The composition of antibiotic resistance genes is not affected by grazing but is determined by microorganisms in grassland soils

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Highlights

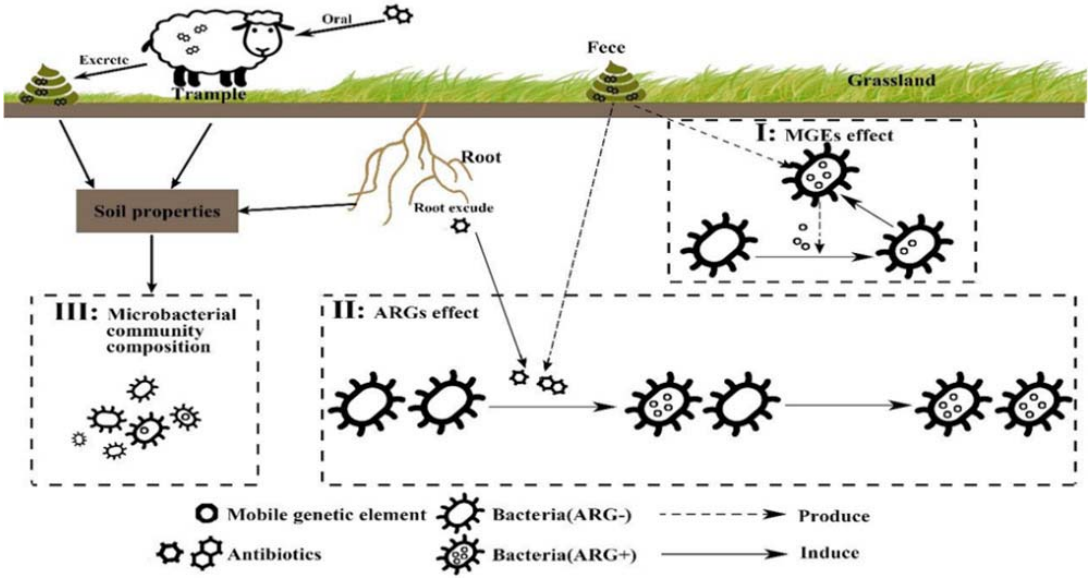
•ARGs related with resistance to cephalosporin and tetracycline were dominant.
•ARGs driven by factors affecting the distribution of soil microorganisms in grasslands.
•Long-term historic grazing had no effect on ARGs in grassland soils.
•ARGs shaped by the initial plant, soil environmental parameters and microbiomes in grassland.

Abstract

Grazing is expected to exert a substantial influence on antibiotic resistance genes (ARGs) in grassland ecosystems. However, the precise effects of grazing on the composition of ARGs in grassland soils remain unclear. This is especially the case for grassland soils subject to long-term grazing. Here, we investigated ARGs and bacterial community composition in soils subject to long-term historic grazing (13–39 years) and corresponding ungrazed samples. Using a combination of shotgun metagenomics, amplicon analyses and associated soil physicochemical data, we provide novel insights regarding the structure of ARGs in grassland soils. Interestingly, our analysis revealed that long-term historic grazing had no impacts on the composition of ARGs in grassland soils. An average of 378 ARGs, conferring resistance to 14 major categories of antibiotics (80%), were identified in both grazing and ungrazed sites. *Actinobacteria*, *Proteobacteria* and *Acidobacteria* were the most prevalent predicted hosts in these soils and were also shown to harbour genetic capacity for multiple-resistant ARGs. Our results suggested that positive effects of bacterial community

composition on ARGs could potentially be controlled by affecting MGEs. Soil properties had direct effects on the composition of ARGs through affecting the frequency of horizontal gene transfer among bacteria. Twelve novel ARGs were found in *S. grandis* steppe grasslands, indicating that different vegetation types might induce shifts in soil ARGs. Collectively, these findings suggest that soil properties, plants and microorganisms play critical roles in shaping ARG patterns in grasslands. Together, these data establish a solid baseline for understanding environmental antibiotic resistance in grasslands.

Graphical abstract



Keywords:

Grazing management; Metagenomics; Antibiotic resistance genes; Soil microbes; Eurasian steppe; Grassland soils

1. Introduction

Antibiotic resistance is widespread across ecosystems and pre-dates the exploration and application of modern antibiotics (D'Costa et al., 2011; Pawlowski et al., 2016a). However, there is now consensus that the emerging prevalence and dissemination of antibiotic resistance genes (ARGs) poses a serious threat to public health in the 21st century (Berendonk et al., 2015; Udikovic-Kolic et al., 2014). The selective pressure exerted by residual antibiotics and ARGs was considered the primary driver of microbial communities in terrestrial environments (Allen et al., 2010). Several studies have shown that ARGs are introduced to natural environments via several mechanisms including atmospheric transport, sedimentation of dust and dissipation by animals (Allen et al., 2010; Stalder et al., 2014; Mceachran et al., 2015). ARGs are also dispersed through horizontal gene transfer (HGT) mediated by MGEs, such as plasmids, integrons and transposons (Gillings et al., 2015; Prober et al., 2015; Surette and Wright, 2017).

Soils are major environmental reservoirs of ARGs, accounting for approximately 30% of known annotated sequences in public databases (Nesme and Simonet, 2015; Pawlowski et al., 2016a; Surette and Wright, 2017). The widespread occurrence of ARGs in natural ecosystems may be attributed to the inherent presence of antibiotics in soils (Yergeau et al., 2014). Several recent studies have reported that microbial community composition in soils may be the main determinant of ARG composition (Forsberg et al., 2014; Prober et al., 2015; F. Wang et al., 2016; J.T. Wang et al., 2016). Ultimately, changes in soil microbial communities, which host various ARGs, may affect their composition and diversity (Chen et al., 2016). Shifts in plant community composition may also affect the structure of microbial communities as a result of differences in plant litter, root exudates, soil types and properties including soil nutrient status and pH (Prober et al., 2015; F. Wang et al., 2016; J.T. Wang et al., 2016). Therefore, these shifts may indirectly alter the composition of the antibiotic resistome, and affect the structure of microorganisms which harbour ARGs and result in distinct soil ARG profiles (Forsberg et al., 2014; Hu et al., 2018; J.T. Wang et al., 2016). Furthermore, the co-occurrence patterns of various compounds found in soils, for instance heavy metals, may promote the dissemination of antibiotics and ARGs (Bicudo and Goyal, 2003; Zhu et al., 2013; Prober et al., 2015; Zhang et al., 2017). Previous studies have suggested that the dissemination of ARGs in environments under anthropogenic disturbance including soils irrigated with sewage, amended by manure (Zhang et al., 2017) and those contaminated with heavy metals (H. Hu et al., 2016). However, there is a lack of understanding regarding the key determinants shaping the composition and diversity of ARGs in natural ecosystems. Reducing this knowledge deficit is critical for predicting and managing the spread of environmental ARGs.

Grassland ecosystems occupy approximately 40% of the Earth's terrestrial surface and provide key ecosystem services (Aminov, 2009). Currently, antibiotics are widely used to prevent and treat livestock diseases (Zhu et al., 2013). In China, the use of antibiotics is estimated to be approximately 162,000 tons annually (Li, 2014), and roughly 52% of this is ascribed to veterinary antibiotics (Liu et al., 2015). Antibiotics are also used to treat and prevent diseases in grassland animals (Lin et al., 2019). However, the most widely used antibiotics are poorly absorbed by animals (Kyselkova et al., 2015). Approximately 75% of antibiotics, of which a large proportion consists of manure-derived ARG-harbouring bacteria, are excreted into soils (Halling-Sørensen, 2001). This would significantly exacerbate the development of multi-resistance in pathogenic and non-pathogenic bacteria as a result of selection in grassland soils (Davies and Davies, 2010; Van Goethem et al., 2018). In addition, grazing by livestock may alter ARGs through impacts on the soil microbiome and the resulting effects in plant communities (Bai et al., 2012; Milchunas and Lauenroth, 1993), physicochemical properties (Semmartin et al., 2009; Y. Zhou et al., 2017; X. Zhou et al., 2017), animal trampling, and the deposition of manure (Liu et al., 2015; McNaughton and McNaughton, 1997; Yang et al., 2013). Although grazing is expected to exert a substantial influence on ARGs through multiple ways (Fig. S1), the effects on the composition and diversity of ARGs in soils subject to long-term grazing remains poorly understood.

The temperate steppe in northern China is a prominent landscape in the Eurasian steppe and has been described as the largest natural ecosystem on earth (Xu et al., 2016). Recently, a new global map of AMR (antimicrobial resistance) showed that the north-eastern region of China may be a resistance hotspot (Van Boeckel et al., 2019). In this region, ivermectin is widely used to prevent livestock infectious diseases (Guli et al., 2020). In addition, grazing and ungrazed patterns are the two principal management strategies employed in these grassland ecosystems. To assess the effects of long-term grazing on ARGs, we collected soil

samples from nine grasslands across the Eurasian steppes. Each of these grasslands contained at least one continuously grazing site and one or two corresponding long-term ungrazed grasslands (13–39 years). We applied metagenomic analyses to detect the composition and diversity of ARGs. In addition, we measured the structure of microbial and plant communities as well as soil physiochemical properties, allowing us to gain mechanistic insights. The objectives of this study were to (1) explore whether grazing can alter soil ARGs by effecting shifts in plant and microbe community composition due to grazing; (2) identify whether animal feces contain ARGs which may be directly transmitted in soils through plasmids or other mobile genetic elements (MGEs).

2. Materials and methods

2.1. Location, field sites and sampling

In total, 81 soil samples were collected from nine sites across Eurasian steppes in China (Fig. 1, Table S1). These samples include three sub-sites (from each site) and three soil replicates for each sub-site. Among these samples, 54 soil samples (6 sites) are semiarid temperate grasslands from the Inner Mongolia Plateau. They were subject to different grazing-exclusion durations, including three long-term ungrazed grasslands (19–39 years: UN-39, UN-38 and UN-19 respectively) and three historic long-term grazing grasslands (GN-1, GN-3 and GN-2 respectively). A total of 27 soil samples (from 3 sites) were collected from sub-alpine meadows in Sinkiang, including two long-term ungrazed grasslands (33 and 13 years: US-33 and US-13, respectively) and one historic long-term grazing grassland (GS). DNA from three sub-sites including one grassland site was pooled and used for library construction and shotgun metagenomic sequencing. From each grazing site, 100 fecal samples were collected and transported on dry ice to Beijing and stored at −80 °C until processing. DNA samples from the same site were pooled after DNA extraction.

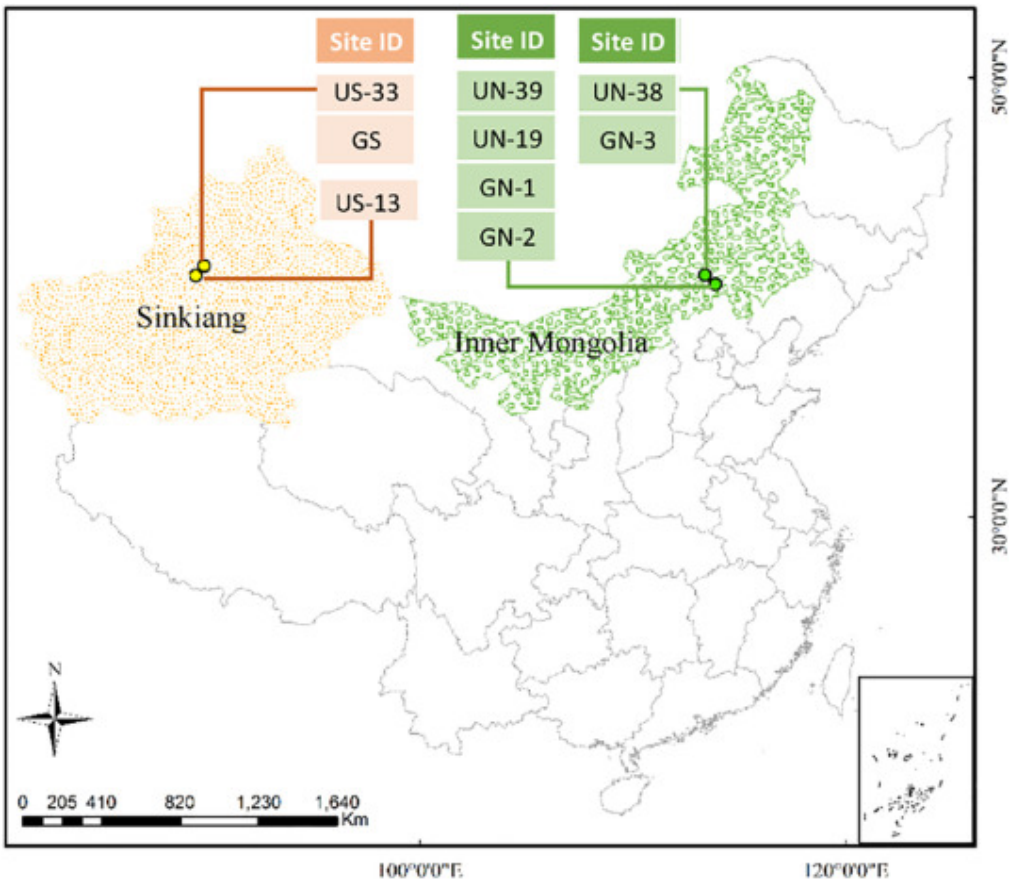


Fig. 1. Geographic locations of samples collected from nine grazing and ungrazed sites in the Eurasian steppe. The sites were categorized into four groups as follows; the first group in Sinkiang composed of one grazing (GS) and two ungrazed (US-33, US-13) sites. The second was comprised of 39 ungrazed (UN-39) and grazing (GN-1) sites. The third included 38 ungrazed (UN-38) and grazing (GN-3) sites. The fourth included 19 ungrazed (UN-19) and grazing (GN-2) sites.

From each sub-site, three plots (1 m × 1 m) were selected in a horizontal line with an equal distance (100 m). Above-ground samples were harvested from each plot by cutting standing biomass in each plot before soil sampling (n = 3). Within each plot, the % plant cover was visually estimated for all vascular plant species. Individual plant species were recorded to calculate species diversity (Hao et al., 2017). A soil auger (with a 5 cm diameter) was used to drill three replicate soil cores (from a depth of 0–15 cm) and mixed. A 2-mm sieve was used to remove roots and stones from soil samples. All soil samples were transported on dry ice to the laboratory in Beijing. On arrival, sieved soil samples were stochastically divided into two subsamples. A portion of the soil was used to analyse pH, total carbon, nitrogen, and phosphorus using standard quality control procedures (Fig. S2) (Che et al., 2018). Metallic element concentrations were measured using iCAP Q ICP-MS technique (Thermo Fisher Scientific, Fig. S3). The remaining soil portion was stored at −80 °C for DNA extraction.

2.2. Soil physicochemical characterization

The pH was measured by a Delta 320 pH-meter (Mettler-Toledo Instruments, Columbus, OH, USA) using a 1:2.5 ratio of soil to water. Soil total nitrogen (TN) was determined using an auto-analyzer (SEAL Analytical GmbH, Norderstedt, Germany). Soil total phosphorus (TP) was measured by UV-VIS spectrophotometer (UV2700, SHIMADZU, Japan). Soil inorganic

nitrogen (including NH_4^+-N and NO_3^--N) was extracted from soils with 2 M KCl and measured by continuous-flow ion auto-analyzer (SEAL Analytical GmbH, Norderstedt, Germany). Soil total organic carbon (TOC) was measured by a TOC Analyzer (Liqui TOC II; Elementar Analysensysteme GmbH, Hanau, Germany).

2.3. DNA extraction, library preparation and metagenomic sequencing

DNA was extracted from 0.25 g of soil using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) as specified in the manufacturer's protocol. A NanoDrop spectrophotometer (Thermo Scientific, Germany) was used to quantify DNA concentrations and purity. Samples with DNA concentrations above 1 μg/ml were used to construct libraries in the lab. The libraries were transported, on dry-ice, to Novegene (Beijing, China) for sequencing.

Libraries were generated using the Nextera XT DNA Library Preparation Kit (Illumina Inc., CA) with index sequences added to each sample, as per manufacturer's recommendations. Briefly, DNA was randomly fragmented by Covaris sonication to roughly 350 bp before fragments were end-polished, A-tailed, and ligated with the full-length adaptors for Illumina sequencing with further PCR amplification (Schmieder and Edwards, 2011). The PCR products were purified (AMPure XP system) and the library were analysed for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). At last, libraries were quantified using real-time PCR. High quality libraries were sequenced using the Illumina HiSeq platform.

2.4. Metagenomic assembly

Raw reads were quality-filtered, trimmed and screened using Prinseq-lite v0.20.4 (Schmieder and Edwards, 2011). High-quality paired-end (PE) reads were aligned using FLASH v1.2.11 (Magoc and Salzberg, 2011). We used metaSPAdes v3.9.0 for de novo assembly of metagenomic sequences (Nurk et al., 2017). For each sample, high quality reads were assembled into scaffolds. These scaffolds were used to obtain sequence fragments without ambiguous Ns (scaftigs). Processed data for each sample was mapped against these scaftigs to generate PE reads using SoapAligner (version 2.21, using similarity criteria ≥90%, -m 200, -x 400). These PE reads were then combined for hybrid assemblies, using the same assembly parameters detailed as for the individual samples. Only scaftigs longer than 500 bp were used for further analysis. The quality of each assembled metagenome (n = 9) was assessed by MetaQUAST v4.3. In order to provide taxonomic assignments, all contigs were compared to the complete NCBI's NR (Non-Redundant Protein Sequence Database) using DIAMOND v0.7.9.58, at an E-value cutoff of 1×10^{-5}.

2.5. Gene prediction from scaftigs

Open Reading Frame (ORF) predictions were performed using MetaGeneMark (V2.10 using default setting) for each sample and mixed-assembled Scaftigs (≥500 bp) (Shi et al., 2017; Zeller et al., 2014), omitting all sequences with length less than 100 nt (Sunagawa et al., 2015). De-redundancy was performed using CD-HIT (V4.5.8, set as -c 0.95, -G 0, -aS 0.9, -g 1, -d 0) (Li and Godzik, 2006) to obtain and construct non-redundant initial gene catalogue. Here, operationally non-redundant continuous genes encoding nucleic acid sequences were called genes. These genes were clustered based on 95% identity and 90% coverage, respectively, with the longest sequences selected as the representative sequence. Using

SoapAligner (set as identity ≥95%, -m 200, -x 400), the processed data for each sample was compared to the initial gene catalogue. The number of reads in the alignment was calculated for each gene across all samples. Only genes with ≥2 mapped reads were selected for further analysis. We calculated gene abundances by counting the number of reads prior to normalization by gene length using the following formula:

$$G_k = \frac{r_k}{L_k} \cdot \frac{1}{\sum_{i=1}^{n} \frac{r_i}{L_i}} \quad (1)$$

where G_k is the relative abundance of the k gene, r is the reads of genes, L is the length of genes, n is total number of genes.

2.6. Taxonomic classification

Comparisons of Unigenes with sequences for bacteria, fungi, archaea, and viruses retrieved from NCBI's NR database were done using DIAMOND v0.7.9.58 (Buchfink et al., 2015). For each sequence, results with E-value ≤1 e-10 were selected for downstream analysis (Qin et al., 2010). After filtering, these analyses may produce affiliations to multiple species as a result of the available classifications. The LCA algorithm, which applies systematic classification in MEGAN (Version 6.0) software (Qin et al., 2010), was used to measure biological significance. These appeared as the first branch prior to taxonomic classification. The relative abundance of taxonomic groups was calculated by summing the abundance of genes annotated to specific features. ARG affiliations for each species was obtained based on the corresponding species classifications. The composition and diversity of each antibiotic resistant bacteria (ARB) was determined for each sample.

2.7. ARGs and MEGs analysis

We aligned Unigenes to the CARD database by using the Resistance Gene Identifier (RGI) software implemented in the archive (RGI built-in blastp, default E-value ≤1 e-30) (Kristiansson et al., 2011). Based on RGI comparisons and the combined abundance data from Unigenes, the relative abundance of each ARO (Antibiotic Resistance Ontology) was calculated from the abundance data. Unigenes for each ARO species assignment was generated using Circos (Krzywinski et al., 2009).

All sequence reads, which were identified in the metagenomes, were aligned to INTEGRALL and NCBI RefSeq database using BLASTn (McArthur et al., 2013). Integrator BLAST and NCBI RefSeq database alignment threshold was set at 90% of nucleic acid sequence similarity, with a length of at least 25 amino acids per alignment sequence (Guo et al., 2017).

2.8. Sequencing of 16S rRNA gene amplicons

The V4-V5 region of the bacterial 16S rRNA gene was amplified using the primer set 515F (5-G T G Y C A G C M G C C G C G G T A-3)/909R (5-C C C C G Y C A A T T C M T T T R A G T-3) (Bates et al., 2011). Linked with the Illumina adaptor and a 12-bp barcode sequence, PCR amplification was performed in 50 μl reactions containing 0.5 μl ExTaq (TakaraBio Inc. Shiga, Japan), 4 μl Mg^{2+}, 4 μl dNTP, 5 μl buffer, 1 μl of each primer and 10 ng of template DNA. Purified PCR products were sequenced using the Illumina MiSeq instrument with Reagent Kitv3 (2 × 300 bp) at the Chengdu Institute of Biology (Chinese

Academy of Sciences). The generated sequences were processed and clustered into operational taxonomic units (OTUs) using QIIME (Edgar, 2013). Raw and paired end sequences were merged by Fast Length Adjustment of Short reads (FLASH) (Magoc and Salzberg, 2011), while quality filtering and phylotype clustering were executed using the UPARSE pipeline with USEARCH v8.0.1623 (Edgar, 2013). After singletons were discarded, the unique representative sequences were used to cluster into operational taxonomic units (OTUs) at the 97% sequence identity by cluster OTUs command UPARSE (Edgar, 2013).

2.9. Network analysis

All possible pair-wise Spearman's rank correlations between ARGs, MGEs and bacterial genera were calculated by using the "psych" package in R (Oksanen et al., 2010). The outputs were used to generate correlation matrices. The top 30 ARGs, MGEs and bacterial genera, respectively, were selected to reduce computational burden and to avoid spurious correlation bias. A correlation was considered statistically significant when it met conditions for the Spearman's correlation coefficient (ρ) > 0.7 and the *P*-value <0.01 (Xie et al., 2018). The pairwise correlations were imported into Gephi 0.9.2 (Gou et al., 2018) for network visualization.

2.10. Statistical analysis

The Shannon diversity index was used to measure plant species and soil bacterial diversity of each sample as:

$$H' = -\sum[P_i \times log\, P_i] \quad (2)$$

with P_i representing the proportional abundance of plant species and of OTUs with 97% sequence similarity and a sequencing depth of 45,070 per sample.

One-way ANOVA and *t*-test were performed to assess differences in the diversity of ARGs across ungrazed and grazing samples in SPSS 20.0 (SPSS Inc. Chicago, USA). All data were checked to confirm assumptions for ANOVA prior to downstream analysis. Differences in ARGs among soil and feces samples were visualized by non-metric multidimensional scaling (NMDS) ordinations plots. These NMDS were calculated based on Bray-Curtis distances using the "vegan" package in R (Oksanen et al., 2010). Linear regression analyses were performed to test the relationships between the diversity of ARGs with bacteria diversity, MGEs, plant diversity and soil properties using the 'ggplot2' package in R version 3.3.1. Mantel tests were performed to assess correlations between metallic elements and ARGs. The threshold for significance was $P \leq 0.05$. Structural equation models (SEM) were constructed to determine both of the direct and indirect effects of the bacterial abundance, bacterial diversity, soil properties (pH, total carbon, soil NH_4 - nitrogen, soil NO_3 - nitrogen, and phosphorus), and MGEs on ARG types using AMOS software version 24 (IBM Corp. USA).

3. Results

3.1. Diversity and relative abundance of ARGs in ungrazed and grazing grassland soils

An average of 446,987 and 476,747 open reading frames per metagenome were observed in the ungrazed and grazing sites, respectively. Of these, 370 to 393 ARGs were annotated as

potentially encoding antibiotic resistance in ungrazed and grazing sites, respectively (Table 1). The detected ARGs may confer resistance to 14 major classes of antibiotics including multidrug, tetracycline, macrolide-lincosamide-streptogramin (MLS), bacitracin, chloramphenicol and aminoglycoside resistance (Fig. 2). These major classes constituted 80% of the total ARG relative abundances across all soils.

Table 1. The richness and relative abundances of antibiotic resistance genes (ARGs) in samples retrieved from the Eurasian steppe.

Management pattern	Sample site	ARGs richness		Relative abundance ($\times 10^{-2}$)[a]
		Unique ARGs	Total ARGs	
Ungrazed	UN-39	4	386	1.62
	UN-38	1	376	1.50
	UN-19	3	376	1.40
	US-33	5	370	1.70
	US-13	0	370	1.75
Grazing	GN-1	5	393	1.32
	GN-2	5	381	1.44
	GN-3	12	387	1.62
	GS	2	370	1.71

[a]Relative abundance was calculated as the total amount of ARG divided by the number of genes predicted per sample site. Three long-term ungrazed grasslands in the Inner Mongolia (19–39 years: UN-39, UN-38, and UN-19, respectively) and corresponding historic long-term grazing grasslands (GN-1, GN-3, and GN-2, respectively). Samples were collected from sub-alpine meadows in Sinkiang, including two long-term ungrazed grasslands (33 and 13 years: US-33 and US-13, respectively) and one historic long-term grazing grassland (GS).

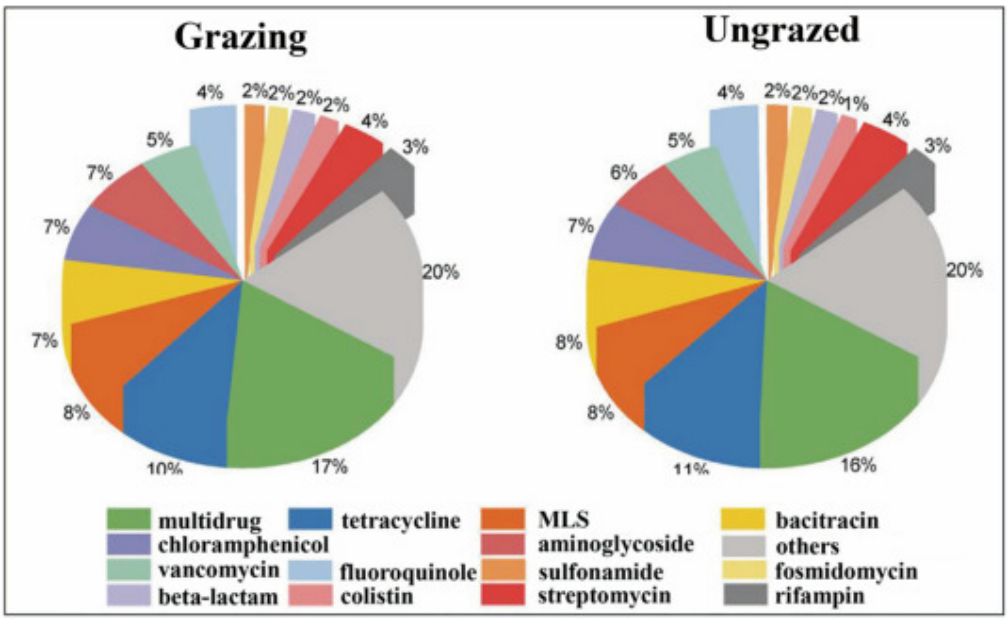


Fig. 2. The relative abundances of ARG families in Grazing and Ungrazed plots. The pie charts show the proportions of ARG families to the total identified from ungrazed and grazing grassland soils.

We found no significant differences in species diversity and the total ARGs found in ungrazed and grazing sites ($F = 0.67$, $P = 0.52$). However, the frequency of unique ARGs per site was highest in samples from grazing site in Inner Mongolia (GN-3, $n = 12$) (Tables 1, S2). The total relative abundance of ARGs was not significantly different ($t = 1.09$, $P > 0.05$) between ungrazed and the corresponding grazing grassland. Moreover, the results of

ANOVA suggested that only four ARGs (i.e. *MexK*, *tet34*, *aph2.llla* and *abeM* of the total ARGs) were significantly different between ungrazed and grazing grassland soils (Fig. S4). Remarkably, we found a significant difference in the species and abundances of fecal ARGs from sheep and soil samples linked to grazing sites and only 91 ARGs in the feces recovered from the grazing plots. The three highest ARGs were *aminocoum-arin_resistant_alaS*, *bifidobacteria_ileS* and *Streptomyces_cinnamoneus_EF-Tu*, which accounted for 19.7%, 16% and 12.8% of the total ARG abundance, respectively (Fig. S5). The NMDS ordination revealed that soil ARGs of Inner Mongolia Plateau were significantly different from those in Sinkiang soils (adonis test, $P < 0.05$), while the antibiotic resistome of grazing and ungrazed soils could not be separated. The results also showed a clear separation (adonis test, $P < 0.001$) between feces and corresponding grazing soils (Fig. 3).

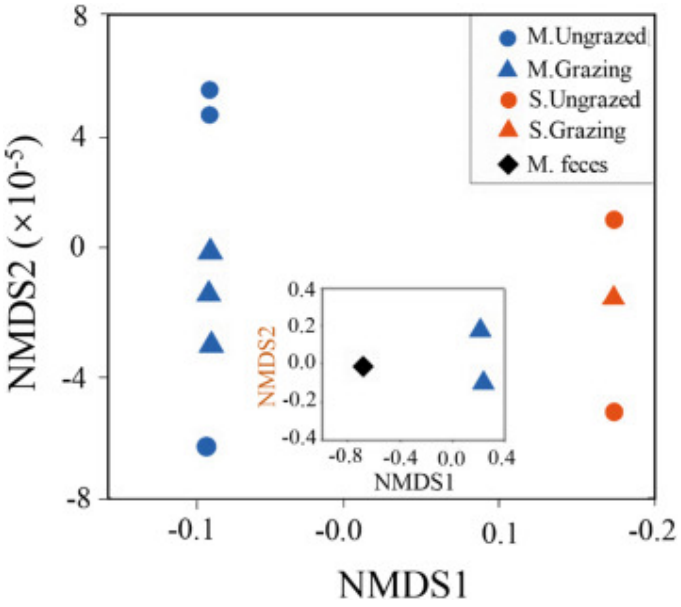


Fig. 3. Non-metric multidimensional scaling (NMDS) ordination plots of ARGs based on Bray-Curtis dissimilarity matrices between soils from Inner Mongolia Plateau and those from Sinkiang. The imbedded NMDS plot shows ARGs between feces and corresponding grazing sites. The stress value is 0.15. (Abbreviations: M.feces, the feces of Inner Mongolia Plateau; M.Grazing, grazing sites of Inner Mongolia Plateau; M.Ungrazed, ungrazed sites of Inner Mongolia Plateau; S.Ungrazed, ungrazed sites of sub-alpine meadows in Sinkiang; S.Grazing, grazing sites of sub-alpine meadows in Sinkiang).

3.2. Co-occurrence patterns of ARGs, MGEs and the bacterial communities

The bacterial community composition and relative abundances at phylum level could be clearly identified at every site (Fig. S6). Among each sample of grassland soils, ten phyla were detected, and the relative abundance of bacterial communities demonstrated no significant difference ($t = 0.16$, $P > 0.05$) between the ungrazed soil and the corresponding grazing sample. *Acidobacteria*, *Proteobacteria* and *Actinobacteria* were three dominant phyla in antibiotic resistance bacteria at phylum level, regardless of whether the soils were grazing or ungrazed (Fig. 4a and b, Outer circle). Similarly, *Actinobacteria*, *Proteobacteria* and *Acidobacteria* were three dominant phyla in antibiotic resistance bacteria, with the percentages of 35%, 22% and 15% in the ungrazed grassland soils (Fig. 4a, Inner circle) and 36%, 23% and 14% in the grazing grassland soils (Fig. 4b, Inner circle), respectively. Especially, the proportion of *Actinobacteria* in the antibiotic resistance bacteria was higher

compared to the total microbial community, suggesting that *Actinobacteria* harbour a greater proportion of resistance genes.

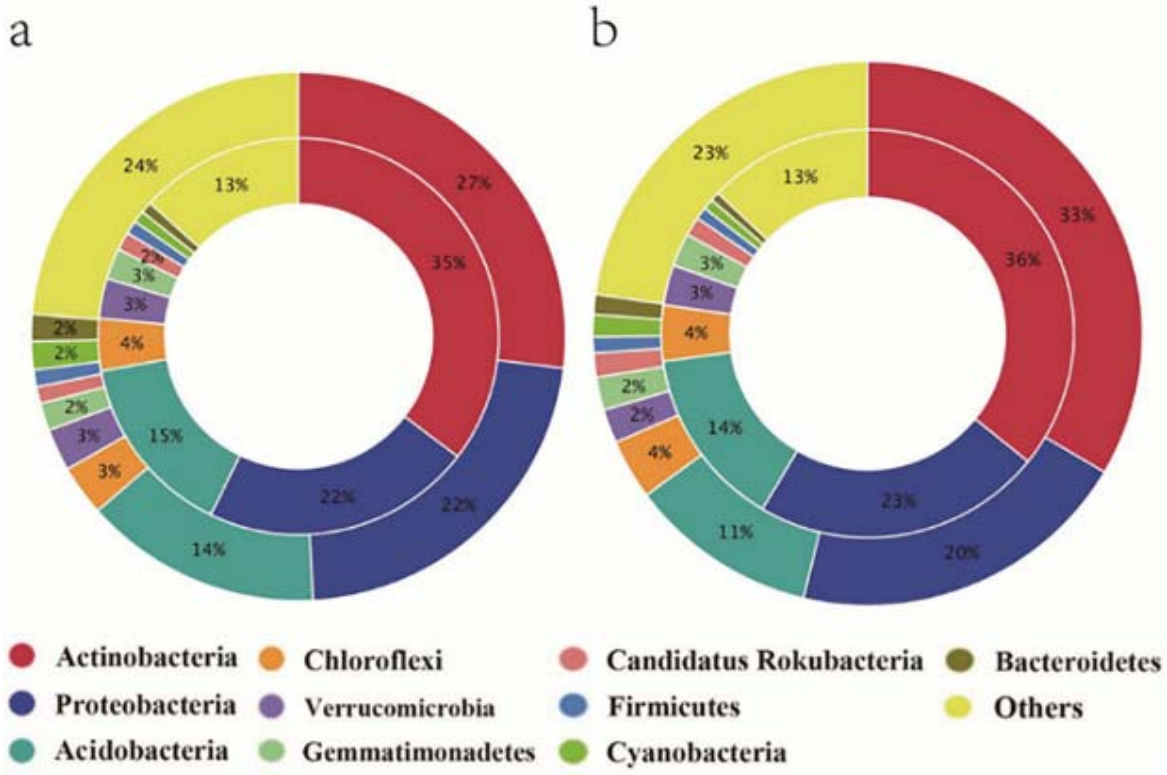


Fig. 4. Donut plots showing the phylogenetic composition between the locations. Ungrazed (a) and grazing (b) grassland soils are shown. The inner and outer circle represent the distribution of bacteria (total ARGs) and the overall distribution of soil bacteria, respectively.

A network consisting of 90 nodes (30 ARGs, 30 MGEs and 30 bacterial genera) was constructed to investigate the interactions among ARGs, MGEs and bacterial genera (Fig. 5). Only 4 bacterial genera including *Mesorhizobium*, *Bradyrhizobium*, *Chthoniobacter* and *Jiangella* were positively related to diverse MGEs (Spearman's $\rho > 0.8$, $P < 0.05$). Only 3 ARGs including *tetA48*, *vanRI* and *bifidobacteria_ileS* had significant relationships with diverse MGEs. *Jiangella*, *Pyrinomonas*, *Chloracido-bacterium*, *Ilumatobacter*, *Conexibacter*, *Pseudolabrys*, *Solirubrobacter*, *Thermo-leophilum*, *Variibacter* and *Rhodoplanes* also had significant relationships with diverse ARGs, especially those conferring resistance to novobiocin, tetracycline and ciprofloxacin. Interestingly, *tetA48*, *vanRI* and *bifidobacteria_ileS* (belonging to ARGs) had the most substantial connections with diverse ARGs, MGEs and bacteria genera, while genera *Jiangella* (belonging to bacteria genera) had the most connections with diverse ARGs, MGEs and bacteria genera.

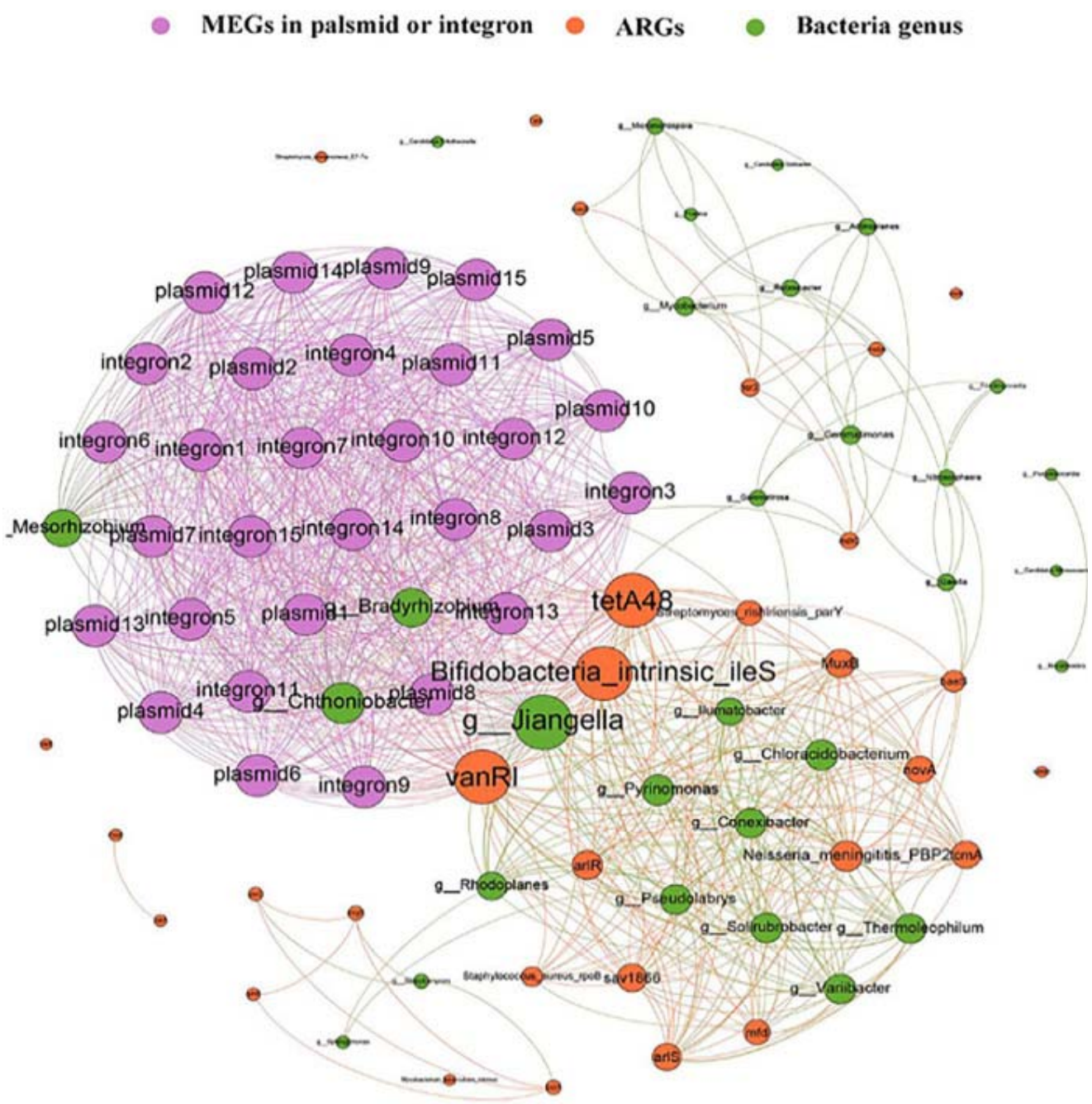


Fig. 5. Co-occurrence patterns among the detected ARG, MGEs and bacterial genera across grassland biomes. The edges indicate strong ($\rho > 0.8$) and significant ($P < 0.05$) correlations between ARG, MGEs and bacterial genera. The nodes, coded with different colours, represent different classes of ARGs, MGEs and bacteria. The edges, connecting nodes, correspond to statistically significant correlations between nodes. Node size is proportional to the number of connections between nodes (degree). The thickness of the edges is proportional to the correlation coefficient.

3.3. Relationships between ARGs with plants, bacteria, environmental factors, MGEs and heavy metals

The richness of ARGs had significantly positive linear relationships with plant richness ($R^2 = 0.83$, $P < 0.01$) and the TOC ($R^2 = 0.50$, $P = 0.02$). However, the richness of ARGs had

strongly negative linear relationships with both bacterial diversity and soil properties (including NO_3^--N, total nitrogen, and total phosphorus) (Fig. 6). It is noteworthy that the relative abundances of the total ARGs had significant positive correlations with MGEs of total integrons and plasmids (Fig. S7). The abundance and diversity of MGEs have no significant difference ($t = 0.19$, $P > 0.05$) between the ungrazed soils and the corresponding grazing samples. Similar results were found when assessing the proportion of ARGs and the concentrations of 10 heavy metals (Table S3).

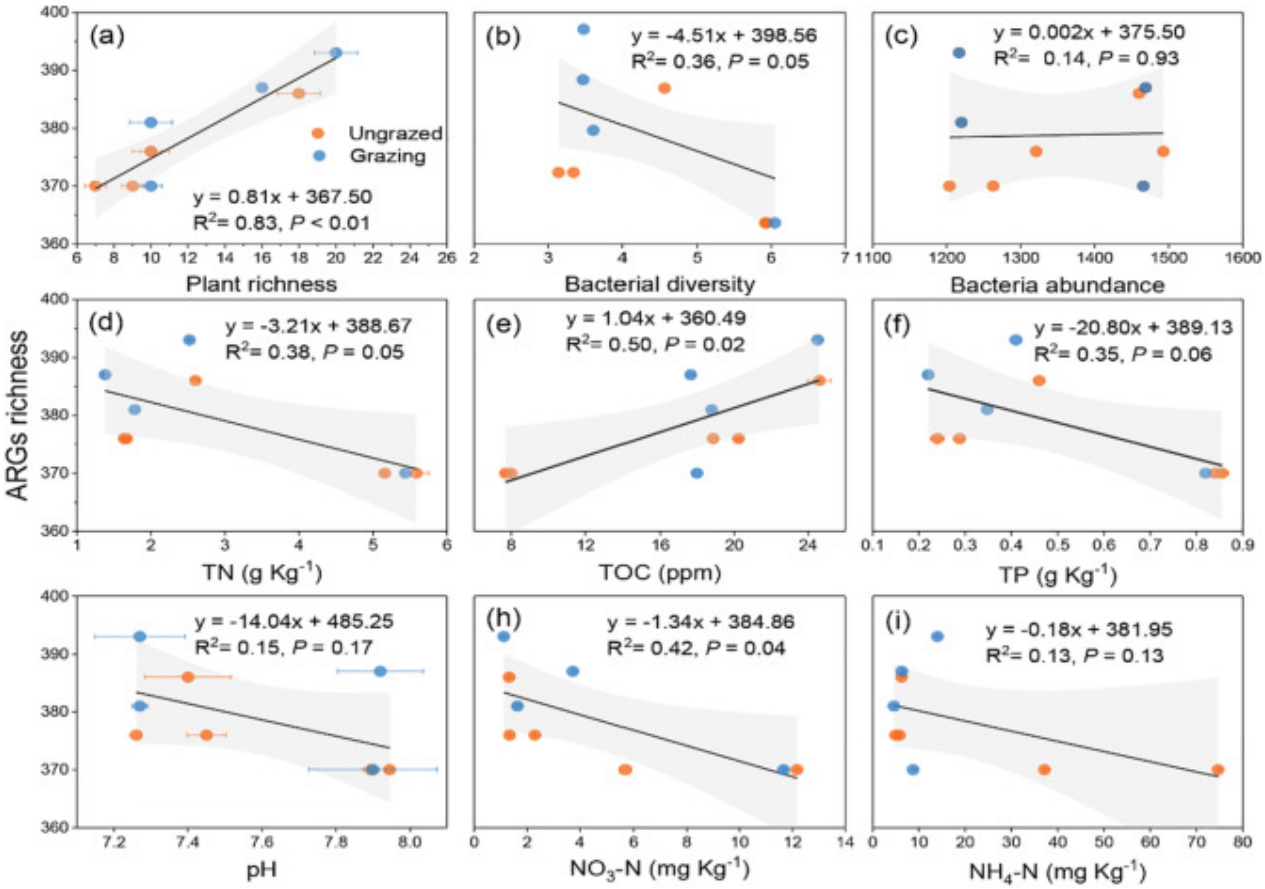


Fig. 6. Relationships between the ARGs richness and the plant richness (a), bacterial diversity (b), bacterial abundance (c), soil total nitrogen (d), total organic carbon (e), and total phosphorus (f), pH (g), soil NO_3-N (h), soil NH_4-N (i). The line in each plot represents the best-fit curve and the shaded area represents its 95% confidence limits. Orange dots represent grazing plots, and blue dots represent ungrazed plots. Values are mean ± SE.

SEM analysis suggested that long-term moderate grazing has no significant effects on the abundances of ARGs and other factors (Fig. 7). Soil pH had negative effects on the abundance of ARGs (Standardized coefficient = −0.79, $P < 0.05$). No significant and direct effects of bacterial diversity and abundance on ARGs were found, but both bacterial diversity and abundance had strongly indirect positive effects on the ARGs through its positive impacts on abundance of MGEs (Standardized coefficient = 0.84 and 0.73, $P < 0.01$ and $P < 0.05$, respectively). MGEs showed a positive effect on the abundance of ARGs (Standardized coefficient = 0.85, $P < 0.05$). However, no significant correlations between soil bacterial community, plants and soil properties were found.

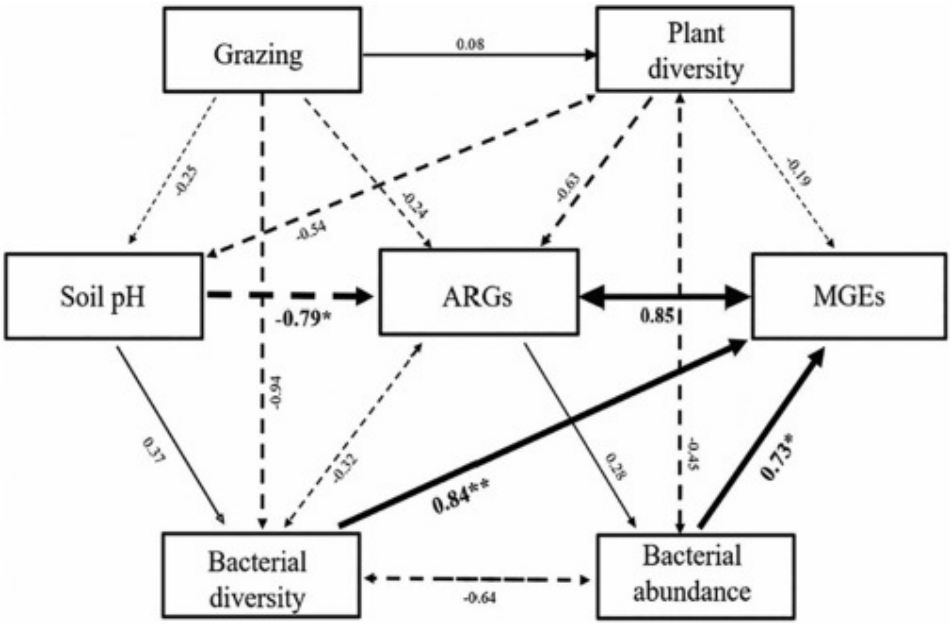


Fig. 7. Structural equation modelling of the direct and indirect effects of bacterial diversity and abundance, plant diversity, soil pH, grazed and MGEs on the richness of ARGs. Solid and dashed lines indicate positive and negative relationships, respectively. The width of the lines is proportional to the strength of path coefficients expressed by the numbers adjacent to arrows (*$P \leq 0.05$, **$P \leq 0.01$). The hypothetical models are consistent with our data, consistent with $\chi^2 = 1.877$, $P = 0.13$, GFI = 0.98, AIC = 40.6 values.

4. Discussion

4.1. Ubiquitous ARGs in natural grassland biomes

A key finding of our study is that ca. 390 ARGs (encoding resistance to fourteen major classes of antibiotics) were detected from grazing and ungrazed grassland soils with minimal anthropogenic disturbance. We found that ARGs in natural grassland ecosystems were richer than those from natural ecosystems including forest soils (Hu et al., 2018) and remote Antarctic surface soils (Van Goethem et al., 2018). In line with previous studies of ARGs in forest (Hu et al., 2018) and agricultural soils (Gou et al., 2018), we also found that the majority of genes conferred multidrug resistance. Additionally, in grassland soils, genes conferring resistance to tetracycline, MLS, bacitracin, chloramphenicol and aminoglycoside were also abundant. ARGs in natural grasslands were more likely to represent historical genes, due to minimal anthropogenic influence (Pawlowski et al., 2016a). Our findings provide evidence that ARGs are pervasive in these relatively primordial habitats (Chen et al., 2013). Furthermore, our findings also show that natural grassland ecosystems may be significant reservoirs of ARGs. These findings may have implications on determining risks due to antibiotic resistance in natural ecosystems.

The occurrence of ARGs in natural grasslands may be ascribed to the inherent presence of antibiotics produced by certain microorganisms in soils. However, measuring the specific concentrations of antibiotics in natural settings is difficult for several reasons (Allen et al., 2010). At high concentrations, these antibiotics are vital for competition in resource limited environments (D'Costa et al., 2011). At low concentrations, these antibiotics may also be used as signalling molecules secreted for communicating between microorganisms (Martinez, 2008). In addition to long-term primordial ARGs, other unknown selection pressures such as

anthropogenic disturbance, may lead to variations in ARGs levels in grasslands. Although the samples used in this study were collected from isolated areas, with no industrial activities and anthropogenic disturbance, we cannot exclude other possible mechanisms for ARG transmission. These alternate diffusion mechanisms may include, for instance, through deposition by ARG-carrying birds (F. Wang et al., 2016).

4.2. Evolutionary patterns of ARGs in grazing and ungrazed grassland soils

Several studies have reported that the application of manure may introduce new ARGs to soils (Han et al., 2018). This introduction may ultimately change the abundance and diversity of ARGs in these soils (H.W. Hu et al., 2016; Chen et al., 2017; Zhang et al., 2017; Gou et al., 2018; Han et al., 2018). It is possible that animal feces, with residual antibiotics retaining some antimicrobial activities, may enhance the proliferation of ARGs. However, in our study, animal feces introduced by grazing had no effect on ARGs. Moreover, no new ARGs were accumulated due to the deposition of animal feces in grazing grasslands. Grazing management, a primary use of grasslands in the Eurasian steppe, is known to affect soil bacterial community composition and may indirectly influence the diversity and prevalence of ARGs (Wang et al., 2019; Yang et al., 2019). However, soil bacterial community composition and abundances had no significant effect in grazing grassland soil compared with ungrazed grassland soils (Fig. S6). Fourteen families of ARGs, with the highest abundance in both the ungrazed and grazing samples, were consistently affiliated to the top ten most abundant bacterial phyla in the corresponding samples. Together, these results suggest that the grazing patterns do not affect the distribution of ARGs (Fig. S1). A possible alternative explanation for this finding may be the intrinsic resistance of environmental bacteria, which are important sources of resistance genes (Aminov, 2009; Davies and Davies, 2010). The long-term effects of exogenous microbiomes, from animal feces, on the resistome may be offset by the original community. This may be due to higher microbial diversity and consequent interactions which may restrict the dissemination of ARGs (Gou et al., 2018; Tyrrell et al., 2019). Another explanation may be the low concentration of swine manure (40 mg g^{-1} soil) in previous studies (Han et al., 2018). These soils may also be severely impacted by cattle, which may result in adverse impacts on vegetation and damage to the soil structure (N. Zhang et al., 2018; J. Zhang et al., 2018). The combined effects on vegetation (e.g. rhizosphere effects) and soil nutrient variables (e.g. pH) may substantially affect the microbiome (Bai et al., 2012). Nevertheless, a previous study demonstrated that the concentration of antibiotics detected in the feces of dairy cows treated with antibiotics to prevent and treat diseases, was less than 1 mg kg^{-1} (Liu et al., 2015). Another study revealed that the concentration of grassland soil exposed to inputs of fresh feces from grazing cattle may be as low as 0.2 mg kg^{-1} (Kyselkova et al., 2015). Regardless of the precise concentrations, moderate grazing results in very little feces per square meter of grassland soils (Chen et al., 2017). Our findings are consistent with the notion that bacterial community composition is a primary determinant of ARGs in grassland soils, especially when low levels of exogenous antibiotics and ARGs are introduced into soils (Hu et al., 2018; Stalder et al., 2014; Van Goethem et al., 2018).

4.3. Potential for horizontal gene transfer via MGEs of ARGs in grassland soils

The diverse and abundant ARGs detected in the grassland soils, along with abundant MGEs, suggest that these ARGs may be horizontally transferred to other bacteria (Hu et al., 2018). The MGEs may rapidly spread within species through HGT with previous studies showing that this may occur among different species or genera (Hall et al., 2017). In our study, the

relative abundance of total ARGs in grassland soils was significantly positively correlated with integrons and plasmids. Of particular interest, we found that the bacterial diversity and abundance had positive direct effects on MGEs. These positive effects on ARGs were maintained despite the effects of multiple drivers. This result is consistent with the view that bacterial abundance has the greatest effect on the composition and abundance of ARGs (Jia et al., 2015; Chen et al., 2016; Han et al., 2018). Bacterial abundance and diversity, which have strong impacts on MGEs, may also influence soil resistomes. This suggests that there may be a potential risk of HGT mediated by MGEs among ARGs in grassland soils. However, it should be noted that although there are significant correlations among ARGs, MGEs and bacteria, the effects of HGT in grassland soils remain unclear.

4.4. Soil properties, bacterial and plant communities as important determinants of ARGs in grassland

The frequency of HGT in grassland ARGs is likely to be affected by soil properties (e.g., pH) (Aminov, 2009). In our study, the richness of ARGs had significantly linear relationships with NO_3^--N, total nitrogen, and total phosphorus. Soil pH had negative effects on the abundance of ARGs in SEM analysis. It is entirely conceivable that soil properties may directly affect ARGs through affecting the frequency of HGT among microbial communities. However, soil pH had no indirect effects on ARG patterns through its positive impacts on bacterial diversity.

Not only are soil bacterial communities the primary sources of antibiotic compounds (Allen et al., 2010), they are also the dominant host organisms of ARGs. For example, *Actinobacteria* are well-known for their capacity to produce antibiotics in soils (Forsberg et al., 2015). Recent metagenomic studies have shown that members of this phyla host multi-resistant ARGs (D'Costa et al., 2011; Forsberg et al., 2015). Previous work suggested that shifts in bacterial communities, may influence their diversity and prevalence of ARGs (Chen et al., 2016). However, soil bacterial composition did not change as a result of grazing. There was no significant difference in bacterial abundance between ungrazed and grazing grasslands in our study. The most abundant prokaryotes in grassland soils (*Actinobacteria*, *Proteobacteria* and *Acidobacteria*) contributed most ARGs. These results contrast our hypothesis that long-term historic grazing pattern may alter the bacterial communities and consequently change their related ARGs. Nevertheless, our results are consistent with studies showing that *Actinobacteria* and *Proteobacteria* harbour the highest number of ARGs in different ecological soil niches (Dantas et al., 2008; Noronha et al., 2017). These results support a view that bacteria may be important predictors of ARGs in natural grassland biomes. Our results support the argument that the distribution of ARG may be broadly consistent with the composition of soil microbial communities (Hu et al., 2018).

Interestingly, the number of novel ARGs in soils of UN-39 (dominated by *Leymus chinensis*) and UN-38 (dominated by *Spectrunculus grandis*) in Inner Mongolia were significantly different, although they belonged to the same environment (they were ca. 15 km apart). Twelve novel ARGs were found in *S. grandis* steppe grasslands (UN-38). Our results indicate that different vegetation types might induce a change in soil ARGs. The richness and abundance of resistance genes in soils vary with plant species. Some studies have shown that plants can affect the structure of soil microbial communities through plant litters and root exudation (Prober et al., 2015; F. Wang et al., 2016; J.T. Wang et al., 2016). This may, in turn, indirectly influence soil resistance composition via altering the classification and the phylogenetic structure of ARGs-bearing microbes. An alternative reason for this finding is

that plants can exude specialized antibiotic compounds and then lead to the changes of ARG contents (Forsberg et al., 2014). However, we did not find direct impacts of plant communities on ARGs in our study. Therefore, it is still unclear how the variations in plant species affect the antibiotic resistance genes in the grassland soils.

5. Conclusions

In summary, we provide the first insights regarding the long-term effects of historic grazing management in the Eurasian steppe. Our data demonstrate that grazing had no significant effects on the diversity and relative abundance of ARGs. Moreover, we also show that grazing had no significant effects on grassland soil bacterial communities. A synthesis of our data suggests that the diversity and relative abundances of ARGs was shaped by several variables, including bacterial community composition, soil properties, and plant community composition. In contrast to previous studies, our results suggest that the accumulation of animal feces in grazing grasslands does not introduce new ARGs to these soils. Understanding relationships between ARGs with MGEs, plants, bacteria and soil properties in this semi-arid grassland ecosystem is critical for predicting patterns of environmental resistomes. These insights are crucial for considering the risks associated with ARGs in natural grassland ecosystems.

CRediT authorship contribution statement

Zhenzhen Zheng: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Linfeng Li:** Investigation, Formal analysis, Data curation, Writing - review & editing. **Thulani P. Makhalanyane:** Writing - review & editing. **Chunming Xu:** Methodology. **Kaihui Li:** Writing - review & editing. **Kai Xue:** Investigation. **Cong Xu:** Investigation. **Ruyan Qian:** Investigation. **Biao Zhang:** Investigation. **Jianqing Du:** Investigation. **Hua Yu:** Visualization. **Xiaoyong Cui:** Conceptualization, Methodology. **Yanfen Wang:** Visualization, Supervision. **Yanbin Hao:** Funding acquisition, Formal analysis, Visualization, Writing - review & editing, Project administration.

Declaration of competing interest

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

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Availability of data

The sequence data are available on the NCBI under the BioSample accession numbers 16076879 through 16076887. Contigs from the assembled metagenomes are deposited on the NCBI under the BioProject PRJNA662109.

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