RESEARCH ARTICLE

Soil bacterial community responses to warming and grazing in a Tibetan alpine meadow

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One sentence summary: The present work is the first to isolate and describe IncP-1μ plasmids in China, it greatly expands their available collection and proposes the striking two phylogenetic subclades within IncP-1μ group.

ABSTRACT

Warming and grazing significantly affect the structure and function of an alpine meadow ecosystem. Yet, the responses of soil microbes to these disturbances are not well understood. Controlled asymmetrical warming (+1.2/1.7 °C during daytime/nighttime) with grazing experiments were conducted to study microbial response to warming, grazing and their interactions. Significant interactive effects of warming and grazing were observed on soil bacterial α-diversity and composition. Warming only caused significant increase in bacterial α-diversity under no-grazing conditions. Grazing induced no substantial differences in bacterial α-diversity and composition irrespective of warming. Warming, regardless of grazing, caused a significant increase in soil bacterial community similarity across space, but grazing only induced significant increases under no-warming conditions. The positive effects of warming on bacterial α-diversity and grazing on community similarity were weakened by grazing and warming, respectively. Soil and plant variables explained well the variations in microbial communities, indicating that changes in soil and plant properties may primarily regulate soil microbial responses to warming in this alpine meadow. The results suggest that bacterial communities may become more similar across space in a future, warmed climate and moderate grazing may potentially offset, at least partially, the effects of global warming on the soil microbial diversity.

Keywords: Tibetan alpine meadow; warming; grazing; interaction; bacterial diversity; bacterial composition
INTRODUCTION

Climate warming is unequivocal and significantly affects the soil microbiome and its biodiversity in terrestrial ecosystems, and plays a critical role in carbon, nitrogen and other nutrient cycles (Sala et al. 2000; Falkowski, Fenchel and Delong 2008; Zhou et al. 2011). Soil temperature and water content, which exert influence over soil microbes, can be directly affected by warming. Temperature directly influences microbial composition and diversity (Schindlbacher et al. 2011; Sheik et al. 2011) and these effects also vary with soil water content (Davidson and Janssens 2006; Sheik et al. 2011). In addition, substantial changes have been observed in grassland above-ground plant composition and biomass due to experimental warming (Zhou et al. 2011; Wang et al. 2012), changes that are also likely to affect the soil microbiome (Bardgett, Freeman and Ostle 2008; el Zahar Haichar et al. 2008). However, due to the complexity of soil microbiome, many questions remain about their responses to climate warming, especially in the Tibetan Plateau.

As the Earth’s largest and highest plateau, the Tibetan Plateau has proven particularly vulnerable to the effects of climate change, and the rise in temperature in this region in the past 50 years is approximately three times the average global warming rate (Qiu 2008). More than 50 million sheep and 13.3 million domestic yaks graze on grasslands of the Tibetan Plateau (Yao et al. 2006), which in combination with the rising temperatures imposes non-negligible disturbance on the soil microbiome and carbon cycles. Because of the large amount of soil carbon contained in this region (representing 23% of China’s total organic soil stored carbon and 2% of the global pool of soil carbon (Wang et al. 2002)), a slight shift in the soil carbon pool would provide a strong feedback to global atmospheric CO$_2$ concentrations, and consequently to global warming. Thus, the response of the soil microbial community to warming and grazing in Tibetan grasslands should be considered in predicting feedbacks among future climate change, the carbon cycle, and ecosystem function on the Tibetan Plateau.

As a major biotic factor influencing Tibetan grassland ecosystems, livestock grazing significantly changes soil geochemical properties and above-ground vegetation, thereby affecting the soil microbial community (Luo et al. 2009; Wang et al. 2012; Yang et al. 2013). Substantial effects of grazing on microbial composition and diversity have been observed (e.g., Yang et al. 2013). Moreover, the opposite effects of grazing and warming on plant and microbial composition were found (Zhou et al. 2011; Wang et al. 2012; Yang et al. 2013). In our study site, warming significantly increased the above-ground biomass and the coverage of graminoid and legume species, but reduced non-legume forb coverage in the plant community, while opposite results were found for the effects of grazing (Wang et al. 2012). The microbes functioning in labile carbon degradation and nitrogen-cycling (denitrification, nitrogen fixation, nitrification, nitrogen mineralization) were potentially increased by warming (Zhou et al. 2011). In contrast, the microbes functioning in soil organic matter degradation and nitrogen-cycling are potentially inhibited by grazing, except for those active in nitrification (Yang et al. 2013). These observations highlight the fact that microbial response to warming and grazing is difficult to understand without knowledge of their potential interactive effects.

Here we conducted a controlled warming-grazing experiment (i.e., no-warming with no-grazing (C), warming with no-grazing (W), no-warming with grazing (G) and warming with grazing (WG)) using the free-air temperature enhancement (FATE) system at the Haibei Alpine Meadow Ecosystem Research Station (HBAMERS) on the Tibetan Plateau from 2006 to study the effects of warming, grazing and their interactions on the soil microbial community. Our previous results found that warming significantly increased soil respiration, CH$_4$ uptake, and the litter degradation rate, and altered plant composition; whereas grazing had little effects and even contrary effects on these factors (Luo et al. 2010; Lin et al. 2011, 2015; Wang et al. 2012). Moreover, a significant interaction between warming and grazing was found on plant diversity, soil nitrogen concentration, carbon/nitrogen ratio, total extractable organic phosphate and CH$_4$ uptake (Luo et al. 2010; Lin et al. 2011; Rui et al. 2012; Wang et al. 2012). In this study, 454 pyrosequencing was used to gain insight into how soil microbial communities respond to 3-year warming and grazing in this alpine meadow. Based on previous observations, we hypothesized that (i) soil bacterial composition and diversity would be changed by warming and grazing; (ii) changes in the soil bacterial community are controlled by a few environmental factors; (iii) the effects of warming and grazing on soil bacteria are not additive, and the impacts of warming on soil bacteria would be modified by grazing and vice versa.

MATERIALS AND METHODS

Experimental site

The experimental site is located at the HBAMERS (37°37’N, 101°12’E). The station lies in the northeast of the Tibetan Plateau in a large valley surrounded by the Qilian Mountains; the mean elevation of the valley bottom is 3200 m. The station experiences a typical plateau continental climate, dominated by the southeast monsoon from May to September in summer and high pressure from Siberia in winter. Summers are short and cool, and winters are long and severely cold. The mean annual temperature is −2°C, and mean annual precipitation is 500 mm, over 80% of which falls during the summer monsoon season.

Aboveground vegetation at the experimental site is dominated by Kobresia humilis, Festuca ovina, Elymus nutans, Poa pratensis, Carex scabrirostris, Scirpus distigma dicus, Gentiana straminea, G. farrei, Blysmus sinocompressus and Potentilla nivea. A detailed site description can be found in Zhao and Zhou (1999).

Controlled warming-grazing experiment

The design of the controlled warming (i.e., FATE system with infrared heaters) with grazing experiment was described previously by Luo et al. (2010). Briefly, in May 2006, eight hexagonal arrays of Mor FTE (1000W, 240V; Mor Electric Heating Association, Comstock Park, Michigan, USA) infrared heaters were deployed over a vegetation canopy that had previously been heavily grazed by sheep during cool seasons from October to May of prior years at the HBAMERS, with eight dummy arrays over reference plots. The heaters were controlled using the proportional-integral-derivative-outputs control system so as to ensure constant warming between heated and reference plots. The set point differences of the vegetation canopy between heated and corresponding reference plots were 1.2°C during the daytime and 1.7°C at night in summer, which falls within the limits of predicted temperature increases for this century (1.5–5°C) (Houghton et al. 2001). A two-way factorial design (warming and grazing) was used with four replicates of each of four treatments: no-warming with no-grazing (C), no-warming with grazing (G), warming with no-grazing (W) and warming with grazing (WG). In total, 16 plots of 3 m diameter were fully randomized throughout the study site.
All experimental sheep were fenced in three additional 5 × 5 m fenced plots for one day before the beginning of the grazing experiment to help them adapt to small plots. The canopy height of the vegetation was measured at 50 points within the plots before and after grazing, and the sheep were removed from the grazing plots when the canopy height was reduced to approximately half of the initial height. Initially, one adult Tibetan sheep was fenced in each of the grazing plots on the morning of 15 August 2006 for approximately 2 h. The canopy height was about 8–9 and 4–5 cm before and after grazing, respectively. Two adult Tibetan sheep were fenced for approximately 1 h in each of the grazing plots on the mornings of 12 July, 3 August and 12 September in 2007, 8 July and 20 August in 2008 and 9 July in 2009. The canopy heights were about 6–7 and 3–4 cm before and after grazing, respectively. A 50 × 50 cm cage was set up inside each plot for each grazing event. The forage utilization rate was calculated using the difference between biomass present inside and outside the cage after each grazing event. The annual cumulative forage utilization rates during the growing seasons were 32%, 44% and 61% for the G treatment, and 32%, 50%, and 56% for the WG treatment in 2006, 2007 and 2008, respectively.

**Soil sampling**

Soil samples were collected on 3 August in 2009 after 3 years of experimental warming. In each plot, five 1.5 cm diameter soil cores of 0–20 cm depth were sampled on a grid basis, and composited and sieved through a 2 mm mesh to remove apparent roots and stones. Then soil samples were stored at –80°C until analysis.

**Soil and vegetation property measurements**

Soil temperatures at depths of 0, 5, 10 and 20 cm and soil moisture at depths of 10 and 20 cm were measured. A detailed description of the method can be found in Wang et al. (2012).

Soil physical and chemical attributes were measured using the method described by Rui et al. (2011). Briefly, total organic carbon (TOC) was measured using a TOC-5000A analyzer (Shimadzu Corp., Kyoto, Japan); total nitrogen (TN) of the soil samples was measured using a Vario EL III Elemental Analyzer (Elementar, Hanau, Germany); and total phosphate (TP) was determined using nitric acid (HNO₃)-perchloric acid (HClO₄) digestion. To measure NH₄⁺-N and NO₃⁻-N, 10 g dry weight of soil samples was suspended in a 50 ml of 2M KCL solution. After shaking at room temperature for 1 h and subsequently standing for 30 min, the supernatant was filtered through a filter paper of 30–50 μm pore size. NH₄⁺-N and NO₃⁻-N were determined using a FIAstar 5000 Analyzer (FOSS, Hillerd, Danmark). To measure vegetation variables, a quadrat in the site was selected. Vegetation species, density, abundance and average height were recorded following procedures described in Wang et al. (2012). Then vegetation was mowed and immediately weighed to provide biomass data. Vegetation diversity was measured using species richness (SR) and the Shannon diversity (SW) index.

**DNA extraction, PCR and DNA sequencing**

DNA was extracted from 0.5 g of soil using a FastDNA spin kit for soil (MP Biomedical, Carlsbad, CA, USA) following the manufacturer’s instructions. DNA quality assessment and quantification was conducted using a Nano-Drop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Then, the DNA extractions were diluted to 10 ng/μl and stored at −80°C. For pyrosequencing analysis, the extracted DNA was amplified with bacterial specific forward 515F (5'-Fusion A-Barcode-CA linker-GTGGYCAGCMGCGCGGTAT-3') and reverse 909R (5'-Fusion B-TC linker-CCCCGGAATTCTTATTAGT-3') primers as described previously (Wang and Qian 2009), which targets the region V4 of the 16S rRNA. Sequences in the V4 region provide comprehensive coverage (Sul et al. 2011) and give results that are among the highest for taxonomical accuracy (Wang et al. 2007). Amplification reactions were performed as previously described (Wang and Qian 2009). Briefly, the 50-μl amplification mix contained 1× buffer, 0.2 μM of each primer, 1.5 mM MgCl₂, 300 ng/μl BSA, 10 ng of template and 1 units of the Pfu polymerase (BioVision, Mountain View, CA, USA). Amplification was initiated for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 45 s, primer annealing at 56°C for 45 s, extension at 72°C for 1 min and final extension for 10 min. Reactions, performed in triplicate, were combined and purified using gel electrophoresis followed by the QIAquick gel extraction kit and the Qiagen PCR purification kit. High-throughput sequencing was performed with the 454 GS FLX Sequencer (454 Life Sciences) at Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign.

**Sequence analysis**

All sequence processing and diversity estimates were performed using the QIME (Caporaso et al. 2010). We followed the recommendations by Huse et al. (2007). In brief, sequences were discarded if they contained ambiguous base calls, were less than 380 nt or more than 450 nt in length, or if they contained more than 20 homopolymers. A chimera check was performed with QIME via ChimeraSlayer. We then assigned sequences to soil samples based on their barcodes. There were four replicate datasets for each treatment (i.e., C, W, G and WG). Pairwise distances between sequences were calculated using the furthest neighbor algorithm, and OTUs were delineated at 97% sequence similarity. The singleton OTUs (with only one read) were removed, and the remaining sequences were sorted into each sample based on OTU (Zhou et al. 2011). The most abundant sequence from each OTU was selected as a representative sequence for that OTU. Taxonomy was assigned to bacterial OTUs using the Basic Local Alignment Search Tool (BLAST) for each representative sequence against a subset of the Silva database. The sequence data are available on the metagenomics RAST server (http://metagenomics.nmpdr.org) (Meyer et al. 2008) through accession number 4624255.3-4624266.3.

**Data analysis**

Data analysis was conducted using the packages vegan (Dixon 2003) and picante (Kembel et al. 2010) with the statistical platform R. Because of unequal numbers of sequences among soil cores, samples were rarefied to 2100 sequences and samples with fewer than 2100 sequences were not included in the analysis. Samples from plot 1 (W), 2 (G), 3 (C) and 4 (WG) met this criterion and were excluded. It has been reported that 2000 denoised sequences per sample can explain more than 80% and 95% of the trends in α- and β-diversity, respectively, among samples observed for 15 000–20 000 bacterial sequences (Lundin et al. 2012). Thus, the rarified datasets should be acceptable when sampling to 2100 denoised sequences. Rarefaction was repeated 30 times, and each subsequent analysis was based on the means of the 30 random trials. Bacterial α-diversity was calculated using the SW, SR and Pielou’s evenness. Bacterial β-diversity was estimated as the average pairwise community dissimilarity within each
treatment using Bray–Curtis distance matrices (Rodrigues et al. 2013). The distribution of OTUs across soil cores were calculated as the number of soil cores that the OTUs averagely distributed in (Rodrigues et al. 2013). For each OTU, its distribution is the number of soil cores that it was detected. Multiple comparisons of the relative abundance of bacterial phyla, \( \alpha \)-diversity and \( \beta \)-diversity among the four treatments were performed using Tukey’s HSD test. Two-way analysis of variance (ANOVA) was used to test the effects of warming, grazing and their interaction on bacterial diversity.

Non-metric multidimensional scaling (NMDS) and non-parametric multivariate analysis of variance (ADONIS) (Anderson 2001) were used to test the differences in overall community composition among treatments. A total of 14 plant and soil variables were analyzed to evaluate possible linkages between bacteria and soil and vegetation variables (Table S1, Supporting Information). Stepwise regression analysis was performed to find the important factors influencing bacterial \( \alpha \)- and \( \beta \)-diversity. For bacterial composition, the most meaningful variables were selected based on the Bio-Env procedure and variance inflation factors (VIF < 20) with 999 Monte Carlo permutations, as well as Mantel test and biology (Zhou et al. 2011). Finally, eight variables were selected and divided into groups of variables based on soil (pH, TOC, TN and TP), plants (plant SR and below-ground biomass) and soil temperature and moisture (Tm&MS). The selected variables were fitted as vectors onto the NMDS ordination graphics to elucidate interrelationships among vegetation, soil variables, and the bacterial community. To better understand how much each environmental variable influences the functional community structure, variation partitioning analysis (VPA) (Ramette and Tiedje 2007) was performed using the selected variables.

RESULTS

Effects of warming and grazing on soil physicochemical and plant properties

There were no significant changes in soil pH, moisture, TOC, TN, C/N, TP, NH\(_4\)\(^+\)-N, NO\(_3\)\(^-\)-N, plant total coverage or below-ground biomass caused by warming, grazing and their interaction (Table S1, Supporting Information). Warming alone caused a significant increase in soil temperature by 10%, but induced a significant decrease in plant SR and plant SW by 13% and 4%, respectively (Table S1, Supporting Information). Significant interactive effects between warming and grazing were found on plant height and above-ground biomass (Table S1, Supporting Information). Warming caused a significant increase in plant height by 30% in no-grazing plots, but did not have significant effects in grazing plots. Grazing caused a significant decrease in plant height by 19% and 31% in no-warming and warming plots, respectively. A significant increase in plant above-ground biomass by 29% was found caused by warming in no-grazing plots, but not in grazing plots. Grazing induced a significant decrease in plant above-ground biomass by 20% in no-warming plots and by 29% in warming plots.

Effects of warming and grazing on bacterial community diversity

Significant interactive effects between warming and grazing were found on bacterial \( \alpha \)-diversity (i.e., SW, SR and Pielou’s evenness) (Table 1). Warming caused a significant increase in the bacterial SW, SR and Pielou’s evenness by 2%, 1% and 6% (\( P < 0.05 \)), respectively, in no-grazing plots but did not cause significant changes in these indices in grazing plots (Fig. 1). Grazing regardless of warming did not induce significant changes in bacterial \( \alpha \)-diversity (Fig. 1). The response of bacterial \( \alpha \)-diversity to warming and grazing were found to be significantly correlated with a few soil (e.g., pH, TOC, NH\(_4\)\(^+\)-N and NO\(_3\)\(^-\)-N) and plant (plant SR) factors, which explained more than 70% of the variation in bacterial \( \alpha \)-diversity (Table 2).

No significant interactive effects between warming and grazing on bacterial \( \beta \)-diversity were found (\( P = 0.14 \)) (Table 1). Warming alone caused a significant decrease in bacterial \( \beta \)-diversity by 10% (\( P < 0.01 \)) and grazing alone tended to decrease it by 5% (\( P = 0.07 \)) (Table 1 and Fig. 1). Soil TOC and plant SR were found to be important factors influencing bacterial \( \beta \)-diversity, which explained 15% of the variation in bacterial \( \beta \)-diversity (Table 2).

Effects of warming and grazing on bacterial community composition

The soil bacterial community in this alpine meadow mostly consisted of Actinobacteria followed in decreasing order of relative abundance by Proteobacteria, Acidobacteria, Chloroflexi, Planctomycetes and Bacteroidetes (Fig. 2). There were significant interactive effects between warming and grazing on the composition of overall and rare bacterial species (i.e., relative abundance <0.1%) (Table 1), although differences among the four treatments were not significant (Table S2, Supporting Information). NMDS analysis also did not show a clear distinction among the four treatments (Fig. 3A). At the phylum level, significant interactive effects between warming and grazing on the relative abundance of Actinobacteria and Nitrospirae were found (Table 1). Warming caused a substantial increase in the relative abundance of Actinobacteria by 15% and decrease in the relative abundance of Nitrospirae by 29% in grazing plots, but not in the no-grazing plots (Fig. 2). Grazing caused a significant increase in the relative abundance of Actinobacteria by 12% in warming plots, but did not result in significant changes in no-grazing plots (Fig. 2). A significant increase in the relative abundance of Nitrospirae by 87% was induced by grazing in no-warming plots, but not in warming plots (Fig. 2). There was no significant interactive effect between warming and grazing on the relative abundance of the other bacterial phyla (Table 1). Grazing alone induced a

<table>
<thead>
<tr>
<th></th>
<th>W</th>
<th>G</th>
<th>W×G</th>
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<tbody>
<tr>
<td>SW</td>
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<tr>
<td>SR</td>
<td>0.17</td>
<td>0.22</td>
<td>0.02</td>
</tr>
<tr>
<td>Pielou’s evenness</td>
<td>0.14</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>( \beta )-diversity</td>
<td>&lt;0.01</td>
<td>0.07</td>
<td>0.14</td>
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<td>Overall bacterial composition</td>
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<tr>
<td>Rare species composition</td>
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<tr>
<td>Gemmatimonadetes</td>
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<td>0.85</td>
<td>0.06</td>
</tr>
<tr>
<td>Nitrospirae</td>
<td>0.05</td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
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</table>

Table 1 Significance tests of the treatment effects on bacterial community diversity, overall community and rare species (i.e. relative abundance <0.1%) composition and phyla groups using warming (W) and grazing (G) as main factors. Bacterial \( \beta \)-diversity was estimated as the average pairwise community dissimilarity within each treatment. Only significantly shifted phyla groups are shown. \( P \) values indicate the statistical significance. Bold values represent significant effects (\( P < 0.05 \)) of treatments.
Figure 1. Bacterial SW (log transformed data), SR, Pielou’s evenness and bacterial community similarity for no-warming with no-grazing (C), warming with no-grazing (W), no-warming with grazing (G) and warming with grazing (WG) treatment. Different letters indicate significant difference at 0.05 level. Error bars represent standard error (n = 3).

Table 2. Stepwise regression analysis of factors influencing bacterial SW, SR, Pielou’s evenness and β-diversity. Beta value is standardized regression coefficient, which shows the rate of change in the dependent variable brought about by each independent variable. Bold values represent significant correlation (P < 0.05).

<table>
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<th>Beta</th>
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<tr>
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<tr>
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<tr>
<td>Plant SR</td>
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</table>

significant decrease in the relative abundance of Bacteroidetes by 17% (Fig. 2).

At the 97% identity level, the resampled 25 200 sequences were distributed into 2214 OTUs. A total of 75% of the OTUs (85% of reads) were found shared in no-warming (C and G) and warming (W and WG) treatments (Fig. 4A). The OTUs unique for no-warming treatments and warming treatments accounted for 13% (8% of reads) and 12% (8% of reads) of the overall OTUs, respectively (Fig. 4A). To find out the reason for significant increased β-diversity under warming conditions, the distribution ranges of these OTUs were analyzed. Warming caused a significant increase in the distribution ranges of the shared OTUs by 10% (P < 0.05) (Fig. 4B). In addition, the ranges of the OTUs unique to warming treatments were found to be significantly higher by 13% than that of the OTUs unique to no-warming treatments (Fig. 4C). The top two most abundant OTUs detected in the treatments were affiliated with the genus Solirubrobacter (order Solirubrobacterales, phylum Actinobacteria) and Bradyrhizobium (order Rhizobiales, class Alphaproteobacteria) (Fig. S1, Supporting Information). There were no significant effects of warming or grazing on the relative abundance of the top 10 most abundant OTUs (Fig. S1, Supporting Information).

Table 2. Stepwise regression analysis of factors influencing bacterial SW, SR, Pielou’s evenness and β-diversity. Beta value is standardized regression coefficient, which shows the rate of change in the dependent variable brought about by each independent variable. Bold values represent significant correlation (P < 0.05).

Relationship between shift in bacterial community composition and environmental variables

A total of 14 plant and soil variables were measured in this study (Table S1, Supporting Information). Based on forward selection, four soil chemical variables (pH, TOC, TN, TP), two
vegetation variables (plant SR and below-ground biomass) and two soil physical variables (soil temperature and moisture) were selected to elucidate interrelationships among environmental variables and bacterial community. NMDS analysis showed that soil chemical variables and moisture were significantly related to bacterial community composition (Fig. 3A), which was also supported by the Mantel test (Table 3). A total of 73% of the community variations could be explained by these selected variables (Fig. 3B). Soil chemical variables, plant and soil physical variables contributed to 35%, 18% and 18% of the total variance, respectively (Fig. 3B).

**DISCUSSION**

The bacterial composition in this alpine meadow appears to be resistant to warming or require a longer time to exhibit a response, which is consistent with the unchanged composition of the methanotrophic community in this meadow under warming conditions (Zheng et al. 2012). It has been found that warming-induced changes in soil edaphic and plant properties rather than the temperature increase itself may primarily regulate soil microbial response to warming (Zhang et al. 2005; Rinnan et al. 2007; Rui et al. 2015). Thus, the response pattern of soil microbes to warming might be different among ecosystems with different soil and above-ground vegetation properties. Rapid responses of microbial composition to experimental warming have been frequently found previously (reviewed by Allison and Martiny 2008). In contrast, warming experiments (+1.2–2°C) in the arctic showed that it can take more than a decade to detect the first changes in soil microbial community composition (Rinnan et al. 2007; Rinnan, Stark and Tolvanen 2009). Soil chemical variables (e.g., pH, TOC, TN and TP), which were the best predictors of bacterial community composition compared to plant and soil physical characteristics that were measured in this study (Fig. 3B), were not significantly changed by warming alone (Table S1, Supporting Information). The unchanged soil chemical variables and plant below-ground biomass under warming conditions indicated an unchanged supply rate of root litter and labile carbon from root exudates and might explain the unchanged microbial composition. Although, the above ground biomass was significantly increased by warming, the low amount of litter produced annually in combination with the slow decomposition in this alpine ecosystem (Luo et al. 2010) might cause a long time lag before the microbial composition could be affected. In addition, the relative dryness in this meadow (mean annual precipitation is 500 mm) might also contribute to the composition resistance to warming. Sheik et al. (2011) found that more than 4 years of warming significantly shifted the bacterial community...
Figure 3. NMDS of bacterial community composition (A). Abbreviations: P.R, plant species richness; MS, soil moisture; Tm, soil temperature; B.M: below-ground biomass. C: No-warming with no-grazing (black circles); W: warming with no-grazing (dark gray squares); G: no-warming with grazing (gray triangles); WG: warming with grazing (white inverted triangles). (B): CCA-based VPA. Environmental variables were divided into groups of soil (pH, TOC, TN and TP), plant (plant SR and below-ground biomass) and soil temperature and moisture (Tm&MS) variables. The circles show the variation explained by each group of environmental factors alone. The numbers between the circles show the interactions of the two factors on either side and the number in the center of the triangle represents interactions of all three factors.

Figure 4. Distribution of bacterial OTUs across soil cores. (A) Venn diagrams showing the number of OTUs unique to no-warming (C and G, NW) and warming (W and WG, W) plots and that shared between them. (B) The number of soil cores that the shared OTUs averagely distributed in for NW and W plots. Error bars indicate standard error of the data (n = 1659). (C) The number of soil cores that the unique OTUs averagely distributed in for NW and W plots. Different letters indicate significant difference at 0.05 level.

composition in normal water conditions (i.e. mean annual precipitation of 965 mm). However, warming did not affect bacterial composition in relatively dry conditions (i.e. mean annual precipitation of 515 mm). To discern how long this resistance will be maintained, and what changes will ultimately occur among bacterial communities, future investigations should be conducted.

A warming-related increase in the bacterial community similarity observed in our work was previously reported for a fungal response to warming in a Tibetan plateau grassland (Xiong et al. 2014) and for a bacterial community response in the process of forest-to-pasture conversion (Rodrigues et al. 2013). Increased bacterial community similarity could be caused by an increase
in the distribution ranges of existing bacterial species (Olden et al. 2004; Rodrigues et al. 2013). Results consistently show that, there is a significant increase in bacterial distribution ranges caused by warming (Fig. 4). Increasing temperatures induce a warm-adapted microbial community (Allison and Martiny 2008; Zhou et al. 2011; Rui et al. 2015) and helps to create a competitive advantage and promotes their spread (Pascual et al. 2006; van der Putten, Klironomos and Wardle et al. 2007; Wiedner et al. 2007). Though no significant shifts in bacterial community composition were found in our study, certain taxa may adapt to the increased temperature and increase their chances of dispersal. Therefore, the increased bacterial community similarity caused by warming might be due to the increase in distribution ranges of warm-adapted microbial taxa. The increased bacterial distribution might enhance the chance for horizontal gene transfer rates in this microenvironment (Troxler et al. 1997; Storfer 1999), with the end result affecting the feedbacks between this alpine ecosystem and future warming (Olden et al. 2004; Hewitt et al. 2010).

Three years of grazing alone had no significant effects on overall bacterial composition or diversity, but the relative abundance of Bacteroidetes and Nitrospirae were significantly decreased and increased, respectively. Grazing caused no significant changes in the measured soil and plant variables except decreasing plant height and above-ground biomass (Table S1, Supporting Information). There were no significant correlations between bacterial community characteristics and plant height and above-ground biomass. Therefore, the unchanged soil properties caused by grazing might be the explanation of the unchanged microbial composition. Bacteroidetes have been found to play important roles in plant litter decomposition (Rui, Peng and Lu 2009). Thus, a plausible explanation for their decrease caused by grazing might be the reduced plant above ground biomass. A large proportion of bacteria in the phylum Nitrospirae can transform nitrite into nitrate (Freitag et al. 2005; Lücke et al. 2010). Thus, the increased Nitrospirae following grazing suggests that their N-cycling function may be induced, which has been found in previous studies (Ingram et al. 2008; Yang et al. 2013).

Our results showed significant interactions between warming and grazing on bacterial α-diversity and composition (Table 1). For example, a significant increase in bacterial α-diversity was caused only by warming in no-grazing plots, not in grazing plots, indicating that grazing could potentially weaken the positive effects of warming on bacterial α-diversity. This significant interaction might be due to the different effects of warming and grazing on soil and plant properties, and thereby on microbial groups (Zhou et al. 2011; Yang et al. 2013). Opposite effects of warming and grazing on microbial functional groups have been found (Zhou et al. 2011; Yang et al. 2013). Warming caused decrease in soil water content (Luo et al. 2009; Rui et al. 2011), while grazing could constrain water loss through trampling (Warren et al. 1986). There is evidence that warming decreased or had no effect on bacterial richness and evenness in non-drought conditions, while in drought conditions, warming increased them (Sheik et al. 2011; Zhou et al. 2011). Soil pH, NH$_4^+$–N and moisture were important in predicting the response of bacterial α-diversity to warming and grazing in our study (Table 2). Although not at a significant level, warming caused an increase in soil pH by 4%, a decrease in NH$_4^+$–N and moisture by 26% and 16% in no-grazing plots, respectively; however, warming caused few changes in these factors in grazing plots (Table S1, Supporting Information). In addition, an contrasting response pattern of plant functional groups to warming and grazing have been found in this meadow (Wang et al. 2012), which may induce different response of soil carbon quality (Väisänen et al. 2015) and contribute to their interactions on the bacterial community. The existing interaction between warming and grazing on soil bacterial community indicates that feedbacks between climate warming and microbial community are likely to be complicated by land use. Similar to bacterial α-diversity, plant aboveground net primary production and microbial methanotrophic activity were significantly increased by warming in no-grazing plots, but few changes were found in grazing plots (Wang et al. 2012; Zheng et al. 2012). In Arctic tundra, warming-induced increases in soil respiration were also limited by grazing (Väisänen et al. 2014; Väisänen et al. 2015). Therefore, moderate grazing may offset, at least partially, the effects of global warming on the soil microbial community and ecosystem function.

The significant increase in bacterial α-diversity and decrease in β-diversity (Fig. 1) caused by warming under no-grazing conditions implies an ‘early warning signal’ for future biodiversity loss (Rodrigues et al. 2013). However, these results should be cautiously interpreted, since this knowledge was obtained mainly from plant community studies. The response patterns of plant community to warming might be different from those of microbial communities (Hewitt et al. 2010). Soil bacterial and plant SR were positively correlated with ecosystem multifunctionality (Jing et al. 2015; Lefcheck et al. 2015). Although we observed that warming resulted in a significant reduction of plant SR by about 17%, this could be due to transient occurrences or disappearance of certain plant species as demonstrated in our previous work (Wang et al. 2012). Thus, the significantly increased bacterial SR caused by warming under no-grazing conditions may benefit the ecosystem multifunctionality (Jing et al. 2015), which can be proved by increased soil respiration (Lin et al. 2011), litter decomposition rate (Luo et al. 2010) and methanotrophic activity (Zheng et al. 2012). In addition, these increased processes may also be caused by changes in microbial activity, which represent physiological responses to warming (Allison and Martiny 2008). However, the effects of these warming-caused physiological shifts of microbial community on soil carbon are difficult to predict due to their potential thermal acclimation (Luo et al. 2001; Allison, Wallenstein and Bradford 2010).

### CONCLUSIONS

The soil bacterial composition in this alpine meadow appeared to be resistant to 3 years of warming and grazing or require a longer time to exhibit a response to them. Soil bacterial community similarity was significantly increased by warming.
regardless of grazing, indicating that bacterial communities may become more similar across space in a future, warmed climate. Significant interactive effects between warming and grazing on bacterial composition and diversity were found. The positive effect of warming on bacterial α-diversity could be weakened by grazing. Our results suggest that moderate grazing has the potential to offset, at least partially, the effects of global warming on the soil microbial diversity and stabilize ecosystem functions in the alpine meadow.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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