($P<0.01$) and GTP community ($P<0.05$), because of rare species such as Chytridiomycota, Glomeromycota playing an important role in the process of shrub encroachment, but Simpson index of fungal diversity was no significant difference among TGCP, GSMP and GTP community ($P>0.05$)(Fig.14), it indicated that dominant species are the same. As the degree of shrub encroachment increased, the Chao index decreased, Chao index of fungal diversity was extremely significant difference between TGCP community and GSMP community ($P<0.001$), and TGCP community and GTP community ($P<0.001$), GSMP community and GTP community ($P<0.01$), indicating that the richness of soil fungi was affected, leading to the instability of shrub encroachment grassland ecosystems.



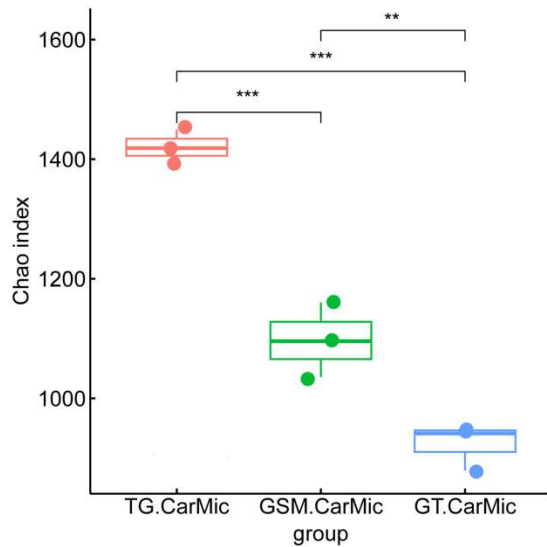


Fig.14 Fungal alpha diversity indices in rhizosphere soil of *Caragana microphylla* in the three shrub encroachment community

Shannon Wiener index of bacterial diversity in rhizosphere soil of *Caragana microphylla* of TGCP community was significantly higher than GSMP community ($P < 0.05$) and GTP community ($P < 0.01$), because rare taxa such as Firmicutes, Rokubacteria, unidentified_Bacteria, Armatimonadetes, Deinococcus-Thermus, Cyanobacteria, Nitrospirae, and Melainabacteria exhibit significant differences, but Simpson index of bacterial diversity was no significant difference among TGCP, GSMP and GTP community ($P > 0.05$)(Fig.15), it indicated that there was no significant difference in dominant species. Chao index of bacterial diversity was extremely significant difference between TGCP community and GSMP community ($P < 0.01$), TGCP community and GTP community ($P < 0.01$), indicating that the richness of soil bacteria was affected, leading to the instability of shrub encroachment grassland ecosystems.

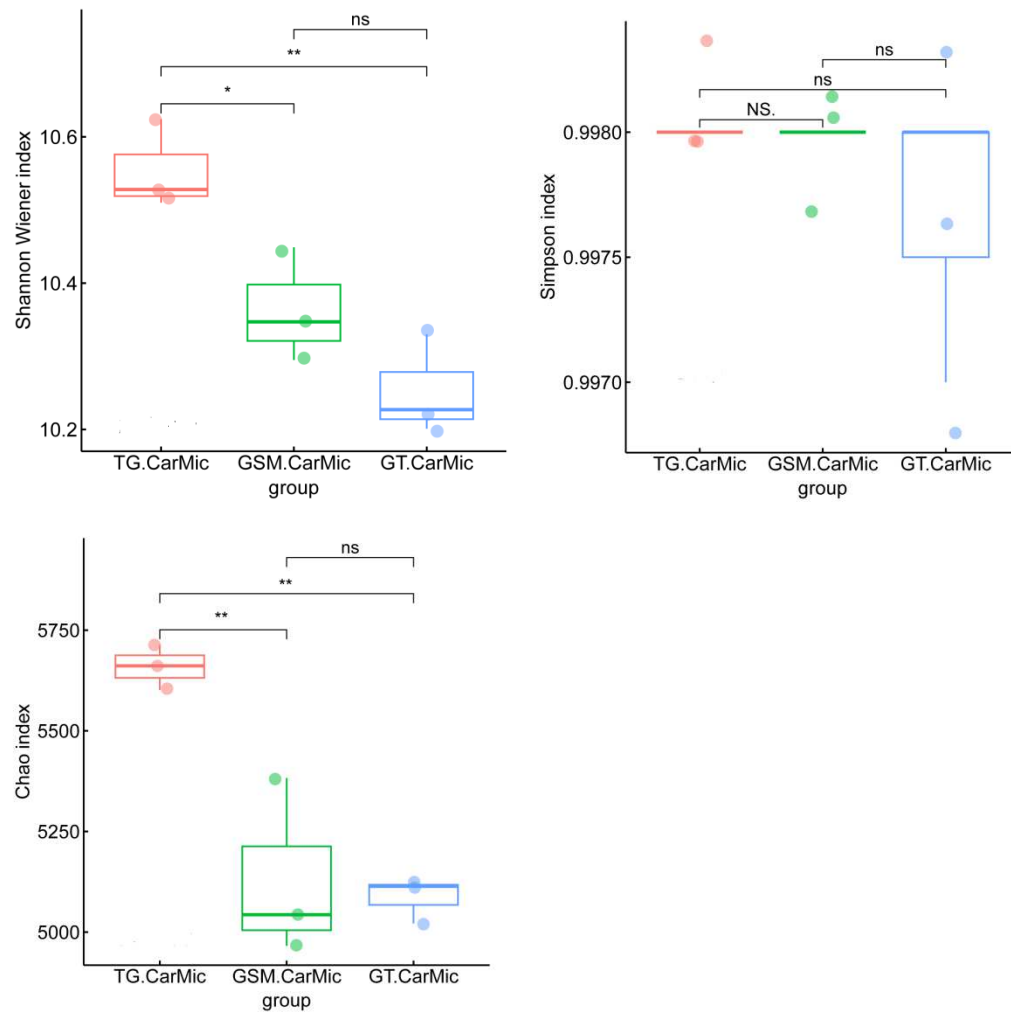


Fig.15 Bacterial alpha diversity indices in rhizosphere soil of *Caragana microphylla* in the three shrub encroachment community

Discussion

The impact of grassland shrub encroachment on plant community structure

The findings of this study indicated that shrub encroachment significantly altered the species composition, vertical structure, diversity, and spatial patterns of plant communities, serving as a key process driving the restructuring of grassland ecosystems. In GSMP and GTP community, plants of Gramineae no longer dominated, but *Caragana microphylla* formed shrub layer, which was occupying an important ecological niche of the community. The reason may be that Shrubs occupy the ecological niche of light, water, and nutrients through interspecific competition, inhibit the growth of shallow rooted herbaceous plants, and promote the succession of communities from typical grasslands to shrub encroachment grasslands, which is

consistent with the universal law of shrubbery in arid and semi-arid grasslands^{16, 17}. However, TGCP community still maintained the species composition characteristics of typical grasslands, and the dominant species were *Stipa grandis*, *Leymus chinensis*, and *Achnatherum sibiricum*. But shrub encroachment has led to a shift in the community from herbaceous dominance to a combination of grass and shrub, resulting in a significant increase in shrub coverage, biomass, and importance values. The coverage and density of plants of Gramineae and dominant herbs have decreased, and dominant species in the community have undergone significant replacement.

Shrub encroachment affects the underground rhizosphere soil microbial community

Shrub encroachment is accompanied by the continuous expansion of *Caragana microphylla*, which not only changes the structure of aboveground plant communities, but also significantly affects the underground rhizosphere soil microbial community, thereby regulating the ecological processes of shrub encroachment grasslands through plant-soil feedback.

First, shrub encroachment of *Caragana microphylla* can alter the community composition and diversity of rhizosphere soil microorganisms. As the coverage of shrubs increases, the diversity of rhizosphere fungi usually show a trend of first decreasing and then increasing, the diversity of rhizosphere bacteria usually show a trend of decreasing, which is closely related to the heterogeneity of soil microhabitats, input of root exudates, and changes in nutrient status during the process of shrub encroachment. Under severe shrub encroachment, shrubs have an advantage in resource competition, and the composition of root exudates tends to be uniform, which may lead to a homogenization of microbial community structure and a decrease in diversity. This result is consistent with the impact of shrub encroachment on rhizosphere microorganisms in arid and semi-arid regions^{17, 18}, indicating that the degree of shrub encroachment is a key factor in regulating the response of rhizosphere microorganisms.

The community composition of rhizosphere soil microorganisms was decreased, with the aggravation of shrub encroachment, the OUTS numbers of soil fungi and

bacteria both showed a decreasing trend, for the soil fungal community, the largest number of fungal OTUs in rhizosphere soil was from TG.CarMic with 1849 OTUs, followed by GSM.CarMic with 1437 OTUs, and the lowest was in GT.CarMic with 1215 OTUs, and species composition of fungal community in TGCP was significant higher than GSMP and GTP; Among them, with the aggravation of shrub encroachment, Basidiomycota decreased first and then increased, Mucoromycota increased first and then decreased, Chytridiomycota decreased. For the soil bacterial community, TG.CarMic had the most bacterial OTUs in the rhizosphere soil (7157), followed by GSM.CarMic (6841) and GT.CarMic (6776). Among them, With the aggravation of shrub encroachment, Actinobacteria decreased first and then increased, Proteobacteria increased first and then decreased, Bacteroidetes decreased. As the degree of shrub encroachment intensifies, the secretion and chemical composition of root exudates undergo significant changes, altering the quality of rhizosphere carbon input and niche differentiation, thereby enriching eutrophic and symbiotic microorganisms and inhibiting the growth of oligotrophic microorganisms adapted to native grassland soils. At the same time, the shrub encroachment of the small leaved golden pheasant will reshape the physicochemical properties of the rhizosphere soil, forming a typical "island effect" and becoming the core environmental screening force driving the construction of microbial communities: the organic matter and available nitrogen and phosphorus content of the rhizosphere soil increase, and the pH value decreases, providing a suitable living microenvironment for eutrophic microorganisms (such as Basidiomycota, Chytridiomycota, Actinobacteria, nitrogen fixing rhizobia, etc.), while inhibiting the original microbial groups that are sensitive to acidity, alkalinity, and nutrients.

Secondly, shrub encroachment of *Caragana microphylla* drives the transformation of root-associated microbial functional groups. As a leguminous shrub, *Caragana microphylla* enhances root nitrogen availability through biological nitrogen fixation, thereby promoting the enrichment of nitrogen cycle-related microbial taxa (e.g., rhizobia, ammonia-oxidizing bacteria, nitrifying microorganisms, etc.). The research findings of Jing, Yudu et al., revealed that shrub encroachment profoundly

enhanced soil multifunctionality¹⁷, bacterial intradomain links-particularly negative links-decreased, resulting in less complex and potentially less competitive bacterial networks, in contrast, the complexity of bacteria-fungi interdomain networks increased significantly, indicating strengthened interdomain connections. This targeted selection of functional microorganisms not only strengthens *Caragana microphylla*'s growth advantages but also alters the nutrient cycling patterns of grassland ecosystems.

Thirdly, the impact of shrub encroachment on rhizosphere microorganisms is closely coupled with changes in soil physicochemical properties. The shrub encroachment of the small leaved pheasant usually reduces the pH of the rhizosphere soil, increases the content of nutrients such as soil organic matter, total nitrogen, and available nitrogen and phosphorus, and forms a significant islands of fertility effect¹⁹. Key factors such as soil pH, moisture content, and carbon nitrogen ratio directly affect the survival and reproduction of microorganisms, and are important environmental screening forces driving changes in microbial community structure. Structural equation analysis also indicates that shrub encroachment can be achieved by altering soil organic matter and pH²⁰, and the changes in microbial community in turn affect soil nutrient mineralization and availability, forming a synergistic response of plants-soil-microorganisms.

In summary, during the process of shrub encroachment, *Caragana microphylla* reshapes the rhizosphere microbial community structure through host selectivity and the "islands of fertility " effect, enriching nitrogen-fixing, phosphorus-solubilizing, and growth-promoting functional microbial groups. This strengthens nutrient cycling and stress resistance, forming a positive feedback mechanism of "shrub-microbe" synergistic expansion. This study reveals the underground regulatory mechanism of shrub encroachment from a microbiological perspective, providing important theoretical foundations for understanding the evolution of arid and semi-arid grassland ecosystems and formulating scientific management strategies for shrub encroachment.

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Ethics Statement and Sampling Permission

Field collection of *Caragana microphylla* was conducted with written permission from West Ujimqin Banner Forestry and Grassland Bureau. All sampling complied with national and local regulations on wild plant collection and research.

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