Biology and Fertility of Soils
https://doi.org/10.1007/s00374-018-01336-5

ORIGINAL PAPER

Impact of long-term grazing exclusion on soil microbial community composition and nutrient availability

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Received: 14 October 2018 / Revised: 12 December 2018 / Accepted: 14 December 2018

Abstract

While grazing exclusion is thought to drive soil nutrient transport and cycling, and reduce soil compaction, its direct impact on microbial community composition remains unclear. In this study, we examined the impact of grazing exclusion on abundance and composition of soil microbial (bacterial, archaeal, and fungal) communities, especially those associated with nutrient cycling. We surveyed soil physicochemical properties and litter mass, at sites undergoing varying durations of grazing exclusion (0–34 years) in a semiarid grassland. Using next-generation amplicon sequencing, we further characterized variations in the composition and diversity of soil microbial communities associated with grazing exclusion and soil depths, as well as subsequent changes in physicochemical properties. Most soil physicochemical parameter values significantly increased as the result of long-term grazing exclusion, and these properties were associated with variation in composition and diversity of microbial communities. Notably, the relative abundances of microbial families associated with C cycling (e.g., *Chitinophagaceae*) increased with an increase in nutrient availability following grazing exclusion. The abundance of the archaeal ammonia-oxidizing *Nitrososphaerae* increased with decreasing concentration of ammonium among samples. Likewise, fungal communities were also associated with the shifts in nutrient concentrations, although the majority of fungi could not be classified to the species level. Nitrate concentration also played a critical role in shaping bacterial, archaeal, or fungal communities. Moreover, bacterial and archaeal communities had a greater mean Shannon index in 0–10-cm than those in 10–20-cm soil layer. Results of this study provide novel insights regarding how the length of grazing exclusion and soil depth influence nutrient gradients and microbial community composition associated with nutrient cycling.

Keywords Bacteria · Fungi · Next-generation amplicon sequencing · Soil nutrients · Exclusion-grazing

Introduction

Grasslands cover about 40% of the Earth's surface and are an important ecosystem providing goods and services to support flora, fauna, and human populations worldwide. It is postulated that grasslands can also be used to mitigate adverse effects due to global climate change (Murray et al. 2000). However, despite their importance, one third of the world's grasslands are severely degraded, mainly due to overgrazing. This problem has occurred in grasslands from desert, semi-arid, and alpine steppes, and moist alpine meadows (Cui et al. 2005; Deng et al. 2014a; Zhou et al. 2006; Zhu et al. 2016a). Grazing exclusion is an effective approach to preserve and recover vegetation, improve soil quality, increase plant diversity and productivity, and restore degraded grasslands (Feyisa et al. 2017; Listopad et al. 2018). Grazing exclusion can increase soil microbial diversity and abundances of some microbial

Zhe Wang and Qian Zhang contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00374-018-01336-5) contains supplementary material, which is available to authorized users.

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Published online: 05 January 2019

taxa, leading to greater litter decomposition rates in woodland remnants (Lindsay and Cunningham 2009). Microbes, as drivers of biogeochemical cycles, play an important role in influencing nutrient transport and cycling in soils. For example, microbes degrade organic matter and organic C from litter mass, in turn regulating C cycling in soils (Schimel and Schaeffer 2012). Similarly, microbes oxidize ammonia in soils, fix nitrogen (N) from the atmosphere, and drive nitrification and denitrification reactions, driving soil N cycling (Aislabie et al. 2013). Thus, it seems logical that grazing exclusion may increase specific populations of soil microbes involved in autotrophic N cycling, as well as heterotrophic communities that rely on nutrient exchange and physical properties of soils.

Recent studies have examined the influence of grazing exclusion on soil nutrient turnover, plant phenology and diversity, and the reproductive success of vegetation (Medina-Roldán et al. 2012; Zhang et al. 2005; Zhu et al. 2016b, 2016c). Grazing exclusion often leads to increased soil nutrients due, in large part, to the accumulation of plant litter on the soil surface, while soil compaction is reduced due to removal of animal trampling, resulting in an increase in soil air permeability. The effects of grazing exclusion on microbial community composition and function in grasslands remain relatively unexplored. Moreover, there is little information available regarding how the duration of grazing exclusion influences microbial communities and how it changes soil biogeochemistry that likely influences primary productivity (Medina-Roldán et al. 2012). Furthermore, given that the principal source of nutrient input is from above ground materials, abundance and composition of microbial communities are likely influenced by soil depth.

Several studies have analyzed the vertical distribution of microbial communities in different land use types (Griffiths et al. 2003; Nacke et al. 2011; Steven et al. 2013; Leeuwen et al. 2017). However, and to the best of our knowledge, few studies have examined microbial communities at various depths in grassland soils undergoing long-term grazing exclusions. This information is needed to better restore and manage grasslands.

In this study, we used high-throughput DNA sequencing to characterize the bacterial, archaeal, and fungal communities of grasslands subjected to up to 34 years of grazing exclusion. The primary objective of this study was to determine the relationships between the soil microbial community composition, litter mass, and soil chemical properties, at two soil depths (0–10 and 10–20 cm) in a restored grassland. We hypothesized that (1) grazing exclusion most strongly impacts specific members of the microbial community associated with C and N cycling; (2) heterotrophic members of the microbial community shift in response to changes in soil nutrient concentrations, as well as soil physical properties; and (3) the impact of grazing exclusion on the composition of microbial communities differs by soil depths, with greater impact in the near-surface layer. Understanding these interactions will provide a better context to evaluate how the length of grazing exclusion influences nutrient gradients and corresponding microbial diversity and nutrient cycling functions, and will inform future efforts to restore and remediate grassland degradation.

Materials and methods

Study sites

This study was done in the Yunwushan Natural Grassland Protection Zone (36°13′–36°19′ N; 106°24′–106°28′ E) at Guyuan City, Ningxia Hui Autonomous Region, China. The grassland protection zone has an area of 6700 ha and an elevation ranging from 1800 to 2048 m. The study area has a continental monsoon climate with a mean annual temperature of 6.9 °C, and annual precipitation is 425 mm. The maximum and minimum temperatures occur in July (24 °C) and January (− 14 °C), respectively. The frost-free period is ~ 124 days, normally beginning in mid-April and ending in late September. The soil in the study area is a montane gray-cinnamon soil classified as a Calci-Orthic Aridisol (Chinese taxonomic system) and is equivalent to a Haplic Calcisol in the FAO/UNESCO soil classification system.

Field investigation and sampling

The study area was grazed by goats before protection. The intensity of grazing varied over the years. In 1982, before protection, the physicochemical properties of the soil in the study area with the same soil type and natural conditions were not significantly different (Bureau 2000). Five grazer-excluding treatments established at different times were studied. Grassland sites were fenced with goat-proof wire mesh in 1982, 1987, 1992, 1997, and 2002, and consequently goat grazing was excluded for 34, 29, 24, 19, and 14 years, respectively. A grazing area of 3000 ha was maintained as a control. All six areas adjoined each other, and no synthetic fertilizer was applied.

Most parts of the grazed- and grazer-excluded grasslands have similar topography and altitude. Our previous study in the same control area showed that there were no significant changes in soil organic C (OC) and total N (TN) during the past 34 years in the continuously grazed area (CK treatment) (Qiu et al. 2013). Based on previous analyses, the soils in each treatment had similar initial conditions and the existing grazed grassland was used as a control to compare the effects of grazing exclusion on soil microbiota.

Five pseudo-replicated plots (30 m × 30 m) were randomly established within the grazed and grazer-excluded treatment areas in August 2016 (Fig. 1). Since each treatment area was > 400 ha, and distances between each plot were > 500 m, the

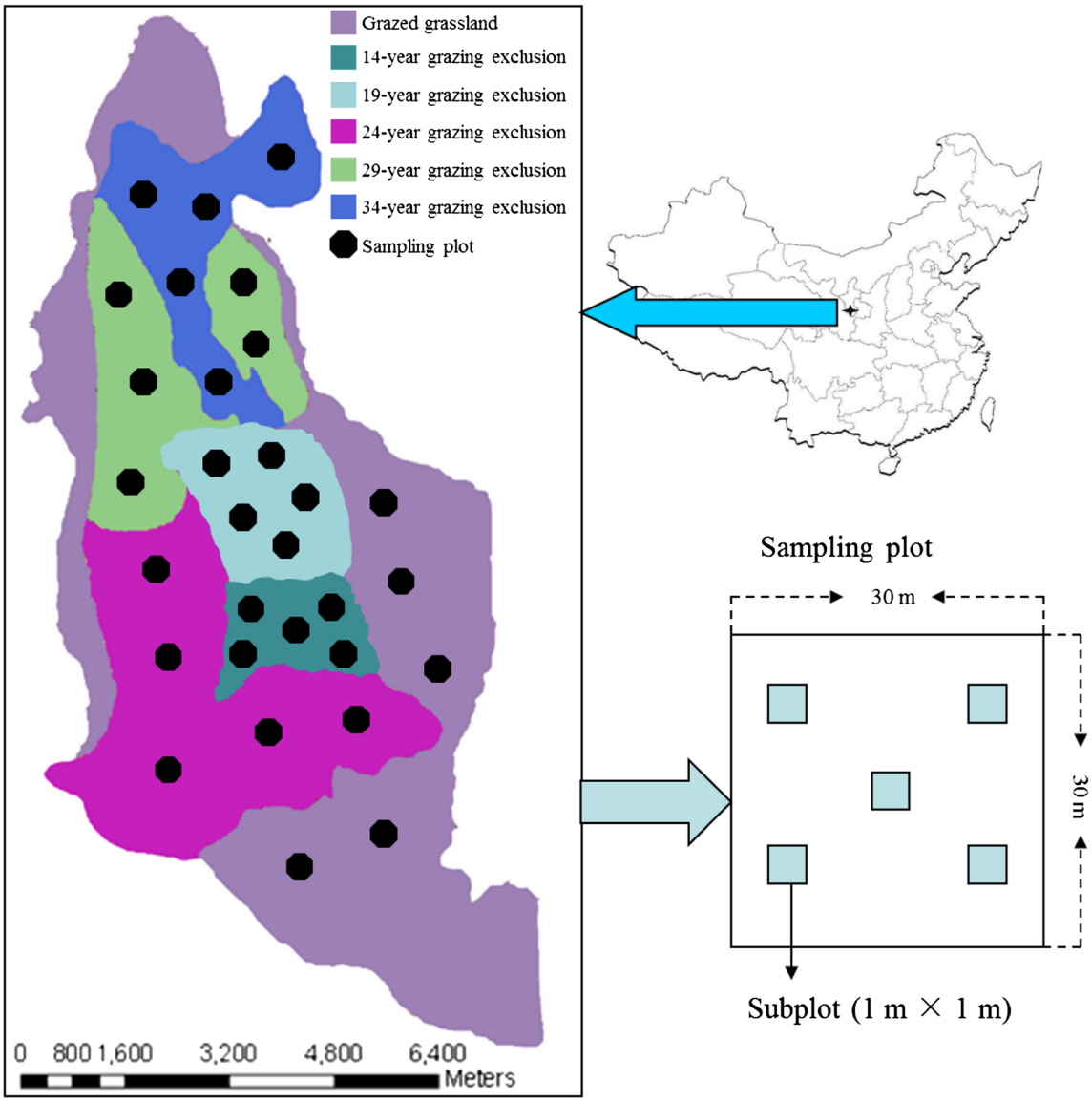

Fig. 1 Location of the study site in the Yunwushan Natural Grassland Protection Zone and the sampling scheme

sampling design reflected the effects of grazing exclusion on soil microbes in this larger ecosystem. All plots for both grazed and grazer-excluded treatments had the same soil type and similar physiographic conditions (slope degree and direction, topography, and altitude).

Three 1 m × 1 m subplots were established in each larger plot for field investigation and sampling. The subplots were at least 15 m apart and did not differ in physiographic conditions. The mean number of each plant species within each subplot was determined from three replicate subplots. Litter layer depth and the total amount of litter were measured in each subplot. The details of each exclusion site are presented in Table 1. Soil samples were collected at the 0–10- and 10–20-cm depths, using a 9.0-cm (diameter) tube auger, to determine whether soil layers at different depths varied in soil physicochemical properties and microbial community composition. Roots were separated from the soil samples by shaking and hand picking, and oven dried at 65 °C to measure root biomass at the 0–10- and 10–20-cm depths. All visible pieces of organic material were removed from soil samples. The moist soil samples were brought to the laboratory and stored at − 20 °C prior to DNA extraction. Separate soil samples were air-dried for analysis of edaphic properties.

Laboratory analysis

Soil OC was determined by using the Walkley-Black method (Nelson and Sommers 1982), and TN was measured by using the Kjeldahl method (Bremner and Mulvaney 1982). Total phosphorus (TP) was determined by colorimetric analysis after wet digestion with sulfuric acid and perchloric acid, and available phosphorus (AP) was determined by the Olsen

Table 1 Altitude, area, litter characteristics, and dominate plant species at each grazing exclusion sample site

Years of exclusion	Altitude (m)	Area (ha)	Litter layer depth (cm)	Litter mass (g m^{-2})	Dominate species
0	1969–2013	3000	0.2 ± 0.02	120 ± 19	*Carex aridula, Thymus mongolicus*
14	2073–2097	420	0.5 ± 0.07	199 ± 24	*Stipa grandis, Artemisia frigida*
19	1876–1899	820	0.8 ± 0.25	221 ± 30	*Stipa grandis*
24	1955–1975	1000	1.4 ± 0.39	197 ± 52	*Stipa grandis, Thymus mongolicus*
29	1948–1969	855	2.2 ± 0.49	276 ± 82	*Stipa grandis, Thymus mongolicus*
34	2013–2048	460	3.0 ± 0.29	692 ± 143	*Stipa przewalskyi, Artemisia sacrorum*

method (Olsen and Sommers 1982). The soil was extracted using potassium chloride, and the extract was analyzed immediately for ammonium (NH_4^+) and nitrate (NO_3^-) with a Lachat Flow Analyzer (AutoAnalyzer3-AA3, Seal Analytical) (Kachurina et al. 2000). Available potassium (AK) was extracted with neutral ammonium acetate and measured by atomic absorption spectrometry (SpectrAA-220 Zeeman, Varian Inc., Palo Alto, CA) (Doll and Lucas 1973). Soil pH was measured in a 1:2 soil/water suspension using a glass electrode.

DNA extraction and sequencing

The total DNA was extracted from soil samples using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA). We have followed Schöler et al. (2017) and Vestergaard et al. (2017) for sequencing as it concerns number of replicates, checking the absence of polluted DNA in the used kits and solution, etc. The V3-V4 hypervariable region of the 16S rRNA was amplified for bacteria and archaea using primer set 341F (5′-ACTCCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTAVVGGGTATCTAATC-3′) (Sinclair et al. 2015). Fungal communities were characterized by amplification and sequencing of the internal transcribed spacer region 1 (ITS1), between the 18S and 5.8S rRNA genes, using ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTC TTCATCGATGC-3′) primers (Mueller et al. 2014). Briefly, samples were amplified through thermal cycling conditions: 95 °C for 5 min, followed by 25 cycles of 98 °C for 20 s, 55 °C for 15 s, and 72 °C for 1 min. Adapters and barcodes were added for an additional 10 PCR cycles. Amplicons were gel purified, pooled, and paired-end sequenced by the Realbio Genomics Institute (Shanghai, China) at a read length of 250 nucleotides (nt), on an Illumina HiSeq platform (Illumina, Inc., San Diego, CA, USA). Raw sequencing data were deposited in the Sequence Read Archive (SRA) of NCBI under Accession Number SRP139861.

Sequence quality control

Quality control of sequence data was done using the SHI7 program (Al-Ghalith et al. 2018). Nextera adaptors were trimmed through the script "*axe_adaptors_nextera_noL.sh*". The script "*create_flasher_general.sh*" was used to generate uniform, full-length consensus sequences, and assembled sequences were trimmed using a cutoff quality score of Q30 and read length longer than 300 nt. Trimmed sequence reads were further subjected to denoising, deduplication, compaction, and read cleanup, as described previously (Al-Ghalith et al. 2016). High quality sequence reads were clustered into operational taxonomic units (OTUs) at a 97% similarity value using the SILVA123 database for bacteria and archaea (Pruesse et al. 2007), and a 99% similarity for fungi using the UNITE database version 7 under the bootstrap cutoff 60 (Kõljalg et al. 2013). For statistical comparisons, samples were rarefied, by random subsampling to 30,000 reads per sample for bacteria and archaea, and 4300 reads per sample for fungi.

Statistical analysis

Diversities and compositions of the microbial communities were analyzed using mothur software version 1.39 (Schloss et al. 2009). Alpha diversity was calculated using the abundance-based coverage estimate (ACE), Shannon index, and non-parametric Shannon index. Differences in beta diversity were evaluated among samples using analysis of similarity (ANOSIM), which employs Bray-Curtis dissimilarity matrices (BC) (Bray and Curtis 1957; Clarke 1993). Phylogenetic beta diversity (phylobetadiversity) was analyzed using unweighted UniFrac distances (Lozupone and Knight 2005). Ordination of Bray-Curtis matrices was performed using principal coordinate analysis (PCoA) (Anderson and Willis 2003). Visualization of the taxonomic distribution of microbial communities was performed using the "ggplot2" package in R (Al-Masaudi et al. 2017). Variations of taxa by duration of grazing exclusion were evaluated using linear discriminant analysis of effect sizes (LEfSe) (Segata and Huttenhower 2011), which employs Kruskal-Wallis and Wilcoxon rank-abundance tests and then utilizes linear discriminant analysis (LDA) to estimate effect sizes of the features.

A distance-based linear model (distLM) was constructed to examine the relationship among microbial community composition, physicochemical properties, and duration of grazing

exclusion (Anderson and Robinson 2003). Microbial community composition (e.g., bacteria and archaea classified to families and fungi classified to genera) was square-root-transformed and calculated into a lower triangular resemblance matrix on the basis of S17 Bray-Curtis similarity (Clarke and Gorley 2006). The predictor variables (e.g., physicochemical properties) were normalized to fit different types of variables into a common measurement scale matching a normal distribution, between − 2 and 2, and Euclidean distance was calculated (Primer-E Ltd., Plymouth, UK). A selection of predictor variables was performed using the corrected Akaike's information criterion (AIC) and Monte Carlo simulations ($n = 999$ permutations). Distance-based redundancy analysis (dbRDA) was used for visualization (Anderson et al. 2006; Legendre and Anderson 1999). Statistical analyses were also done by using XLSTAT-Ecology (Addinsoft, Belmont, MA) and the PERMANOVA subroutine of Primer 6 (Primer-E Ltd., Plymouth, UK). All statistical analyses were done at $\alpha = 0.05$, with Bonferroni correction for multiple comparisons, where applicable.

Results

Physicochemical properties

When averaged across grazing exclusion durations, OC, TN, AK, AP, exchangeable NH_4^+, C/N ratio, and root biomass were significantly greater in the 0–10-cm soil layer than in the 10–20-cm soil layer ($P < 0.05$). In contrast, NO_3^- and pH were significantly lower in the 0–10-cm soil layer than that in the 10–20 cm ($P < 0.05$). However, TP was similar between two soil depths (Table 2, $P = 0.140$). Duration of grazing exclusion had a significant impact on the physicochemical and plant properties. Some of the nutrient concentrations (TP, AK, and NO_3^-) showed positive and significant correlations with the duration of grazing exclusion ($\rho = 0.385$ to 0.744, $P < 0.003$). In contrast, the concentrations of exchangeable NH_4^+, C/N ratio, and pH were negatively correlated with the duration of grazing exclusion ($\rho = -0.457$ to -0.260, $P < 0.05$). There was no significant difference in root biomass during long-term grazing exclusion ($\rho = 0.188$, $P = 0.154$). The C/N ratio, root biomass, and all other properties were significantly greater in samples with longer durations (≥ 19 years) of grazing exclusion than those with shorter durations (≤ 19 years), with the exception of exchangeable NH_4^+ concentration (Table 2). Furthermore, soil pH was significantly greater after 19 years of exclusion (pH = 8.53 and 8.62 in the 0–10-cm and 10–20-cm soil layer, respectively) than it was for all other grazing exclusion times (8.32–8.53 and 8.35–8.62 in the two layers) (Table 2, $P < 0.001$). Aboveground litter biomass was significantly greater at the

Table 2 Effects of grazing exclusion on soil properties at 0–10-cm and 10–20-cm depth in a semiarid grassland of the Loess Plateau

Layer	0–10 cm					10–20 cm					$P > F$		
Years of exclusion	0 year	14 years	19 years	24 years	29 years	34 years	0 year	14 years	19 years	24 years	29 years	34 years	Layer	Age	Layer × age
[a]TN (g kg^{-1})	1.90 ± 0.03	2.27 ± 0.17	1.01 ± 0.13	1.53 ± 0.14	2.64 ± 0.07	2.61 ± 0.09	1.59 ± 0.05	2.17 ± 0.16	0.87 ± 0.15	1.44 ± 0.04	2.40 ± 0.05	2.54 ± 0.04	*0.012*	*< 0.001*	0.838
TP (g kg^{-1})	0.64 ± 0.02	0.65 ± 0.01	0.59 ± 0.00	0.60 ± 0.03	0.66 ± 0.01	0.76 ± 0.03	0.59 ± 0.02	0.61 ± 0.03	0.58 ± 0.01	0.59 ± 0.02	0.64 ± 0.01	0.74 ± 0.07	0.140	*< 0.001*	0.986
AK (mg kg^{-1})	212 ± 30	265 ± 14	140 ± 27	232 ± 21	280 ± 19	322 ± 21	168 ± 10	155 ± 17	95 ± 8	176 ± 12	164 ± 7	286 ± 25	*< 0.001*	*< 0.001*	0.136
AP (mg kg^{-1})	6.20 ± 0.97	5.37 ± 1.05	3.74 ± 0.70	4.67 ± 1.28	6.05 ± 0.59	6.30 ± 0.39	4.44 ± 0.31	3.81 ± 0.25	3.28 ± 0.24	2.16 ± 0.14	5.03 ± 0.20	6.55 ± 0.38	*0.003*	*< 0.001*	0.348
NO_3^- (mg kg^{-1})	2.95 ± 0.41	8.98 ± 2.33	3.78 ± 1.28	7.87 ± 0.35	12.8 ± 2.1	16.5 ± 1.9	3.56 ± 0.43	10.8 ± 1.8	4.82 ± 0.62	13.4 ± 0.6	14.6 ± 0.9	17.2 ± 1.5	*0.021*	*< 0.001*	0.481
NH_4^+ (mg kg^{-1})	6.34 ± 0.38	5.48 ± 0.58	4.20 ± 0.82	4.04 ± 0.60	5.02 ± 0.89	3.71 ± 0.14	5.52 ± 0.49	5.29 ± 0.94	2.86 ± 0.44	3.25 ± 0.19	3.42 ± 0.17	3.44 ± 0.10	*0.011*	*< 0.001*	0.756
OC (g kg^{-1})	21.4 ± 0.9	24.6 ± 2.1	10.1 ± 1.4	16.1 ± 1.3	27.8 ± 1.3	27.3 ± 1.0	17.5 ± 0.5	22.2 ± 1.5	8.3 ± 1.7	14.8 ± 0.4	24.3 ± 0.5	25.3 ± 0.9	*0.001*	*< 0.001*	0.876
C:N (unitless)	11.3 ± 0.3	10.8 ± 0.2	10.0 ± 0.2	10.6 ± 0.3	10.6 ± 0.3	10.4 ± 0.1	11.0 ± 0.1	10.3 ± 0.2	9.3 ± 0.6	10.3 ± 0.2	10.1 ± 0.2	9.9 ± 0.3	*0.007*	*< 0.001*	0.959
pH (unitless)	8.38 ± 0.03	8.32 ± 0.05	8.53 ± 0.02	8.46 ± 0.04	8.37 ± 0.03	8.33 ± 0.01	8.45 ± 0.04	8.44 ± 0.03	8.62 ± 0.03	8.50 ± 0.01	8.44 ± 0.02	8.36 ± 0.02	*< 0.001*	*< 0.001*	0.716
Root (g m^{-2})	4.28 ± 0.60	4.80 ± 1.37	3.55 ± 0.66	4.63 ± 0.52	5.31 ± 0.41	5.19 ± 0.63	1.35 ± 0.19	1.17 ± 0.22	1.82 ± 0.30	1.32 ± 0.16	1.22 ± 0.23	2.07 ± 0.18	*< 0.001*	0.599	0.419

OC organic carbon, *TN* total nitrogen, *TP* total phosphorus, *AK* available potassium, *AP* available phosphorus, NO_3^- nitrate, NH_4^+ ammonium, *C/N* carbon to nitrogen ratio; and root, root biomass

longest duration (34 years) of grazing exclusion (Table 1, $P < 0.001$).

Microbial community composition

Bacterial/archaeal and fungal communities were characterized by sequencing the V3-V4 hypervariable region of the 16S rRNA gene and the ITS1 region, respectively, from a total of 128 samples. Each sample was normalized to 30,000 and 4300 reads for bacterial/archaeal and fungal community analyses, respectively. These clustered into 2742–3171 and 95–136 OTUs (Tables 3 and 4), and resulted in mean Good's coverage estimates of 97.0 ± 0.3% and 99.5 ± 0.2% (M ± SD) for bacterial/archaeal and fungal communities, respectively. Bacterial and archaeal communities had a mean Shannon diversity index value of 6.86 ± 0.18. Shannon indices were greater in the 0–10-cm soil layer than in the 10–20 cm (Table 3, $P < 0.0001$). The diversity was also greater in samples with longer durations of grazing exclusion than those with shorter durations of grazing exclusion (Table 3, $P < 0.0001$). Fungal communities were much less diverse than bacteria and archaea, with a mean Shannon diversity index of 3.07 ± 0.78. The Shannon indices for fungal communities did not significantly differ by soil depth or by the duration of grazing exclusion (Table 4, $P = 0.210$).

Bacterial and archaeal communities were primarily comprised of members of the phyla *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* (Fig. 2a). The richness and relative abundance of members within the phylum *Acidobacteria* significantly increased when comparing year 0 (without grazing exclusion) and 34 years (long-term grazing exclusion) (Tukey's post hoc test, $P < 0.0001$). Conversely, *Actinobacteria* significantly decreased when comparing at 0 year and 34 years (Tukey's post hoc test, $P < 0.0001$). The relative abundance of *Proteobacteria* was greater in samples from 19-year exclusion than the other exclusion periods. At the family level, members of the *Gaiellaceae* (phylum *Actinobacteria*), *Chitinophagaceae* (phylum *Bacteroidetes*), and *Sphingomonadaceae* (class *Alphaproteobacteria*) were most frequently detected and significantly varied during the grazing exclusion (ANOVA, $P < 0.005$, Fig. 2b). Approximately 50–60% of the bacterial and archaeal microbiota could not be classified to the taxonomic level of family.

There were no significant differences in species richness and the relative abundance of *Acidobacteria*, *Actinobacteria*, and *Proteobacteria* among soil layers (ANOVA, $P = 0.19$–0.51, Fig. 2a). However, the richness and relative abundance of microbiota within the phyla *Nitrospirae*, *Chloroflexi*, and *Gemmatimonadetes* were greater in the lower layer (10–20 cm) than those in the upper layer (0–10 cm) (ANOVA, $P < 0.01$, Fig. 2a). At the family level, *Sphingomonadaceae* and *Chitinophagaceae* were observed to be significantly greater in the upper layer than those in the lower stratum, while *Gemmatimonadetes* was significantly greater in the lower layer (ANOVA, $P < 0.001$, $P < 0.05$, and $P < 0.0003$, respectively, Fig. 2b).

Fungal communities were primarily comprised of members of the classes *Agaricomycetes*, *Saccharomycetes*, *Sodariomycetes*, and *Wallemiomycetes* (Fig. 3a). A decrease in the relative abundance and diversity of *Agaricomycetes* during grazing exclusion period was observed, except at the

Table 3 Coverage and alpha diversity (M ± SD) among bacterial communities by duration of grazing exclusion in different depth of soil based on 16S rRNA gene sequence

Soil layer	Age[a]	Coverage (%)	S_{obs}	SI	NPSI	ACE
0–10 cm	0 year	97.1 ± 0.2	2790 ± 202	6.78 ± 0.21	6.85 ± 0.20	3654 ± 207
	14 years	97.1 ± 0.2	2878 ± 148	6.93 ± 0.08	6.99 ± 0.08	3719 ± 211
	19 years	96.9 ± 0.2	3029 ± 89	6.99 ± 0.04	7.06 ± 0.04	3918 ± 204
	24 years	97.0 ± 0.1	3060 ± 115	6.99 ± 0.11	7.06 ± 0.11	3923 ± 96
	29 years	96.8 ± 0.1	3171 ± 149	7.05 ± 0.10	7.13 ± 0.10	4084 ± 163
	34 years	96.6 ± 0.2	3202 ± 191	6.98 ± 0.16	7.06 ± 0.17	4203 ± 202
10–20 cm	0 year	97.1 ± 0.2	2742 ± 126	6.69 ± 0.12	6.76 ± 0.12	3627 ± 188
	14 years	97.6 ± 0.3	2451 ± 240	6.57 ± 0.17	6.63 ± 0.18	3165 ± 354
	19 years	97.1 ± 0.2	2913 ± 175	6.90 ± 0.19	6.97 ± 0.18	3773 ± 168
	24 years	97.0 ± 0.2	2844 ± 128	6.79 ± 0.08	6.86 ± 0.08	3743 ± 239
	29 years	97.3 ± 0.2	2755 ± 168	6.76 ± 0.09	6.83 ± 0.09	3548 ± 224
	34 years	96.9 ± 0.3	3010 ± 279	6.89 ± 0.14	6.96 ± 0.15	3948 ± 385
$P > F$	Layer	0.0005	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	Age	< 0.0001	< 0.0001	0.0012	0.0009	< 0.0001
	Layer × ager	0.0950	0.1113	0.1277	0.1246	0.0791

S_{obs} number of OTUs observed at 97% similarity, *SI* Shannon index, *NPSI* non-parametric Shannon index, *ACE* abundance-based coverage estimate

[a] Duration of grazing exclusion

Table 4 Coverage and alpha diversity (M ± SD) among fungi communities by duration of grazing exclusion in different depth soils based on ITS1 sequence

Soil layer	Age[a]	Coverage (%)	S_{obs}	SI	NPSI	ACE
0–10 cm	0 year	99.5 ± 0.1	95 ± 15	2.25 ± 0.61	2.27 ± 0.01	112 ± 20
	14 years	99.5 ± 0.2	104 ± 27	2.49 ± 1.44	3.02 ± 0.44	124 ± 26
	19 years	99.4 ± 0.2	110 ± 17	2.99 ± 0.45	3.52 ± 0.26	135 ± 23
	24 years	99.6 ± 0.2	104 ± 29	3.20 ± 0.41	2.85 ± 0.46	117 ± 38
	29 years	99.7 ± 0.1	97 ± 9	3.24 ± 0.31	3.59 ± 0.22	103 ± 11
	34 years	99.6 ± 0.1	116 ± 14	3.50 ± 0.27	3.04 ± 1.43	1.432.52 ± 129 ± 13
10–20 cm	0 year	99.5 ± 0.1	108 ± 8	2.83 ± 0.47	2.52 ± 1.43	124 ± 6
	14 years	99.7 ± 0.1	98 ± 16	3.09 ± 0.58	3.22 ± 0.42	108 ± 19
	19 years	99.4 ± 0.2	136 ± 12	3.56 ± 0.22	3.26 ± 0.31	160 ± 22
	24 years	99.5 ± 0.2	124 ± 14	3.45 ± 0.44	3.10 ± 0.58	142 ± 20
	29 years	99.5 ± 0.2	120 ± 37	3.02 ± 1.43	3.48 ± 0.44	134 ± 38
	34 years	99.4 ± 0.2	133 ± 16	3.29 ± 0.92	3.32 ± 0.91	160 ± 11
$P > F$	Layer	0.0877	0.4663	0.6105	0.6116	0.2794
	Age	0.0260	0.0029	0.0281	0.0277	0.0014
	Layer × Age	0.2005	0.4516	0.9532	0.9517	0.1051

S_{obs} number of OTUs observed at 97% similarity, *SI* Shannon index, *NPSI* non-parametric Shannon index, *ACE* abundance-based coverage estimate

[a] Duration of grazing exclusion

24-year period in the 0–10-cm soil layer, which had the greatest relative abundance (53.9%) and the greatest OTU richness (57). The abundance and richness of *Agaricomycetes* generally decreased with longer durations of grazing exclusion in both soil layers, except for samples collected at 0–10-cm depth after 24 years of exclusion (ANOVA, $P = 0.003$). However, the abundance and richness of *Saccharomycetes* were greater in grazing-excluded soils than in grazed soils (ANOVA, $P = 0.004$). In contrast, *Wallemiomycetes* abundance was not significantly altered during grazing exclusion. Members of the classes *Dothieomycetes*, *Eurotiomycetes*, and *Lecanoromycetes* were found at low relative abundance and diversity (Fig. 3a). The relative abundance of *Eurotiomycetes* increased with grazing exclusion (ANOVA, $P = 0.004$), while the *Dothieomycetes* and *Lecanoromycetes* were not significantly affected by grazing exclusion (ANOVA, $P = 0.35$ and 0.32, respectively). No fungal classes significantly differed in abundance between the upper and lower soil layers (ANOVA, $P > 0.05$, Fig. 3a), while the abundance of fungi predominantly was classified as members of the genus *Hygrocybe* (class *Agaricomycetes*) and was significantly different in abundance following grazing exclusion (ANOVA, $P = 0.002$). However, members of the genus *Mortierella* (class *Mortierellomycotina*) were not affected by exclusion (ANOVA, $P = 0.06$). It should be noted, however, that the majority of fungi (16–49%) could not be classified to

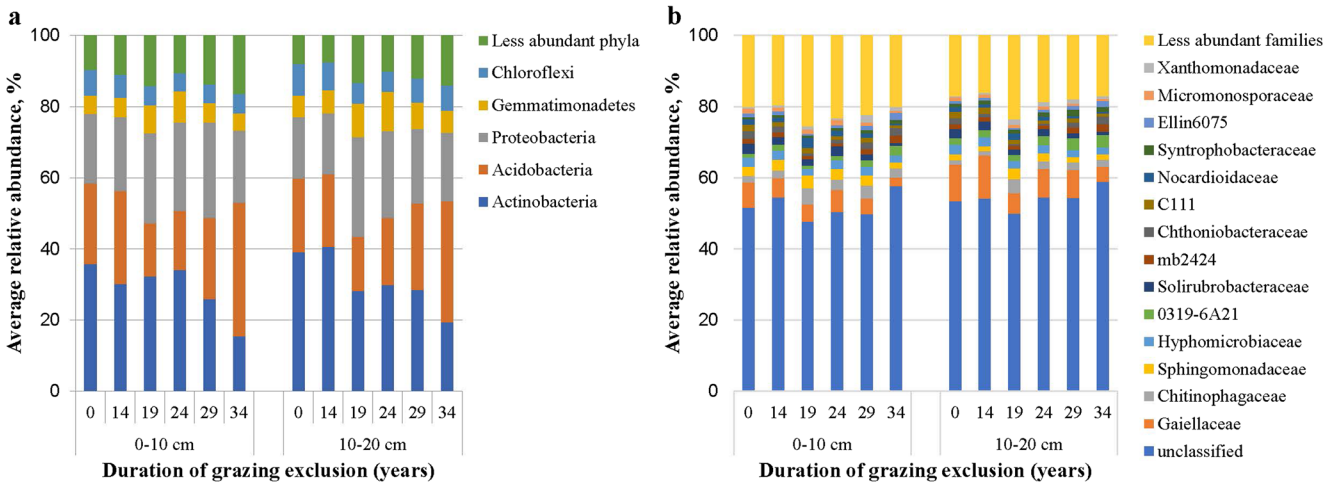

Fig. 2 Relative abundance of bacterial taxa in the soil samples at phylum level (**a**) and at family level (**b**). The *x*-axis represents the upper (0–10 cm) and lower (10–20 cm) soil layers; numbers following soil layer represent the durations of grazing exclusion (0-year, 14-year, 19-year, 24-year, 29-year, and 34-year)

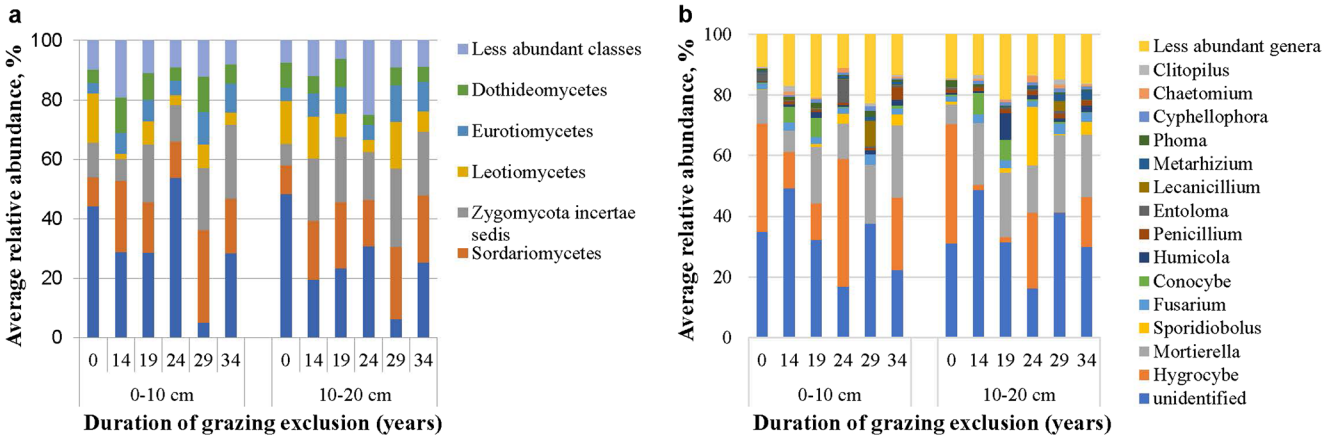

Fig. 3 Relative abundance of fungal taxa in the soil samples at class level (**a**) and at genus level (**b**). The *x*-axis represents the upper (0–10 cm) and lower (10–20 cm) soil layers; numbers following soil layer represent the duration of grazing exclusion (0-year, 14-year, 19-year, 24-year, 29-year, and 34-year)

the genus level (Fig. 3b). None of the fungal classes were significantly different in abundance and diversity between the upper and lower soil layers (ANOVA, $P > 0.05$, Fig. 3a). In addition, the dominant fungal genera (*Hygrocybe* and *Mortierella)* did not significantly differ between the two soil layers. Furthermore, grazing exclusion significantly increased the abundance of *Capronia* (ANOVA, $P < 0.001$) and decreased the abundance of *Cadophora* (ANOVA, $P = 0.004$) in the upper soil layer but did not affect them in the lower layer (Fig. 3b).

The effects of grazing exclusion on the soil microbial community varied with soil depth. Beta diversity of bacterial and archaeal community was significantly and independently affected by grazing exclusion or soil depth ($R = 0.57$, $P < 0.001$ and $R = 0.26$, $P < 0.001$, respectively), as evaluated by analysis of similarity (ANOSIM, Fig. 4a, b). The only pair-wise comparisons that did not show significant differences in community composition were between 0 and 14 years and 14 and 29 years of exclusion (ANOSIM, $R = 0.16$, $P = 0.031$ and $R = 0.22$, 0.013, respectively, at a Bonferroni adjusted $\alpha = 0.003$). Similar to what was found with bacterial and archaeal communities, fungal communities showed differences by duration of grazing exclusion (ANOSIM, $R = 0.47$, $P \leq 0.001$, Fig. 5a, b), but not with soil depth ($R = 0.04$, $P = 0.03$). Phylogenetic differences in bacterial and fungal communities (phylobetadiversity), assessed using the unweighted UniFrac distances, showed significant differences among all years of grazing exclusion ($P < 0.001$, Fig. S1).

Relation of microbial community composition to physicochemical properties

A distance-based linear model was used to identify the physicochemical properties that explained variations in the microbial community composition. Among the 11 physicochemical properties analyzed, litter mass, NO_3^-, root biomass, and OC explained 35% of the observed total variation in the bacterial and archaeal community composition, as determined by dbRDA analysis (Fig. S2a). Based on ordination position, litter mass and nitrate (NO_3^-) were associated with a greater duration of grazing exclusion in influencing the bacterial and archaeal community composition. For fungal communities, OC, TN, NO_3^-, TP, and AK together contributed to 16% of the observed variation (Fig. S2b), with OC, NO_3^-, and TP showing positive associations with greater durations of grazing exclusion in influencing the fungal community.

LEfSe was done to determine which taxa varied with duration of grazing exclusion and soil depth. Several bacterial classes, including *Chloracidobacteria* and *Acidobacteria*-6 (order level), had significantly greater relative abundances in samples collected from the areas with 34 years of grazing exclusion, than did the other samples (Fig. S3a, b). Moreover, these two taxa were consistently seen in both soil layers. The effects of grazing exclusion on archaea and bacteria were greater in the lower than in the upper soil layer (Fig. S3b). The TP and NO_3^- levels were also significantly greater in samples collected from the area with 34 years of grazing exclusion than the other samples (ANOVA, $P < 0.0001$, Table 2) and were significantly positively correlated with the abundances of several genera within these taxa (e.g., *Chloracidobacteria*, Spearman's $\rho = 0.448$, $P < 0.001$ for TP; *Acidobacteria*-6, Spearman's $\rho = 0.476$, $P < 0.001$ for TP and Spearman's $\rho = 0.379$, $P = 0.002$ for NO_3^-, Fig. S4). The relative abundance of *Gemmatimonadetes* was significantly greater in the samples collected from the lower soil layer in the area after 24 years of grazing exclusion than that found in other samples (Fig. S3b). Moreover, the *Gemmatimonadetes* was significantly negatively correlated with AP (Spearman's $\rho = -0.476$, $P < 0.001$, Fig. S4), which was significantly lower due to temporal and spatial differences (Table 2, $P < 0.001$). On the contrary, the LDA score of *Solirubrobacterales* was greater in both soil layers without grazing exclusion than in other samples ($P < 0.001$, Fig. S3a, b). The relative abundance of *Solirubrobacterales* was

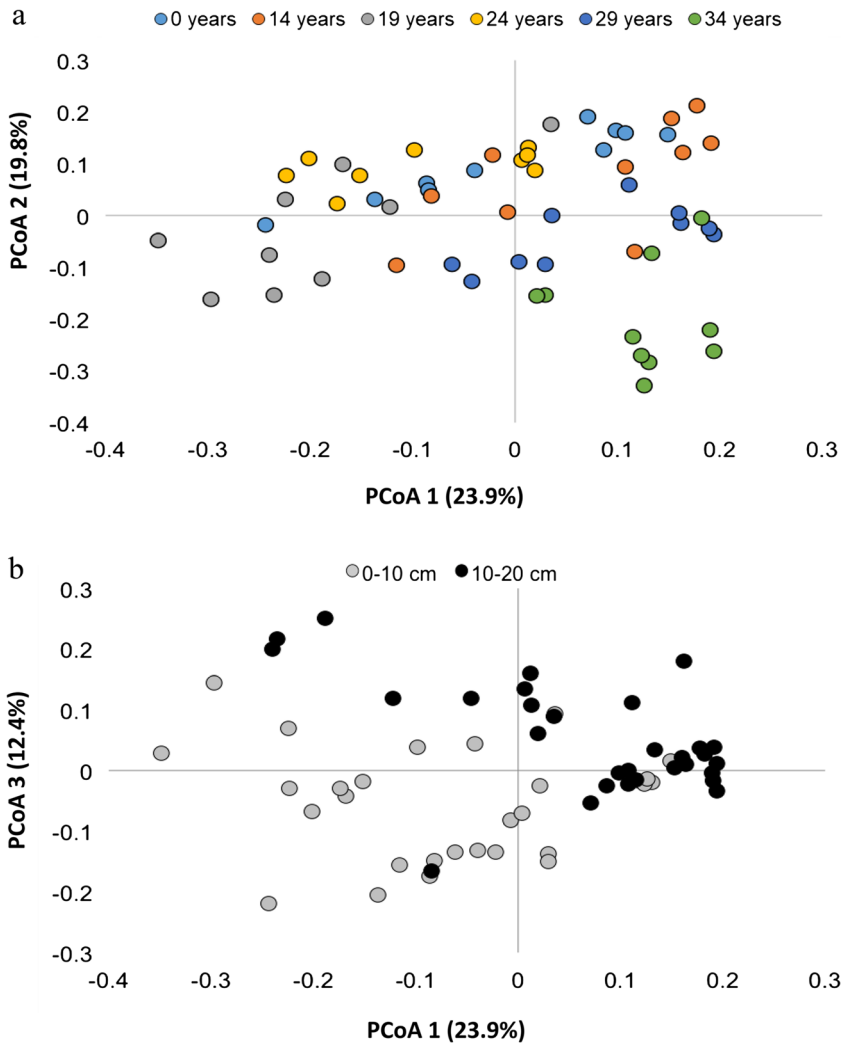

Fig. 4 Principal coordinate analysis (PCoA) of Bray-Curtis similarities among bacterial/archaeal communities ($r^2 = 0.75$) (**a**) and soil depth ($r^2 = 0.62$) (**b**). Each circle of the ordination plot represents the abundance data for each site of collection

significantly and positively correlated with exchangeable NH_4^+ (Spearman's $\rho = 0.572$, $P < 0.0001$) and the C/N ratio (Spearman's $\rho = 0.3$–0.6, $P < 0.02$).

Among the predominant fungal genera, the relative abundance of *Hygrocybe acutoconica* was significantly greater with no grazing exclusion than the other samples, in both layers of soil ($P < 0.0001$, Fig. S3c, d), while the relative abundances of *Mortierella*, and other less abundant fungi, tended to increase significantly during grazing exclusion (Fig. S3c). Similarly, correlations were observed between the abundances of many fungal genera (*Mortierella*, *Humicola*, *Penicillium*, *Lecanicillium*, *Metarhizium*, and *Cyphellophora*) and times of exclusion (Spearman's $\rho =$ 0.299 to 0.617, $P \leq 0.022$), but these correlations were generally weaker than the correlations between the relative abundance of these fungal genera and the nutrient concentrations (Spearman's $\rho = -0.334$ to 0.514, $P < 0.05$). Moreover, variation in fungal genera present with grazing exclusion was greater than bacterial and archaeal genera in both layers of soil (Fig. S3c, d).

Discussion

The use of grazing exclusion as a grassland management strategy has a major impact on grassland ecosystems, improving vegetation cover, litter biomass, and soil fertility (Deng et al. 2014b; Yang et al. 2016). Results of this current study show that long-term grazing exclusion resulted in increased nutrient concentrations, vegetation cover, and litter mass, mainly due to the increased aboveground biomass and organic matter returned to soils (Qiu et al. 2013). Similar studies have also shown that greater duration of exclusion led to greater plant cover and nutrient concentrations when compared between 6 and 2 years of grazing exclusion in grasslands (Pei et al. 2008). Furthermore, several studies have investigated the historical alteration of vegetation cover, above ground biomass, and soil physicochemical properties in grazing grassland (Feurdean et al. 2017; Wang et al. 2017; Wiesmair et al. 2017). Despite this initial information; however, the impact of temporal variation in grazing exclusion (up to 34 years), coupled with spatial variation (vertical distribution), of

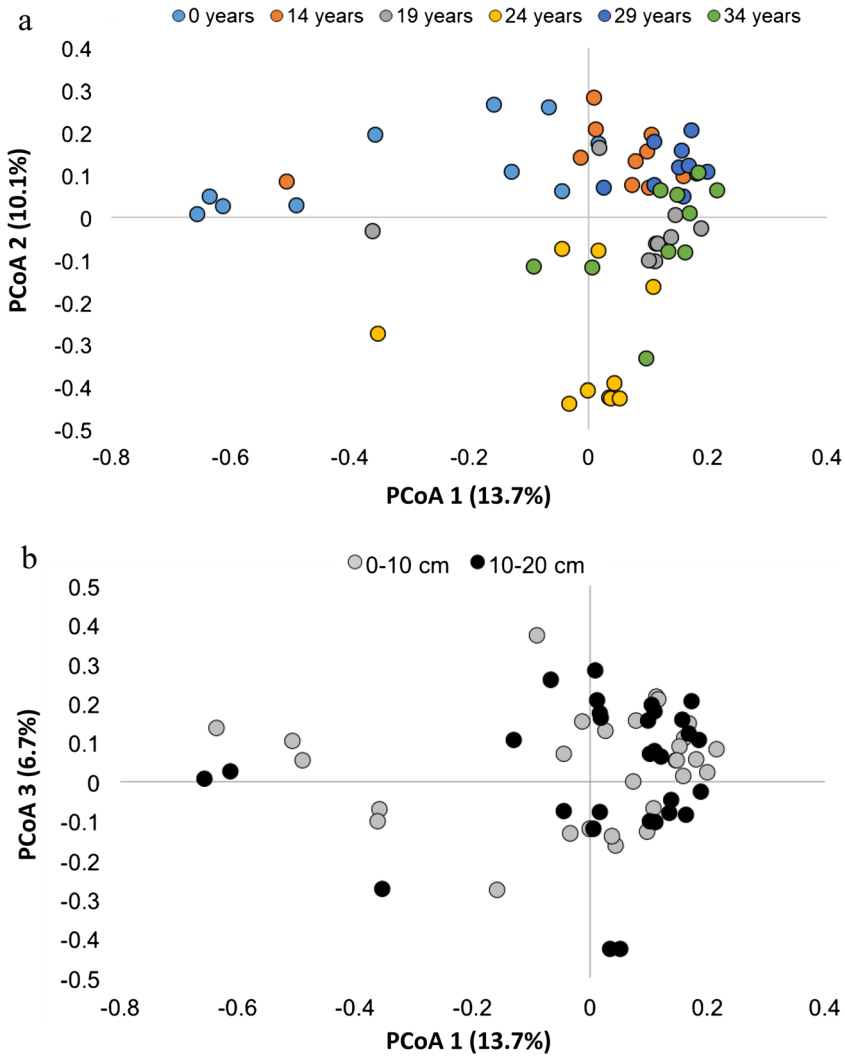

Fig. 5 Principal coordinate analysis (PCoA) of Bray-Curtis similarities among fungal community ($r^2 = 0.38$) (**a**) and in soil depths ($r^2 = 0.35$) (**b**). Each circle of the ordination plot represents the abundance data for each site of collection

microbial community composition associated with grazing exclusion remains less well understood. It was previously reported that grazing exclusion for 7 years resulted in a decline in microbial activity, due to a decrease in C and N cycling (Medina-Roldán et al. 2012). Similar to our findings, a previous study comparing 33 years of grazing exclusion and grazed grassland in the same site as this study showed that grazing exclusion increased soil nutrients and bacterial diversity (Cheng et al. 2016). However, there is currently a paucity of evidence regarding temporal and spatial changes in the composition and diversity of soil microbial communities (both bacterial and fungal) due to the greater species richness and microscale variation in these environments (Robertson et al. 1997; Blackwood et al. 2006; Schmidt and Waldron 2015).

Since microorganisms are essential components in providing ecosystem services and play critical roles in driving biogeochemical cycles (Falkowski et al. 2008), it is important to characterize microbial communities during grazing exclusion, coupled with spatial distribution in grasslands. The results from distance-based linear modeling showed that both NO_3^- and litter mass were key variables linked to microbial community composition. Changes in nutrient concentrations were hypothesized to impact specific members of the microbial community associated with nutrient cycling. Similar to previous studies (Jones et al. 2009; Lauber et al. 2009), we noted a significant negative correlation between *Acidobacteria* and pH (Spearman's $\rho = -0.675$, $P < 0.0001$) (Fig. S4) during grazing exclusion. The *Acidobacteria* have been reported to use NH_4^+ as a preferred N source (Kielak et al. 2016), suggesting that shifts in nutrient concentrations may drive increases in abundance of this group, and vice versa. Similarly, the *Nitrososphaeraceae* had significantly greater relative abundance in samples collected at 19 years of grazing exclusion than in the other samples, and the concentration of exchangeable NH_4^+ was also significantly lower in these samples (ANOVA, $P < 0.001$, Fig. 2b). Members of this family are known to oxidize ammonium (i.e., ammonia oxidizing archaea) and therefore may play an important role in N cycling (Tourna et al. 2011). The *Gemmatimonadetes*, which had been reported to be associated with biological phosphate removal

(Fawaz 2013; Zhang et al. 2003), were also observed to shift with changes in AP during grazing exclusion.

Heterotrophic members of the microbial community also shifted in response to changes in soil nutrient concentrations. Members of the family *Chthoniobacteraceae*, which are associated with the C cycle in soil (Leschine and Canale-Parola 1989; Sangwan et al. 2004; Thomas et al. 2011), were present in significantly greater relative abundances in samples collected from the area with 34 years of grazing exclusion (ANOVA, $P < 0.001$), which is consistent with our observation that the relative abundance and diversity of *Chthoniobacteraceae* were positively correlated with soil OC (Spearman $\rho = 0.569$ and 0.569, $P < 0.0001$, respectively, Fig. S4). Shifts in the abundance of other common soil bacteria (*Chitinophagaceae* and *Sphingomonadaceae*) may reflect changes in the soil physical environment including, for example, increased oxygen availability as result of removal of animal trampling and decreased soil bulk density, and increased OC availability associated with greater litter mass.

Moreover, archaeal and bacterial community composition varied significantly with soil depth together with grazing exclusion (Fig. S3a, b). Variation in the abundances of *Gaiellales*, Gitt-GS-136, MB-A2-108, *Acidimicrobiales*, and MND1 observed in soil depths, coupled with grazing exclusion, significantly correlated with an increase in pH in the deeper layer (Spearman's, $P < 0.05$). However, while pH has been reported to affect on the abundance and composition of soil microbial communities (Rousk et al. 2010), exchangeable NH_4^+ was strongly related to the abundance of ammonia-oxidizers (e.g., ammonia-oxidizing archaea) in soil. Previous studies showed that ammonia-oxidizing archaea were dominant in soils with low ammonia (Norman and Barrett 2014; Yang et al. 2017). Other physicochemical and plant factors are also likely to covary, which would influence the composition and diversity of microbial communities. Our data revealed that the lower soil layer contained significantly greater NO_3^- and lower AP than did the upper layer. While these shifts in nutrient availability likely influence the abundance and composition of microbial communities themselves, they also influence plants, which in turn affect the microbiota by providing microenvironments and nutrients for growth and survival (Denton et al. 1998; Haichar et al. 2008). Not surprisingly, grazing exclusion also contributed to changes in microbial community composition between the surface layer (0–10 cm) and lower layers (10–20 cm) soil profiles. Animal traffic (e.g., sheep or cow) highly reduces soil aeration in the surface layer of soil, while hardly affecting the oxygen profile in the lower layer (Donkor et al. 2002). Furthermore, degradation of animal feces following grazing exclusion could also explain the high concentration of nutrients observed in the surface layer.

Similar to the bacteria and archaea, the abundance and composition of fungal communities were also influenced by grazing exclusion and soil depth. However, fungi were poorly classified at lower taxonomic levels (e.g., genus and species), complicating more sophisticated analyses of potential responses to nutrient availability. This was in part due to underrepresentation of many soil fungi in databases, as well as the short sequence reads obtained using NGS technology. Of note, the distance-based linear model indicated that five nutrient properties explained a moderate percentage (16%) of observed total variation in the abundance and composition of fungal communities, suggesting a distinct species sorting response to changes in nutrient concentrations among the community as the result of grazing exclusion (Leibold et al. 2004; Staley et al. 2015). Similar to the current study, it was previously reported that there was an increase in fungal diversity following grazing exclusion (Zhao et al. 2017), and compositional shifts in the community depended significantly on the covering plant species (Guo et al. 2016). Future directed studies, including the culture of fungal species, are required to better characterize the response of these communities to grazing and grazing exclusion.

Conclusion

The data presented here provide important and novel information regarding microbial community composition at different soil depths following long-term grazing exclusion. Our results demonstrate that long-term grazing exclusion results in increased nutrient concentrations, improved litter mass, and increased diversity within the bacterial, archaeal, and fungal communities. Shifts in specific families of bacteria and archaea related to nitrogen cycling corresponded to similar shifts in NH_4^+ concentration, suggesting that soil microbiota drive soil improvements by converting NH_4^+ to biologically available NO_3^-. Shifts in composition of fungal communities also changed in conjunction with changes in nutrient availability and bacterial community composition, although further study is required to elucidate the dynamics of the interactions among these factors. Moreover, we observed large shifts in the composition of archaeal and bacterial communities in different soil depths coupled with grazing exclusion, which may suggest that microbial communities located in the subsurface could be used to distinguish the temporal variation of grazing exclusion.

Acknowledgements Sequence processing and analyses were performed, in part, using the resources of the Minnesota Supercomputing Institute.

Funding information This study was finally supported by the National Key Research Program of China (2016YFC0500700), the National Natural Science Foundation of China (41622105, 41571130082, and 41571296) and the Programs from Chinese Academy of Sciences (QYZDB-SSW-DQC039), and Northwest A&F University (2452017028).

Abbreviations ACE, abundance-based coverage estimate; AIC, Akaike's information criterion; ANOSIM, analysis of similarity; AK, available potassium; AP, available phosphorus; dbRDA, distance-based redundancy analysis; distLM, distance-based linear model; ITS 1, internal transcribed spacer region 1; LDA, linear discriminant analysis; NH_4^+, ammonium; NO_3^-, nitrate; OTU, operational taxonomic unit; OC, organic carbon; PCoA, principal coordination analysis; TN, total nitrogen; TP, total phosphorus

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