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Research Article

# Microbial quality of soil from the Pampa biome in response to different grazing pressures

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## Abstract

The aim of this study was to evaluate the impact of different grazing pressures on the activity and diversity of soil bacteria. We performed a long-term experiment in Eldorado do Sul, southern Brazil, that assessed three levels of grazing pressure: high pressure (HP), with 4% herbage allowance (HA), moderate pressure (MP), with 12% HA, and low pressure (LP), with 16% HA. Two reference areas were also assessed, one of never-grazed native vegetation (NG) and another of regenerated vegetation after two years of grazing (RG). Soil samples were evaluated for microbial biomass and enzymatic (β-glucosidase, arylsulfatase and urease) activities. The structure of the bacterial community and the population of diazotrophic bacteria were evaluated by RFLP of the 16S rRNA and nifH genes, respectively. The diversity of diazotrophic bacteria was assessed by partial sequencing of the 16S rDNA gene. The presence of grazing animals increased soil microbial biomass in MP and HP. The structures of the bacterial community and the populations of diazotrophic bacteria were altered by the different grazing managements, with a greater diversity of diazotrophic bacteria in the LP treatment. Based on the characteristics evaluated, the MP treatment was the most appropriate for animal production and conservation of the Pampa biome.

*Keywords*: bacterial diversity, diazotrophic bacteria, diversity, grasslands, soil microbial communities.

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## Introduction

The Pampa biome is located between latitudes 24° and 35° S and covers an area of 500,000 km2 in Uruguay, northeastern Argentina, southern Brazil and part of Paraguay (Pallarés *et al.*, 2005). This biome is composed of herbaceous native plants classified as steppe in the international phytogeographic system (Berreta, 2001) and is recognized for its great species diversity that includes approximately 450 grasses and 200 forage legumes (Boldrini, 2007).

Since European immigrants introduced the first herds in the 17th century (Bilenca and Miñarro, 2004), livestock production has been one of the main economic activities of the region, with the natural grassland serving as the feeding basis for animal production (Carvalho and Batello, 2009). Historically, the natural grasslands of the Pampa biome have been used in extensive systems of beef cattle breeding characterized by low environmental impact, with little or no contribution of external inputs (Viglizzo *et al.*, 2001). However, in recent decades, the ecosystem has been threatened by the introduction of exotic forage species, the exploitation of planted forests and the introduction of annual crops (Carvalho and Batello, 2009). Even in areas still managed by the traditional livestock system, there are risks associated with overstocking (Carvalho *et al.*, 2006). Conte *et al.* (2011) claimed that adjustment of the number of animals to herbage allowance is critical to the sustainability of natural pastures. These authors found a decrease in labile carbon and an increase in soil density associated with a reduction in herbage allowance in an area of the Pampa biome. In addition to the loss of soil physical quality, often resulting in water erosion and compaction of the surface layers (Bertol *et al.*, 2000), excessive stocking may also result in decreased plant diversity (Soares *et al.*, 2003).

In natural environments or environments with little human intervention, such as traditional grazing systems of the Pampa biome, the soil microbiota plays a vital role in maintaining the ecosystem. The cycling of organic matter, nutrient availability and formation and stabilization of aggregates are a direct result of microbial activity that in turn

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influences the productivity, diversity and composition of plant communities (Van Der Heijden *et al.*, 2008). Pastures grown without fertilization, in soils with low natural fertility (Streck *et al.*, 2008), are nutritionally poor environments in which symbiotic microorganisms are responsible for the acquisition of scarce nutrients by plants (Cleveland *et al.*, 1999; Van Der Heijden *et al.*, 2008). In such cases, diazotrophic bacteria assume an even more relevant role than in other agricultural systems and are the main source of nitrogen for the vegetation (Van der Heijden *et al.*, 2006).

Like the physical quality, the soil microbiological quality can also be affected by pasture management. Northup *et al.* (1999) found a decrease in the microbial biomass in soils under intensive grazing and attributed the result to a lower input of organic carbon with increasing grazing intensity. Similarly, Holt (1997) noted that, in addition to the microbial biomass, enzyme activity was also reduced with excessive grazing in an Australian soil. Moreover, changes in the floristic composition of the pasture may result in changes in the structure of the soil microbial community, with consequences in its functionality (Johnson *et al.*, 2003). Since pastures are formed by a rich diversity of plants, the consequences of changes in the diversity and activity of soil microorganisms become more unpredictable than in less complex environments, such as annual crops.

In view of the importance of microbiological processes for preservation of the Pampa biome while at the same time allowing adequate economic exploitation, in this study we examined the impact of different grazing pressures on the microbial activity and diversity of soil bacteria.

## Materials and Methods

### Soil sampling

This study is part of a long-term experiment that has been ongoing at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul (30°05' S, 51°40' W, and 46 m altitude), in Eldorado do Sul, Brazil, since 1986. This experiment involves different levels of grazing pressure in an area of natural grassland representative of the phyto-physiognomy of fields in the center of Rio Grande do Sul state (Boldrini *et al.*, 2010), which is part of the Pampa biome. The soil of the experimental location is classified as Paleudult. The area has been maintained without any form of human intervention, except for adjustment of the grazing pressures, which are assessed on average every 28 days using the put-and-take technique.

Soil samples were collected in triplicate in the 0-5 cm layer based on a completely randomized design. Some of the samples were used to evaluate microbial soil quality while others were used to characterize the chemical properties of the soil by standard methods described in Sparks (1996) (Table 1).

The treatments consisted of three levels of grazing pressure: high grazing pressure (HP), with 4% herbage allowance (HA), moderate grazing pressure (MP), with 12% HA, and low grazing pressure (LP), with 16% HA. Two reference areas were also evaluated: an area of never-grazed native vegetation (NG) and another with regenerated vegetation (RG) that was grazed for two years and then excluded from grazing since 1988. In the experimental unit subjected to HP, there was only one layer of vegetation; this layer was homogeneous and had a low canopy profile, with the most frequent species belonging to the genera *Paspalum*, *Axonopus*, *Piptochaetium* and *Coelorachis*. In the other experimental units, there was an upper layer formed mainly by species of the genera *Aristida*, *Eryngium*, *Andropogon*, *Baccharis* and *Vernonia* that resulted in a bimodal pasture structure and mosaic pattern (Corrêa and Maraschin, 1994).

### Biochemical characteristics of the soil

Soil samples were evaluated for microbial biomass (MB), according to the method proposed by Horwath *et al.* (1996). The methods proposed by Dick *et al.* (1996) were used to assess the activities of the enzymes β-glucosidase (carbon cycle), arylsulfatase (sulfur cycle) and urease (nitrogen cycle). Determination of β-glucosidase and arylsulfatase activities was based on the actions of the enzymes on their specific substrates, with the reaction product (ρ-nitrophenol) being quantified colorimetrically. Urease was assessed based on the release of ammonium ($NH_4^+$) by the action of the enzyme on urea. $NH_4^+$ was quantified by distillation and titration according to Sparks (1996).

A geometric mean of the biochemical characteristics, adapted from Hinojosa *et al.* (2004) and used as a general indicator of soil quality, was calculated using the

**Table 1** - Soil chemical properties under different grazing pressures.

| Sample | Clay (%) | OM (%) | pH ($H_2O$) | $P_{exc}$ (mg/dm$^3$) | $K_{exc}$ (mg/dm$^3$) | $Al_{exc}$ (Cmol$_c$/dm$^3$) | $Ca_{exc}$ (Cmol$_c$/dm$^3$) | $Mg_{exc}$ (Cmol$_c$/dm$^3$) |
|---|---|---|---|---|---|---|---|---|
| NG | 21 | 1.9 | 4.8 | 1.4 | 104 | 0.9 | 0.1 | 0.6 |
| RG | 30 | 2.9 | 4.4 | 2.2 | 128 | 1.4 | 0.3 | 0.7 |
| LP | 28 | 2.5 | 4.7 | 1.4 | 138 | 0.8 | 0.9 | 0.8 |
| MP | 19 | 3.6 | 5.0 | 2.2 | 79 | 1.3 | 0.1 | 0.5 |
| HP | 22 | 2.5 | 4.9 | 1.4 | 75 | 1.2 | 0.3 | 0.7 |

Exc - exchangeable, HP - high grazing pressure, LP - low grazing pressure, MP -moderate grazing pressure, NG - never-grazed native vegetation, OM - organic matter content, RG - regenerated vegetation.

formula GMba = (MB x AS x ßG x Ur), in which MB, AS, ßG and Ur represent the microbial biomass and the activities of arylsulfatase, β-glucosidase and urease, respectively. The data for the biochemical characteristics and GMba were assessed by analysis of variance (ANOVA) and the means were compared using the Scott-Knott test (Sisvar 5.1 Build 72), with $p < 0.05$ indicating significance (Ferreira, 2011).

### DNA extraction from soil and evaluation of the bacterial community structure

DNA was extracted from 300 mg of soil using a NucleoSpin® Soil kit (Macherey-Nagel), according to the manufacturer's instructions. The purified DNA was used for the amplification reactions with primers specific for the 16S rRNA (Felske *et al.*, 1999) and nifH (Poly *et al.*, 2001) genes. PCR mixes contained 50 ng of template DNA, 1x reaction buffer, 1 U of *Taq* DNA polymerase (Invitrogen), 100 μM of each deoxynucleotide, 1 μM of each primer, 50 mM $MgCl_2$ and ultrapure water to a final volume of 25 μL. Amplifications were done using an initial cycle of denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 56 °C (for 16S rDNA) or 55 °C (for nifH) and extension at 72 °C for 1 min, followed by a final extension cycle at 72 °C for 5 min. The PCR products were visualized on a 1% agarose gel stained with Blue Green Loading Dye I (LGC Biotecnologia) and then subjected to restriction fragment length polymorphism (RFLP) analysis.

The restriction procedure was adapted from Widmer *et al.* (1999) with the addition of 6 μL of PCR product to 40 μL of a mixture of ultrapure water, 2 U of the restriction enzyme and its corresponding buffer. The enzymes used were *Hae*III, *Hind*III and *Msp*I, and the incubation was done at 37 °C for at least 16 h to ensure complete digestion of the PCR products. The digestion products were resolved by electrophoresis for 3 h with a current of 200 V in 10% polyacrylamide gels in 1x TBE buffer stained with silver nitrate. Gel images were analyzed using Gel-Pro Analyzer 3.1 software and used to generate a binary matrix. The matrices were analyzed with Paleontological Statistics (PAST) software (Hammer *et al.*, 2007) and the similarity dendrograms were quantified using the Jaccard coefficient.

### Isolation, identification and diversity of culturable diazotrophs

Ten grams of soil sample was suspended in sterile saline solution (0.85% NaCl) and incubated at 28°C with shaking for 16 h. Next, serial dilutions (up to $10^{-3}$) were done and 0.1 mL aliquots were removed and inoculated on three semi-solid media lacking N: nitrogen-free malate (NFb), LGI and LGI-P. Bacterial isolation was done as described by Döbereiner *et al.* (1995). The 16S rRNA gene of 100 bacterial isolates was partially amplified (~450 bp) by PCR (Felske *et al.*, 1999). The amplification products were sequenced in the ACT-Gene Laboratory (Center of Biotechnology, UFRGS, RS, Brazil) using an automatic sequencer (ABI-PRISM 3100 Genetic Analyzer 50 cm capillary, Applied Biosystems). The 16S rRNA sequences were analyzed using the BLASTN program (NCBI BLAST) and were deposited in the GenBank database under accession numbers KM032779 to KM032874.

PAST software was used to calculate the Shannon diversity index (*H*', Shannon and Weaver, 1949), dominance (D) and equitability (J) based on the number of isolates belonging to each taxon. Principal coordinate analysis (PCA) was used to assess the correlation between soil properties and population diversity.

## Results

### Biochemical characteristics indicative of soil quality

The biochemical properties of the soils were significantly influenced by the different grazing management schemes, as summarized in Table 2. NG vegetation had the lowest values for all biochemical parameters evaluated, similar to those observed in the LP treatment. The overall geometric mean for the biochemical characteristics was significantly higher in the MP and HP treatments.

**Table 2** - Biochemical characteristics of the soil under different grazing pressures.

| Sample | Microbial biomass (mg $kg^{-1}$) | Arylsulfatase (mg ρ-nitrophenol kg $soil^{-1}h^{-1}$) | β-Glucosidase (mg ρ-nitrophenol kg $soil^{-1}h^{-1}$) | Urease (mg $NH_4^+$ kg $soil^{-1}h^{-1}$) | GMba |
|---|---|---|---|---|---|
| NG | 165[b] | 12.9[c] | 3.1[b] | 29.4[b] | 21.0[b] |
| RG | 255[a] | 20.2[a] | 5.0[a] | 21.2[b] | 27.2[b] |
| LP | 191[b] | 16.3[b] | 3.4[b] | 31.1[b] | 23.9[b] |
| MP | 249[a] | 21.6[a] | 6.9[a] | 31.1[b] | 32.7[a] |
| HP | 250[a] | 16.2[b] | 5.0[a] | 46.7[a] | 31.1[a] |

Means in the same column followed by the same letter do not differ from each other by the Scott-Knott test at $p < 0.05$. GMba - geometric mean of the biochemical characteristics, HP - high grazing pressure, LP - low grazing pressure, MP - moderate grazing pressure, NG - never-grazed native vegetation, RG - regenerated vegetation.

## Soil bacterial diversity

The structure of the soil bacterial community was assessed by RFLP based on the amplification products of the 16S rRNA and nifH genes (Figure 1). The diversity of culturable diazotrophic bacteria was also analyzed by partial sequencing of the 16S rRNA gene (Table 3). Analysis of the RFLP profiles of the 16S rRNA gene revealed a distinct structure of bacterial communities in the different treatments. The NG treatment replicates were grouped in an isolated cluster and shared approximately 60% similarity with the other profiles. A second subdivision, with nearly 70% similarity, contained the samples of the HP treatment. Samples of the MP, LP and NG treatments had profiles that were more similar to each other (> 80% similarity) than to the other groups.

The RFLP profiles of the nifH gene showed a more complex pattern, with greater variability between treatments and between replicates of some treatments. The LP treatment differed from the others and formed a separate cluster, with little more than 20% similarity. The NG treatment also formed an isolated cluster and shared ~50% similarity with the other treatments; the latter showed > 60% similarity among themselves.

Assessment of the diversity of culturable diazotrophic bacteria confirmed the presence of diverse populations in the different grazing treatments. *Burkholderia* and *Enterobacter* were the most ubiquitous bacteria, being identified in all treatments, with 50 and 26 isolates, respectively. The smallest number of taxa was observed in HP, where only these two genera were identified, while in the LP treatment eight taxa were found. The LP treatment also showed the highest *H*', the lowest D and the highest J, contrasting again with HP.

## Relationships between the chemical properties and biochemical characteristics of the soil

PCA was used to investigate the relationships between the biochemical characteristics and chemical properties of the soil under different treatments. The main components of PCA (PC1 and PC2) explained 86.2% of the total data variation, with PC1 accounting for 49.1% and PC2 for 37.1% (Figure 2). The MP and HP treatments were positioned on the left in PC1 and were more associated with the biochemical characteristics. Urease activity was associated with the soil pH, while the other biochemical characteristics were associated with the organic matter (OM) and phosphorous (P) content of the soil. This observation was supported by the significant positive correlations between OM and the activities of arylsulfatase ($r = 0.96$; $p = 0.0078$) and β-glucosidase ($r = 0.92$; $p = 0.0254$). In the opposite position in PC1 were the NG and LP treatments. The diversity of diazotrophic bacteria was related to the clay and basic cation ($Ca^{2+}$, $Mg^{2+}$ and $K^{+}$) contents.

## Discussion

In this study, we investigated the long-term effects of increasing grazing pressures on the microbial quality of a soil compared with two control areas maintained without

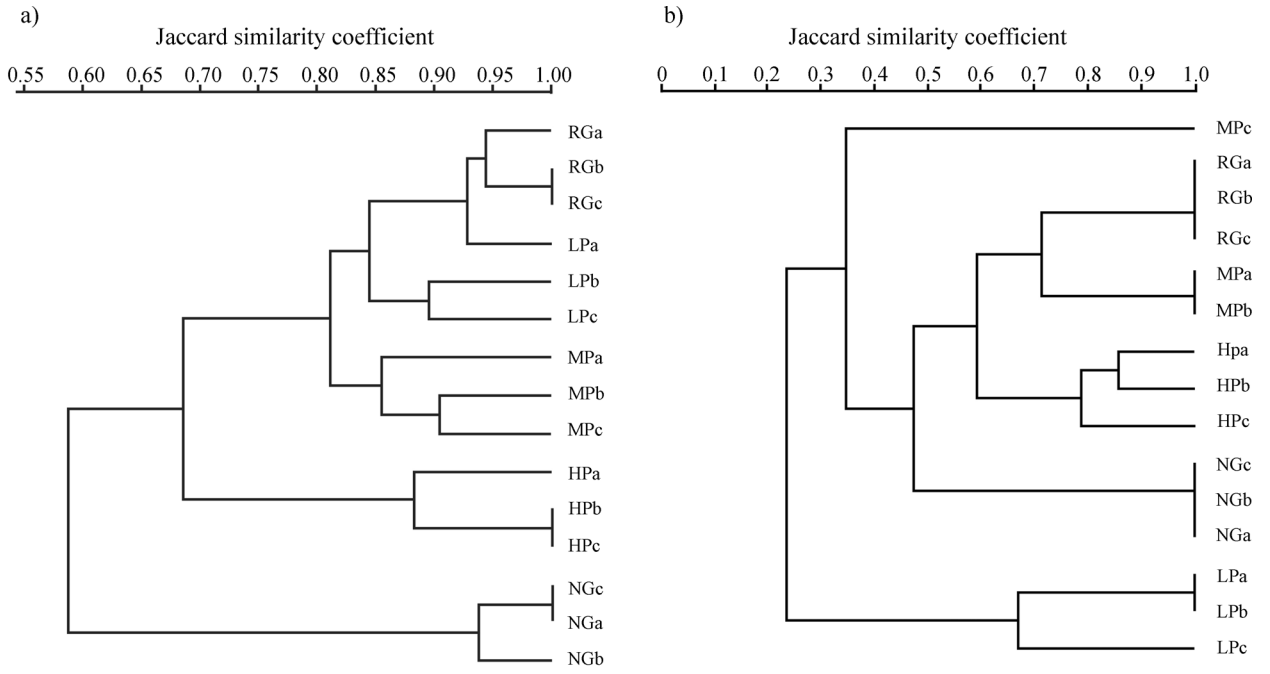


**Figure 1** - Dendrograms generated from RFLP profiles of the 16S rDNA (A) and nifH (B) genes from DNA isolated from soil under different grazing intensities. HP - high grazing pressure, LP - low grazing pressure, MP - moderate grazing pressure, NG - never-grazed native vegetation, RG - regenerated vegetation.

**Table 3** - Diversity of diazotrophic bacteria in soil under different grazing pressures.

| | Sample | | | | | |
|---|---|---|---|---|---|---|
| | NG | RG | LP | MP | HP | Total |
| Taxa | Number of individuals | | | | | |
| *Achromobacter* sp. | - | 2 | - | - | - | 2 |
| *Burkholderia* sp. | 6 | 10 | 6 | 13 | 15 | 50 |
| *Enterobacter* sp. | 7 | 5 | 5 | 5 | 4 | 26 |
| *Klebsiella* sp. | 1 | - | - | - | - | 1 |
| *Gluconacetobacter* sp. | - | - | 2 | - | - | 2 |
| *Ochrobactrum* sp. | - | - | 3 | - | - | 3 |
| *Pantoea* sp. | 1 | 1 | - | - | - | 2 |
| *Pseudomonas* sp. | - | 1 | 1 | 1 | - | 3 |
| *Serratia* sp. | 4 | - | - | - | - | 4 |
| *Stenotrophomonas* sp. | - | - | - | - | 1 | 1 |
| Uncultured Enterobacteriaceae | 1 | - | 1 | 1 | - | 3 |
| Uncultured | - | - | 1 | - | - | 1 |
| Unidentified | - | 1 | 1 | - | - | 2 |
| Total | 20 | 20 | 20 | 20 | 20 | 100 |
| Diversity index | | | | | | |
| *H'* | 1.5 | 1.37 | 1.82 | 0.93 | 0.69 | |
| *D* | 0.26 | 0.33 | 0.19 | 0.49 | 0.60 | |
| *J* | 0.84 | 0.77 | 0.88 | 0.67 | 0.63 | |

*D* - dominance, *J* - equitability, *H'* - Shannon diversity index, HP - high grazing pressure, LP - low grazing pressure, MP - moderate grazing pressure, NG - never-grazed native vegetation, RG - regenerated vegetation.

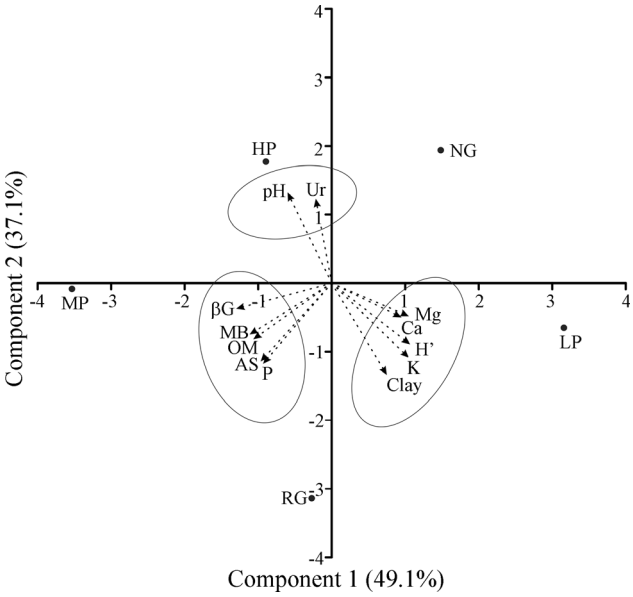


**Figure 2** - Principal component analysis of the chemical, biochemical and microbiological characteristics of soil under different grazing intensities. AS - arylsulfatase, βG - β-glucosidase, HP - high grazing pressure, LP - low grazing pressure, MB - microbial biomass, MP - moderate grazing pressure, NG - never-grazed native vegetation, RG - regenerated vegetation, Ur - urease.

grazing. All of the variables analyzed were significantly affected by the treatments, indicating that the presence of cattle was beneficial in terms of microbiological quality of the soil.

The microbial biomass increased with increasing grazing intensity and with the number of animals per area in the MP and HP plots. Wang *et al.* (2006) also observed an increase in microbial biomass in areas under intensive grazing compared with an area excluded from grazing. Likewise, Iyyemperumal *et al.* (2007) reported an increase in microbial biomass with increasing animal waste deposition in grassland ecosystems, which in turn increased with the number of animals per area. The waste deposited in soil by grazing animals may stimulate the microbiota by providing more readily available labile organic matter compared to original plant material (Prieto *et al.*, 2011). Grazing also promotes the growth and renewal of the plant root system, with a consequent increase in the rhizosphere effect. Hamilton *et al.* (2008) observed that defoliation, which simulates what occurs during grazing, increased the production of root exudates in *Poapratensis* by 1.5 fold, resulting in a proportional increase in the microbial biomass.

The soil enzymatic activity controls the cycling of nutrients through the mineralization of OM and constitutes an important indicator of the functional capacity of soil (Mi-

jangos *et al.*, 2006). ß-Glucosidase, urease and arylsulfatase participate in the C, N and S cycles, respectively. The activities of these hydrolytic enzymes have been widely used in assessments of soil quality because they are highly sensitive to disturbances in the soil (Bandick and Dick, 1999; Matsuoka *et al.*, 2003). Our results show that the activities of these three enzymes followed the same trend as microbial biomass and the geometric mean of the biochemical characteristics (GMba), *i.e.*, higher in the MP and HP treatments compared with the LP and NG treatments. Similar results were obtained by Esch *et al.* (2013), who observed a linear increase in enzymatic activity with increasing grazing pressure. As discussed for microbial biomass, enzymatic activity is favored by the deposition of animal waste (Bol *et al.*, 2003) and the rhizosphere effect (Reddy *et al.*, 1987). A previous investigation done in the same area showed a linear increase in the root mass of native grassland with increasing grazing pressure (Conte *et al.*, 2011), and this was apparently accompanied by increased enzymatic activity.

The microbial community structure was also altered by the treatments. With regard to the bacterial community in general, the NG area had a completely different structure. Indeed, non-grazed areas often have microbial communities that differ from those of grazed areas (Frank *et al.*, 2003; Ford *et al.*, 2013), and the changes caused by grazing seem to occur more rapidly than an eventual recovery of the original structure after interruption of grazing (Attard *et al.*, 2008).

HP grazing was distinguishable from the other groups in terms of microbial community structure. A similar result was described by Zhou *et al.* (2010), who found that microbial communities of non-grazed areas and intensely grazed areas were different from those of areas under moderate and low intensities of grazing. The presence of grazing animals is expected to alter the microbial community structure mainly by modifying the composition of the plant community and the contribution of organic C, especially the labile fraction (Attard *et al.*, 2008). As revealed by previous studies done in this same experimental area, the treatments applied over the years differed with respect to their floristic composition and in relation to the availability of labile C. In both aspects (considered crucial for microbial community structure), the HP treatment differed dramatically from the others, with lower plant diversity (Corrêa and Maraschin, 1994) and a reduction in the soil labile organic C (Conte *et al.*, 2011).

In view of the importance of diazotrophic bacteria for the functioning of a managed ecosystem without the contribution of external inputs, the structure of this bacterial community was assessed as proposed by Poly *et al.* (2001). In a manner similar to those authors, who observed that the nifH gene pool was different in soil under cultivation compared with a natural pasture, we observed that grazing affected the structure of the nitrogen-fixing community. Compared with the 16S rRNA gene, the RFLP amplification of the nifH gene products produced results with greater variability. Additionally, more than the NG treatment, LP soil differed more from the others than did NG treatment. These results are consistent with reports that the composition of the nifH gene pool varies between soils and between microenvironments within a soil (Widmer *et al.*, 1999; Poly *et al.*, 2001; Soares *et al.*, 2006).

The presence of a distinct community in the LP treatment was confirmed by the analysis of culturable diazotrophic bacteria. In this treatment, the largest number of genera, the highest *H'*, the lowest D and the highest J were identified, indicating that the nitrogen-fixing microorganisms benefitted from the diversity of plant species and the presence of animals in this plot. The diversity of diazotrophic bacteria has been attributed to several abiotic factors (Reardon *et al.*, 2014). In this study, PCA revealed an association with the soil texture and aspects related to soil fertility, most notably the presence of the basic cations Ca, Mg and K.

In a previous study done in the same area, Conte *et al.* (2011) observed an increase in density and a reduction in labile carbon with the most intensive grazing, suggesting that this treatment would result in a loss of soil quality. This trend was not confirmed in our study, possibly because the natural grasslands of the Pampa biome are complex systems in which a reduction in one soil quality indicator will not necessarily be accompanied by a reduction in the others. However, considering the biochemical and microbiological characteristics evaluated here and the history of the area in terms of individual animal performance (Mezzalira *et al.*, 2012), chemical and physical characteristics of the soil (Conte *et al.*, 2011) and floristic composition (Corrêa and Maraschin, 1994), the adoption of MP grazing appears to the most appropriate for animal production and conservation of the Pampa biome.

In conclusion, this study evaluated the microbiological quality of soil from the Pampa biome under different grazing intensities. In general, the presence of grazing animals was beneficial for the soil microbial community. Higher intensities of grazing favored an increase in microbial biomass and enzymatic activity. The lowest grazing level showed a greater diversity of diazotrophic bacteria. Additional studies on the microbial diversity in pastures of the Pampa biome are underway and will improve our understanding of the effects of different grazing intensities on the microbial community in soils of this complex ecosystem.

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