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Short communication

Sheep grazing and soil bacterial diversity in shrublands of the Patagonian Monte, Argentina

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ABSTRACT

Our objective was to assess whether long-term continuous sheep grazing was associated with the bacterial community of the Patagonian Monte soils. We randomly extracted soil samples from 5 plant-covered patches (PCP) and the nearest inter-canopy areas (IC) at sites with low (L), moderate (M) and heavy (H) grazing intensity. Bacterial communities were evaluated by denaturing gradient gel electrophoresis (DGGE) and dominant bands were sequenced. Dendrograms showed a different association among grazing sites in PCP compared to IC areas. In PCP, M and H sites were clustered and DGGE revealed a high similarity in the bacterial community regardless of grazing intensity. In IC areas, analyses clustered L and M sites. Band sequencing revealed that members of these communities belonged to *Sphingobacteria*, *Solibacteres*, *Gammaproteobacteria*, *Betaproteobacteria* and *Bacilli.* Some of the band sequences related to *Nitrosococcus oceani* and *Nitrosococcus watsonii* which are restricted to marine environments and salt lakes. *Microcoleus vaginatus* and diatom chloroplast-related sequences were mainly detected in IC areas from L and M. Heavy grazing could alter autotrophic bacteria/diatom composition in IC areas possibly due to physical disturbance of biological crusts by trampling, threatening the potential of crust re-formation. Grazing intensity affected soil bacterial communities differently in PCP and IC areas with soil crust, highlighting the importance of improving our understanding of microbial diversity responses to land use.

1. Introduction

Domestic grazing in native shrubland ecosystems may induce the reduction of grass and total plant cover as well as changes in plant species composition leading to plant canopies with low leaf turnover as well as low nutrient and high secondary defense metabolite concentrations (Wardle et al., 2004). Thus, reduced quantity and quality of plant litterfall could have negative effects on decomposition/mineralization processes and soil organic matter dynamics (Carrera and Bertiller, 2013). Other consequences associated with livestock grazing disturbance are shifts in rhizosphere exudation, nutrient return into the soil through animal urine and faeces, and animal trampling which may affect physical soil properties. Therefore, direct and indirect effects of herbivores on the ecosystem may alter soil nutrient availability and physical-chemical conditions, and hence soil microbial communities. However, little is known about the impact of grazing on the composition of soil microbial communities in resource-poor habitats. Some studies have reported a decrease of both the soil catabolic diversity and N mineralization potential in heavily grazed areas with low plant biomass (Lawrence and Vadakattu, 2007). This suggests that in low-fertility soils, belowground microbial diversity is influenced by aboveground grazing impacts and the quality and quantity of C inputs from plants (Lawrence and Vadakattu, 2007). In a short-term trial, exclusion of grazing increased N cycling and bacterial diversity in the Inner Mongolian steppe, whereas the latter diminished after long-term grazing exclusion (Zhou et al., 2012). Similar to other soil biological attributes, the response of microbial diversity to grazing is not easy to generalize because of its dependence on environmental conditions, the inherent soil properties, plant litter quality and quantity, grazing intensity, animal type and management practices (Bardgett et al., 2001).

In Patagonia, sheep are the predominant domestic herbivore which were introduced at the beginning of the 1900's. At our

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research site, the vegetation (canopy cover < 60% of the soil) is distributed in patches formed by shrubs and grasses on a matrix of bare soil or scarce vegetation (Bisigato and Bertiller, 1997). Our previous studies in the Patagonian Monte demonstrated that long-term grazing induced a reduction of plant litter quantity and quality which negatively affected soil enzyme activities and microbial biomass-C under canopies (Olivera et al., 2014). However, in inter-canopy bare areas, microbial biomass-C and soil enzyme activity (particularly those closely related to microbial metabolism such as dehydrogenase and alkaline phosphatase) increased with heavy grazing intensity, presumably due to a localized nutrient input from animal excreta which promotes belowground microsites with higher microbial activity (Olivera et al., 2014). These results suggest that grazing could promote changes in soil microbial communities which are the main source of soil enzymes. There is a need to further our understanding of grazing effects on the microbiology of Patagonian soils given that, sheep raising for wool and meat production is the predominant land use on native Patagonian shrubland. The aim of this study was to assess whether long-term continuous sheep grazing was associated with changes in the bacterial community of the soils. We hypothesized that intensive long-term grazing, through its effects on plants and soil attributes (Prieto et al., 2011; Olivera et al., 2014) would disturb soil bacterial diversity.

2. Materials and methods

Soil samples (0–10 cm depth and 10 cm in diameter) were collected from a typical Patagonian Monte paddock (42°39′ S, 65°23′ W, 115 m a.s.l., Argentina) that had been stocked at a rate of 0.14 sheep ha^{-1} yr^{-1} over the last 23 years. Within the paddock, we selected 3 sampling sites (about 2 ha each) located at 3,000, 300 and 100 m from an artificial watering point, which correspond to low (L), moderate (M) and heavy (H) grazing intensities, respectively (Prieto et al., 2011; Olivera et al., 2014). We randomly sampled 5 modal size (height: >1 m, diameter 1.5–2.5 m) plant-covered patches (PCP) per site, and the nearest respective neighboring inter-canopy area of bare soil (IC) as previously described in Prieto et al. (2011). Soil samples were sieved through a 1 mm mesh screen to remove plant litter fragments. The quantity of plant litter was gravimetrically determined after separating attached soil particles with a brush and drying at 60 °C for 48 h. Soil organic-C, total soil-N, soil pH, bulk density and microbial biomass-C were determined in the same soil samples in a previous study (Table 1), (Prieto et al., 2011). Silt content ranged from 17.5 to 21.6% at L to 16.8–21.9 at H and soils of IC areas had 8% less sand and 94.6% more clay than PCP areas (Olivera et al., 2014). Differences among grazing sites in PCP or IC areas were evaluated using one-way ANOVA and Fisher Least Significant Difference, and Spearman correlation analysis.

Aliquots (0.5 g) of the soil samples collected in PCP and IC areas at the 3 grazing sites were used to extract total community DNA with the UltraClean Soil DNA Isolation Kit (MOBio Laboratorios, Inc, USA). The quantity of DNA suspensions was estimated by electrophoresis on 1.0% agarose gels followed by staining with GelRed (Biotium, Hayward, CA), using the DNA Precision Molecular Mass Standard (BIO-RAD, USA). Preliminary experiments indicated almost identical DGGE patterns of individual soil samples from each patch type (PCP, IC) and grazing site (L, M, H). Therefore, in order to run all samples in one gel, DNA samples were pooled before PCR amplification. The V6–V8 region of the 16S rRNA gene was amplified using the eubacteria primer set GC-984F/1378R (Heuer et al., 1997) and the V3–V5 region with GC-341F/907R (Muyzer et al., 1998). For the primers GC-984F/1378R, PCR mixture consisted of 30 ng of soil DNA, 0.25 mM of each primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5% DMSO, and 2.5 U of DNA Taq polymerase (Invitrogen, Brazil), and the buffer (1X) provided with the enzyme. Amplification was performed in a Multigene (Labnet, USA) thermocycler with the following program: 5 min at 95 °C, 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 2 minmin at 72 °C, and finally 30 min at 72 °C. The amplicons were checked by electrophoresis on 2% (w/v) agarose gels after staining with GelRed (Biotium, Hayward, CA). DGGE was conducted using a D-Code system (Bio-Rad Laboratories, Inc., Hercules, CA). Acrylamide gel (8%) consisting of a 30–70% gradient of urea and formamide was prepared using denaturant that consisted of 40% (v/v) formamide and 7 M urea. About 20 μl (approximately 800 ng) of PCR products were then subjected to electrophoresis at a constant voltage of 200 V at 60 °C for 4.5 h in 0.5X TAE buffer (1X TAE = 0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA; pH adjusted to 7.4). The gel was then stained with SYBR Gold (Molecular Probes, Eugene, OR) and registered. GC-341F/907R amplification and DGGE analysis were performed according to Ibarrolaza et al. (2009). Dominant DGGE bands from GC-341F/907R gel were excised using a sterile cutter and eluted in 30 μl de buffer TE (Tris–EDTA 1 mM pH = 8.0) at 20 °C overnight. The reamplification of DNA eluted from DGGE bands was performed using 341F (without the GC-clamp) and 907R primers, using the following protocol: 94 °C for 5 min, 30 cycles of 94 °C 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were checked by electrophoresis in 1% agarose gels, and then purified using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). Sequencing on both strands of PCR fragments was performed using the dideoxy chain termination method by the commercial services of the CENPAT Molecular Biology Laboratory (Argentina). DGGE band profiles were analyzed according to Marasco et al. (2012). Briefly, band profiles were converted to line plots with ImageJ software, and the x/y values obtained were imported into a MS Excel worksheet. The matrix of x/y values of 16S rRNA line profiles was subjected to cluster analysis using the Pearson correlation coefficient in SPSS 7.0 (Norusis, 1997). As DGGE patterns corresponded to composite DNA samples of each site no further statistical analyses were performed. The sequences were compared with those deposited in the GenBank (using the online software BLAST) and the EzTaxon-extended (Kim et al., 2012) databases. Partial 16S rRNA sequences were deposited in the GenBank database under the accession numbers KC997797 to KC997810.

3. Results and discussion

Dendrograms resulting from bacterial PCR-DGGE banding patterns showed a different association among grazing sites in PCP (Fig. 1a and b) compared to IC areas (Fig. 1c and d). This was consistent for both 16S rRNA regions (V3–V5 and V6–V8) analyzed (Fig. 1a–d). Cluster analyses of DGGE profiles revealed a high similarity in the soil bacterial community structure of PCP regardless of grazing intensity (within a range of similarity of 90%), (Fig. 1a and b). For V3–V5 and V6–V8 regions of the 16S rRNA gene, these analyses clustered M and H grazing sites in PCP (Fig. 1a and b). In IC areas, the analyses clustered L and M sites (Fig. 1c and d).

Interestingly, our previous studies also detected different responses to grazing in PCP and IC areas for physical-chemical and biological soil properties (Prieto et al., 2011; Olivera et al., 2014). In PCP, similar values of microbial biomass-C were observed among sites; the only significant difference was detected between L and M grazing sites (Table 1). In contrast, microbial biomass-C significantly increased with grazing intensity in IC areas (Table 1), possibly due to a localized positive effect of labile N and C from animal urine and dung (Prieto et al., 2011). In addition, grazing was

Table 1
Mean values ± one standard error of soil properties in PCP (plant-covered patches) and IC (inter-canopy) areas by grazing intensity (L: low, M: moderate, H: heavy). Different lowercase letters indicate significant differences among grazing intensities ($p < 0.05$).

PCP	L	M	H	Reference
Plant litter mass (g m^{-2})	1818.89 ± 384.05b	610.37 ± 79.51a	659.26 ± 117.22a	This study
Soil organic-C (mg C g^{-1} soil)	12.10 ± 2.06b	6.81 ± 0.41a	6.69 ± 0.66a	Prieto et al., 2011
Soil N (mg N g^{-1} soil)	1.15 ± 0.16b	0.60 ± 0.03a	0.63 ± 0.05a	Prieto et al., 2011
Bulk density (g cm^{-3})	1.17 ± 0.03a	1.30 ± 0.03b	1.41 ± 0.02c	Prieto et al., 2011
pH	8.23 ± 0.06a	8.30 ± 0.02a	8.31 ± 0.07a	Prieto et al., 2011
Microbial biomass-C (μg C g^{-1}soil)	494.74 ± 43.34c	369.38 ± 33.68ab	450.65 ± 20.30bc	Prieto et al., 2011
IC				
Plant litter mass (g m^{-2})	59.41 ± 15.84 a	85.77 ± 14.42 a	241.06 ± 43.11 b	This study
Soil organic-C (mg C g^{-1} soil)	4.47 ± 0.26a	4.19 ± 0.30a	4.95 ± 0.30a	Prieto et al., 2011
Soil N (mg N g^{-1} soil)	0.54 ± 0.02a	0.47 ± 0.02a	0.49 ± 0.03a	Prieto et al., 2011
Bulk density (g cm^{-3})	1.31 ± 0.03a	1.41 ± 0.03b	1.46 ± 0.02b	Prieto et al., 2011
pH	8.50 ± 0.02a	8.61 ± 0.04b	8.61 ± 0.02b	Prieto et al., 2011
Microbial biomass-C (μg C g^{-1} soil)	146.40 ± 20.29a	171.18 ± 23.95a	289.56 ± 27.21b	Prieto et al., 2011

associated with a decrease plant litter mass, soil organic-C and soil-N in PCP (Table 1), which may affect the availability of soil resources for microorganisms. A significant positive correlation was found among plant litter mass, soil organic-C, soil-N and microbial biomass-C, while most of these properties were negatively correlated with soil pH and bulk density (Supplementary data, Table A.1). The increase of soil bulk density with grazing is consistent with higher animal trampling, which could also contribute to plant litter dispersion. DGGE results from this study showed different responses of soil bacterial community to long-term continuous sheep grazing in PCP and IC areas, which could be related to these variations in soil properties with grazing intensity and patch type. Zhou et al. (2010) detected differences in bacterial community structure in a steppe grazed by sheep at different grazing intensities over a long-term period, which were associated with soil moisture, keystone plant species biomass, and soil pH. In a mesocosm experiment, grazing led to a change in the structure of the whole soil bacterial community which was related to the supply of urine and/or the impact of labile C from grazers (Attard et al., 2008).

To gain further insight into the bacterial community, the dominant bands from a V6–V8 DGGE gel were identified by partial 16S rRNA sequencing (Table 2). The major taxa associated to PCP and IC areas were *Sphingobacteriia*, *Solibacteres*, *Gammaproteobacteria*, *Betaproteobacteria* and *Bacilli*, but not all taxa were always represented at all grazing intensities (Table 2; Supplementary data Fig. A.1). *Cytophagia* was found in PCP from the H site (Table 2; Supplementary data Fig. A.1). *Cyanobacteria* and diatom chloroplast sequences were mainly detected in IC areas from L and M sites (Table 2; Supplementary data Fig. A.1). Most of the sequences showed their highest matches to sequences from uncultured bacteria suggesting the existence of novel bacterial species in Patagonian Monte soils (Table 2).

In all soil samples we detected sequences related to ammonia-oxidizing bacteria (AOB), such as *Nitrosospira* (*Betaproteobacteria*) and *Nitrosococcus* (*Gammaproteobacteria*), (Table 2; Supplementary data Fig. A.1). AOB are involved in the initial stage of nitrification when ammonia is aerobically oxidized to nitrite. *Nitrosospira* is thought to be the dominant AOB in many soils (Sylvia et al., 2005). Interestingly, we amplified sequences related to *Nitrosococcus oceani* and *Nitrosococcus watsonii* (Table 2). These microorganisms, in contrast to the omnipresent and diverse betaproteobacterial AOB (e.g. *Nitrosospira* and *Nitrosomonas*), are restricted to marine environments and salt lakes (Sylvia et al., 2005; Campbell et al., 2011).

In IC areas of L and M grazing sites, sequences from bands 9 and 13 were related to cultured members of *Oscillatoriales*, being *Microcoleus vaginatus* their closest related species with 100% of homology (Table 2). In addition, sequences from bands 7, 8 and 12 were closely related to uncultured cyanobacterium and uncultured bacterium (99 and 100% homology, respectively), and chloroplasts from diatoms possibly components of biological soil crusts (Table 2; Supplementary data Fig. A.1). These are photosynthetic, diazotrophic communities of bacteria, fungi, algae, lichens and mosses that colonize the uppermost soil surface in most arid and semi-arid ecosystems, preferably in bare interspaces (Belnap and Lange, 2003; Castillo-Monroy and Maestre, 2011).

M. vaginatus is frequently a dominant *Cyanobacteria* in biological soil crusts of desert areas (Li et al., 2013). Moreover, the formation of biological soil crusts begins with surface soil colonization by this

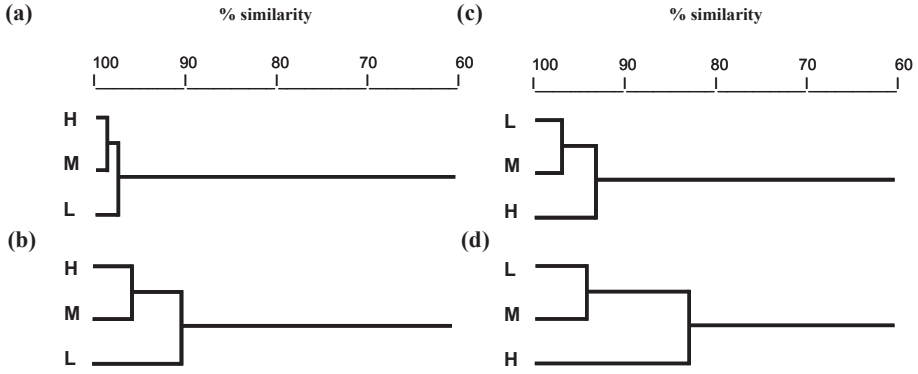

Fig. 1. Cluster analysis of 16S rRNA DGGE band profiles of sites with low (L), moderate (M) and heavy (H) grazing intensity in plant-covered patches (a-variable region V3–V5; b-variable region V6–V8) and inter-canopy areas (c-variable region V3–V5; d-variable region V6–V8).

Table 2
Phylogenetic identification of bacterial sequences corresponding to DGGE bands. Band numbers correspond to sequenced DGGE bands shown in Supplementary material Fig. A.1.

Band number	Class of the closest type strain	Closest type strain or described cultivable strain (EZ-Taxon database)	% Homology	Closest relative (GenBank accesion number)	% Homology
1	*Sphingobacteriia*	*Chitinophaga ginsengisegetis* Gsoil 040 (T)	94	Uncultured bacterium (JX098578.1), soil of Atacama desert	99
3	*Cytophagia*	*Adhaeribacter aerolatus* 6515J-31 (T)	95	Uncultured bacterium (AB696242.1), soil of Gobi desert	99
4	*Solibacteres*	*Candidatus* Solibacter usitatus Ellin 6076	93	Uncultured bacterium (JX255374.1), soil crusts from extrazonal mountain dry steppes in northern Mongolia	99
5	*Betaproteobacteria*	*Nitrosospira tenuis* Nv-1 (T)	92	Uncultured bacterium (GQ397070.1), soil	99
2,14,17	*Gammaproteobacteria*	*Nitrosococcus oceani* ATCC 19707 (T)	91	Uncultured bacterium (JQ738864.1), Lonar crater wall surface rocks	99
10,15		*Nitrosococcus oceani* ATCC 19707 (T)	91	Uncultured bacterium (JQ738864.1), Lonar crater wall surface rocks	99
11		*Nitrosococcus watsonii* C-113 (T)	89	Uncultured bacterium (JQ738864.1), Lonar crater wall surface rocks	96
16		*Nitrosococcus oceani* ATCC 19707 (T)	91	Uncultured bacterium (JQ738864.1), Lonar crater wall surface rocks	99
6	*Bacilli*	*Bacillus foraminis* CV53(T)	94	Uncultured *Bacillus* (JQ793404.1), rhizospheric soil	96
18		*Sporosarcina luteola* Y1 (T)	89	Uncultured bacterium (JN039015.1), rhizospheric soil	93
19		*Bacillus foraminis* CV53 (T)	97	Uncultured *Bacillus* (JQ793404.1), arid soil	99
9,13	*Oscillatoriales*	*Microcoleus vaginatus* PBP-D-KK1	99	*Microcoleus vaginatus* (AF284803.1), soil desert crusts from the Colorado plateau	100
7, 12	*Bacillariophyceae*	cloroplast *Haslea salstonica* (diatom)	98	Uncultured cyanobacterium (JN367221.1), soil	99
8		cloroplast *Phaeodactylum tricornutum* (diatom)	99	Uncultured bacterium (AB657488.1), soil	100

cyanobacterium which can excrete exopolysaccharides that stick soil particles together, increasing soil stability and slowing down water evaporation of dry soils (Kuske et al., 2012). The lack of detection of band 8 and the decrease in intensity of bands 7–12 and 9–13 at the H site (identified as *M. vaginatus* and chloroplast sequences; see Table 2 and line IC-H on Supplementary data Fig. A.1) suggest that heavy grazing intensity could alter autotrophic bacteria/diatom composition in IC areas. This could be related to the physical disturbance of biological crusts by trampling, which may jeopardize crust re-formation. Kuske et al. (2012) found a 23–65% reduction in the proportion of *M. vaginatus* and other *Cyanobacteria* in trampled plots compared to untrampled sites.

4. Conclusions

Grazing may exert opposite effects on soil microorganisms providing nutrients from excreta and simultaneously diminishing plant litter quantity and quality and altering soil physical–chemical properties. This study reveals that in the Patagonian Monte grazing intensity was associated with different soil bacterial communities in PCP and IC areas, including those in biological soil crusts. This highlights the importance of improving our understanding of soil microbial diversity responses to grazing when assessing land management strategies in arid ecosystems. Our results also stress the need for future comprehensive studies, including culture-independent high-throughput approaches (e.g. pyrosequencing), to link the observed changes in the bacterial community to the different grazing effects in soil properties in PCP and IC areas.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaridenv.2015.09.012.

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