



Original article

Cattle excreta on *Lotus tenuis*-promoted grasslands: Exploring soil microbiome and nitrous oxide emissions in the Salado River Basin, Argentina

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ABSTRACT

Cattle production generates inputs such as urine and dung that increase soil microbial diversity by adding organic matter and nitrogen. Legume pastures, such as *Lotus tenuis* improve soil quality by incorporating nitrogen, influencing microbial communities, and contributing to nitrous oxide (N₂O) emissions. This study aimed to investigate the impact of livestock and pasture systems on soil microbiomes and N₂O emissions within grassland ecosystems. We analysed microbial communities in soils of natural and *L. tenuis*-promoted grasslands, using Next Generation Sequencing (NGS) and simulated urine and dung depositions in field mesocosms. The results revealed significant differences in microbial diversity between natural and *L. tenuis*-promoted grasslands across cattle input treatments. Biomarker analysis identified distinct *phyla* in each type of excreta and grassland system. In natural grasslands, *Proteobacteria* and *Actinobacteriota* were prevalent with urine, whereas *Acidobacteriota* and *Verrucomicrobiota* characterised the soils promoted by *L. tenuis*. The indicators of the dung-treated soils were *Pseudonocardia*, *Flavobacterium*, NP-4, *Adhaeribacter*, *Pseudoxanthomonas*, and *Novosphingobium*, while *Brevundimonas*, *Defluviococcus*, *Sphingomonas*, *Sphingomicrobium*, REEP01, *Chthoniobacter*, *Hyphomicrobium*, and PSRF01 were microbial indicators of the urine-treated soils. The N₂O emissions were significantly lower in fields with *L. tenuis* and urine addition compared to natural grasslands under similar conditions, with genera like *Brevundimonas*, *Hyphomicrobium*, and *Nitrosocosmicus* positively correlated with emissions. These findings underscore the role of pasture composition in shaping soil microbiomes and highlight the benefits of legumes, such as *L. tenuis*, in reducing N₂O emissions, providing an alternative for more sustainable livestock management practices.

1. Introduction

The Flooding Pampa is located in the Salad River Basin in Argentina, covering 90,000 km². In this area, agriculture is limited by flooding, salinity and alkalinity [1]. In this context, cattle production has become the main economic activity, with traditional agriculture persisting in areas with better soil conditions. However, the soil limitations of these marginal lands restrict the legume establishment and persistence of high-quality pastures. To address these constraints, the local farmers

have introduced legumes tolerant to abiotic stress conditions into the grassland systems. *Lotus tenuis* was successfully naturalised in the lowlands of the Salado River Basin, and its implementation boosts livestock nutrition by improving access to protein, and strengthening the resilience and sustainability of the grassland ecosystem [2,3]. While legumes improve forage quality, cattle activity generates outputs that contribute to modifying the soil conditions through the deposition of organic residues. These incorporations, mainly represented by urine and dung are sources of organic matter, nitrogen and microorganisms.

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Livestock farming is an activity that releases greenhouse gases into the atmosphere. Among other gases, it contributes to the emission of nitrous oxide (N_2O) [4]. On a global scale, N_2O emissions from excreta deposited on pastures account for 33 % of total N_2O emissions. In Argentina, this value increases to 52 % [5]. As agriculture is a primary contributor to this greenhouse gas, the intervention in nitrogen enrichment challenges is crucial. [6]. Microbial community structure is a better predictor of potential denitrification process than the functional gene abundance [7]. Thus, to investigate the microbial composition of soils under livestock farming, provides critical information to understand the process of N_2O emissions.

We pursue to analyse the effect provoked by cattle activity on the soil bacterial community of two grassland systems: natural and *L. tenuis*-based grassland. As detailed in previous research, the diversity of soil bacteria and fungi was analysed in both pasture systems [8,9]. However, the effect of the animal component combined with the pasture on the soil microbiome remains undetermined. Thus, analysing the impact of cow depositions on soil microbial diversity could improve the holistic approach to understanding the effect of the cattle system on the environment.

Therefore, a comprehensive study of soil microbiota and the determination of biomarkers are essential for understanding the nutrient dynamics and environmental features. We hypothesised that *L. tenuis* when used as a legume-based monoculture for pasture, along with cattle-derived excreta (urine and dung) from animals grazing on the same pasture system, would significantly influence soil microbial communities and impact nitrous oxide emissions. The objective of the study was to analyse the implementation of a full legume-based system in comparison with a poor protein forage, considering the respective soils and excreta of each grassland system and their impact on soil microbiome diversity.

This study used Next-Generation Sequencing technology (NGS) through the metabarcoding strategy to analyse soil samples collected from experimental mesocosms simulating cow depositions in both pasture systems. This approach provides new insights into the microbial and environmental factors related to the use of *L. tenuis* as the main

component of the diet in cattle production.

2. Materials and methods

2.1. Experimental design

The experiment was located at Chacra Experimental Integrada Chascomús (S 35° 45'01"; W 58° 02'22") in the Salado River Basin (Chascomús, Buenos Aires, Argentina). Paddocks with natural grassland cover (*Natural grass*) and promotion of *L. tenuis* (*Lotus tenuis*) conditions were identified as experimental stations. The experiment took place in January 2017, during the summer season in Argentina. The plant composition of both grassland systems was previously described by Nieva et al. [8,9]. The type of soil was *Natraquoll* for both treatments. Representative amounts of urine and dung were added to the soil surface to simulate the effect of cattle waste on the soil bacterial community.

The experimental design (Fig. 1) was completely randomised with three factors according to the type of excreta: "urine", "dung", and "control" without excreta addition. Urine and dung were collected from cows that had grazed exclusively on either *L. tenuis* or natural grassland for 15 days prior to sample collection. Each type of excreta from *L. tenuis*-fed cows was applied to plots with *L. tenuis* cover, while excreta from cows grazing on natural grassland were applied to plots with natural grassland vegetation. This procedure ensured that the excreta reflected the specific diet associated with each pasture system. Quadrants of 0.30 m² were delimited in the selected paddocks. Each quadrant constitutes one replicate. The paddocks had a total area of 425 m², and the distance between quadrants was 4 m. The treatments were performed by adding 0.8 L of urine and 1.5 kg of dung collected from healthy animals, for the urine and dung treatments, respectively. The chosen amounts of urine and dung were based on average volumes/masses collected from healthy animals. For dung, we collected 20 freshly deposited dung pats from the field, measured their individual weights, and calculated the average, which was 1.5 kg. This average value was then used as the standardised amount for the dung treatments. For urine, the volume of 0.8 L was selected to represent a realistic deposition event

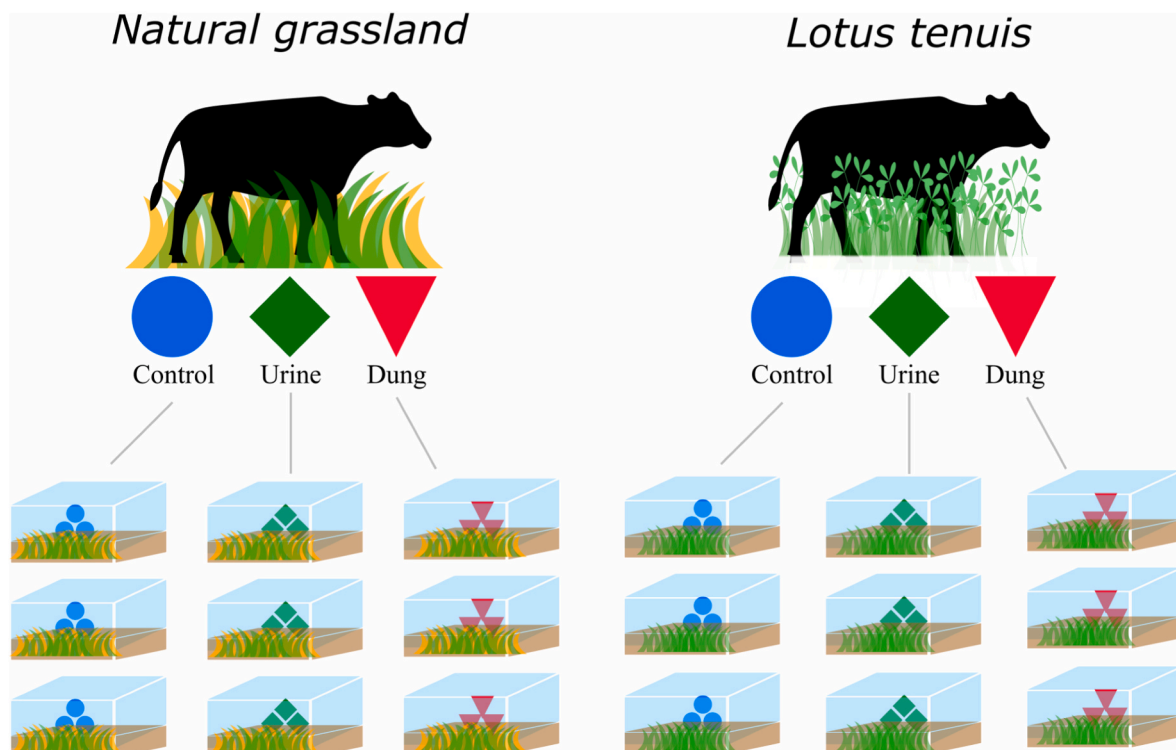


Fig. 1. Experimental design.

for an individual animal, while ensuring that the wetted area did not exceed the limits of the static chambers used for gas flux measurements in order to capture the totality of emissions within the chamber area. This volume falls within the reported range for beef cattle [10,11]. The control condition was performed by adding 1 L of distilled water. Three replicates were used for each combination of treatment and vegetal cover.

After applying the organic materials, the quadrants were left exposed under natural field conditions. Soil samples were collected 10 days after excreta application to capture the microbial response during the early phase following treatment. In each quadrant, five subsamples were taken from the top 15 cm using a borer and then pooled into a composite sample. Samples corresponding to the same treatment and plant coverage condition were homogenised and sieved through a 2 mm mesh. DNA extraction was performed from aliquots of 0.5 g of each soil sample using the *NucleoSpin* soil DNA extraction kit (Macherey-Nagel).

DNA amplification and sequencing were conducted by the Genome Sequencing facilities from the *Centre d'expertise et de services Génome* (Québec, Canada) using the Illumina HiSeq platform. The V3-V4 region of the 16 S rRNA gene was amplified using the universal primers 341 F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATC-TAATCC-3' according to Herlemann et al. [12].

2.2. Sequencing data analysis

Bioinformatic analysis of sequences and further diversity analysis was conducted using the software QIIME2 (q2) version 2020 [13]. The analysis of sequences was performed using the high-resolution sample inference, DADA2 [14], which is available as a plugin in q2. The parameters used for truncation were *-p-trunc-len-f 220* and *-p-trunc-len-r 180*. The unique sequences obtained from denoised data were considered Amplicon Sequence Variants (ASV) based on single nucleotide differences.

Diversity analysis was determined on the resulting ASV feature table after subsampling to the lowest sequences per sample. The diversity analysis was performed using the *q2-core-diversity* plugin from QIIME2 and the *microeco* package for the R environment [15]. Shannon, Simpson, Chao1, and Faith's phylogenetic diversity indices estimated the alpha diversity to infer the richness and distribution of the bacterial soil community of each combination of pasture system and treatment.

Beta diversity was calculated through the weighted UniFrac distances, and the statistical differences were tested using the PERMANOVA test ($p < 0.05$) to compare the diversity between the communities.

The taxonomic classification was performed using a Naive Bayes classifier trained on the Greengenes 13.8 reference database (version 2013) [16]. Features assigned as chloroplast or mitochondria were filtered out from the dataset before the downstream analysis. The classification of the representative sequences was performed by the *q2-feature-classifier* plugin using the *classify-sklearn* strategy.

The selection of microbiome biomarkers was performed by the Linear Discriminant Analysis Effect Size (LEfSe) [17] using the *trans.diff* function included in the *microeco* package. Additionally, the packages *phyloseq* [18], *vegan* [19] and *ggplot2* [20] for the R environment were used for the diversity analysis and data visualisation.

2.3. Gas sampling measurements

Nitrous oxide sampling was carried out during the summer season. In addition, the most important regulating factors of N₂O were measured before sampling. Thus, the soil nitrate content at 0–10 cm depth ranged between 50 and 120 mg N-NO₃ per kg of dry soil, and the water-filled pore space varied between 60 % and 80 %, indicating that the main variables regulating N₂O emissions were not limiting during the sampling period [21]. Closed static chambers (area = 0.13 m², height = 0.295 m) consisting of an iron frame and a PVC cover were used for

sampling. The chamber's headspace was connected to the outside via a two-way valve for gas sampling. The bases were placed in the soil (0.05 m depth) 24 h before the start of sampling and were not moved or rotated in position within the plot [22,23]. Gas samples were taken from the chamber headspace at 0, 20 and 40 min after closing the chambers. After evacuation, the gas was sampled with a syringe (20 ml) and injected into 13 cm³ glass vials using a manual vacuum pump and a manometer. Gas samples were collected between 9 and 11 a.m., as described by Cosentino et al. [21]. The N₂O concentration was measured using a GC equipped with an electron capture detector and a "back-flush system" [24] at the EMBRAPA Agrobiology Laboratory (Brazil). The N₂O fluxes (f) were calculated as:

$$f = \Delta C / \Delta t \times V / A \times m / V_m$$

where $\Delta C / \Delta t$ is the change in the corresponding gas concentration in the chamber during the incubation period (Δt); V is the chamber volume; A is the soil area covered by the chamber; m is the N₂O molecular weight; and V_m is the molar volume of the corresponding gas.

The gas flux was calculated as the slope of a linear function fitted to the change in N₂O concentration during the incubation period. The N₂O emission fluxes over the 10 days of the maximum emission period were accumulated using the trapezoidal rule. This method approximates the area under the flux-time curve by dividing it into trapezoids and adding their areas. By applying this technique, we obtained an accurate estimate of the cumulative N₂O emissions for the experiment.

Correlation analysis was performed to assess the strength of the relationship between N₂O content and ASV abundance. Spearman correlations were calculated using the *trans.env* class of the *microeco* package for the R environment [15].

3. Results

3.1. Diversity analysis

After filtering by quality, 770,925 sequences of the 16 S rRNA gene were obtained, which generated 6791 unique Amplicon Sequence Variants (ASV). The ASV read counts table was subsampled to 27,754 sequences per sample according to the lowest number of reads to assess the bacterial diversity analysis. The sequences of this study are available in the GenBank of the National Center for Biotechnology Information (NCBI) under the project accession code PRJNA1100591. The read counts of each ASV per sample is shown in Supplementary Data.

Alpha diversity indices (Shannon, Simpson, Chao1 and Faith's phylogenetic diversity), calculated from the subsampled ASV table, indicating similar values between the two pasture systems at control conditions (Fig. 2a). However, these indices decreased in soils of *L. tenuis*-based management under the dung treatment. (Kruskal-Wallis pair-wise comparisons, $p < 0.05$, Fig. 2a).

Beta diversity, estimated by weighted UniFrac distances, displayed significant differences between the microbial diversity of soils of *L. tenuis*-based and natural grassland-based pasture systems (Fig. 2b). Patterns observed in the PCoA were statistically verified by the PERMANOVA test (999 permutations), revealing significant differences in soil bacterial communities between pasture systems when considering all management conditions (control, urine, and manure treatments; pseudo- $F = 4.351$, $p = 0.001$). To further evaluate the contribution of individual factors, we performed additional pairwise comparisons. No differences were detected between the Control treatments (without excreta) for the comparisons between natural grassland and *L. tenuis* (pseudo- $F = 1.363$; $p = 0.094$). When comparing the same excreta type between pasture systems, non-significant differences were observed for urine (pseudo- $F = 2.398$, $p = 0.095$) and dung (pseudo- $F = 2.016$, $p = 0.108$). Pairwise comparisons between excreta types (urine vs. dung) were not significant within either pasture system, with no differences observed in the natural grass (pseudo- $F = 1.183$, $p = 0.212$) or in the

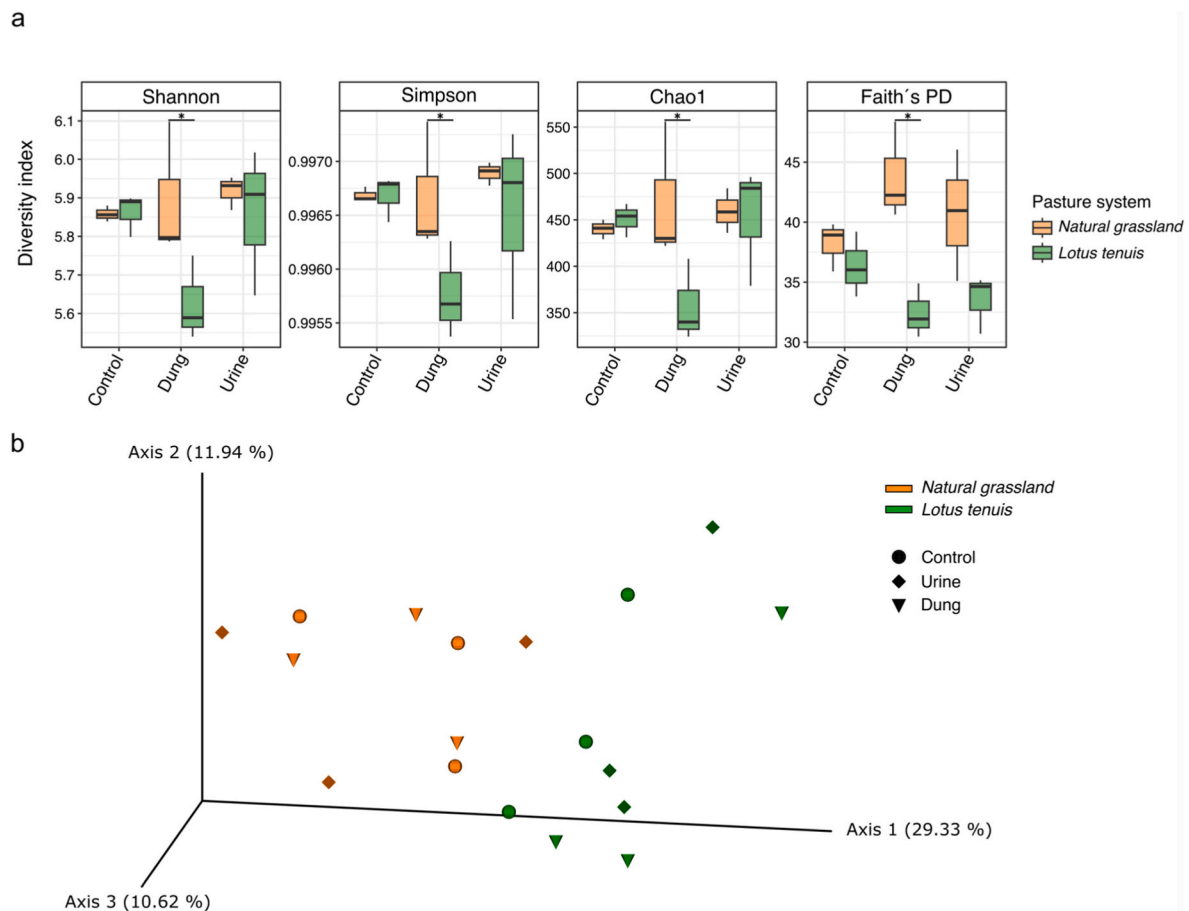


Fig. 2. a) Alpha diversity indices (Shannon, Simpson, Chao1, and Faith's PD) based on rarefied ASV abundance from soil samples under different pasture systems (*Lotus tenuis* and natural grassland) and excreta treatments (control, urine, dung). Differences were tested using pairwise Kruskal-Wallis tests (* $p < 0.05$). b) Ordination by Principal Coordinate Analysis (PCoA) based on weighted UniFrac distances of ASV from 16 S rRNA gene sequences, showing similarities in bacterial community composition across pasture systems (*Lotus tenuis*, natural grassland) and excreta treatments (control, urine, dung).

L. tenuis system (pseudo- $F = 0.812$, $p = 0.604$).

3.2. Taxonomy analysis

According to the *Greengenes* database, the classification of ASV displayed 759,345 sequences belonging to the Kingdom *Bacteria*, which represented 98.5 % of the total sequences, whereas 11,580 were classified as *Archaea*, representing 1.5 % of the total sequences.

The most abundant phyla were classified as *Acidobacteriota*, *Proteobacteria*, *Chloroflexota*, *Actinobacteriota*, *Planctomycetota*, *Gemmatimonadota*, *Methylomirabilota*, *Verrucomicrobiota*, *Firmicutes_D*, *Bacteroidota*, *Desulfobacterota_B*, *Myxococcota_A_473307*, *Thermoproteota (Archaea)*, *Eisenbacteria* and *Nitrospirota_A_437815*. The relative abundance of these phyla was similar among the treatments evaluated (Fig. 3a). We distinguished 14 genera with statistically significant differences among the treatments (Fig. 3b). The genera *Brevundimonas* and *Chthoniobacter* were more abundant in urine treatments, while *Terrimicrobium*, *Flavobacterium*, *Pseudoxanthomonas_A_615337* and *NP-4* were more abundant in soils with dung treatments. The relative abundance of *Sphingomicrobium_483265* and *Novosphingobium_485351* was significantly higher in *L. tenuis* than in natural grassland system, across all the treatments.

3.3. Microbial biomarkers for each combination of excreta and pasture system

To identify the most representative taxa for each combination of

excreta and pasture system, we conducted a LEfSe analysis to detect microbiome biomarkers. The analysis revealed forty-nine significant taxa. LDA scores (Fig. 4) indicated that *Terrimicrobium sacchariphilum* and class *UBA8108* were associated with the natural grassland pasture system at control conditions (without excreta). However, the incorporation of excreta on these soils revealed more bacterial indicators related to this pasture system. In urine treatment, the higher taxonomic level was represented by the class *Actinomycetia*. The genera *Deffluviococcus_A (D. vanus)*, *REEP01*, *Sphingomonas_L_486704* and *Brevundimonas* were detected as biomarkers for the urine condition in the natural grassland pasture system. On the other hand, the genera *Sphingomicrobium_483265*, *PSRF01*, *Chthoniobacter (C. flavus)* and *Hyphomicrobium_A* were significant for the urine treatment of *L. tenuis* pasture system. For the dung treatment, the genera *Pseudonocardia*, *Flavobacterium* and *NP-4* were biomarkers for the natural grassland system. At the same time, *Novosphingobium_485351*, *Adhaeribacter terrigena* and *Pseudoxanthomonas_A_615337* were the biomarkers for the soils of the *L. tenuis* after dung incorporation. There was correspondence between the taxa selected as biomarker and their relative abundance for the combination of excreta and grassland (Supplementary Fig. 1). This is the case of *Flavobacterium* and *Brevundimonas*, also with significant differences in relative abundance (Fig. 3b) and selected as indicators of dung and urine, respectively.

3.4. Correlation between N_2O content and soil bacteria

Our results revealed that soils treated with urine exhibited elevated

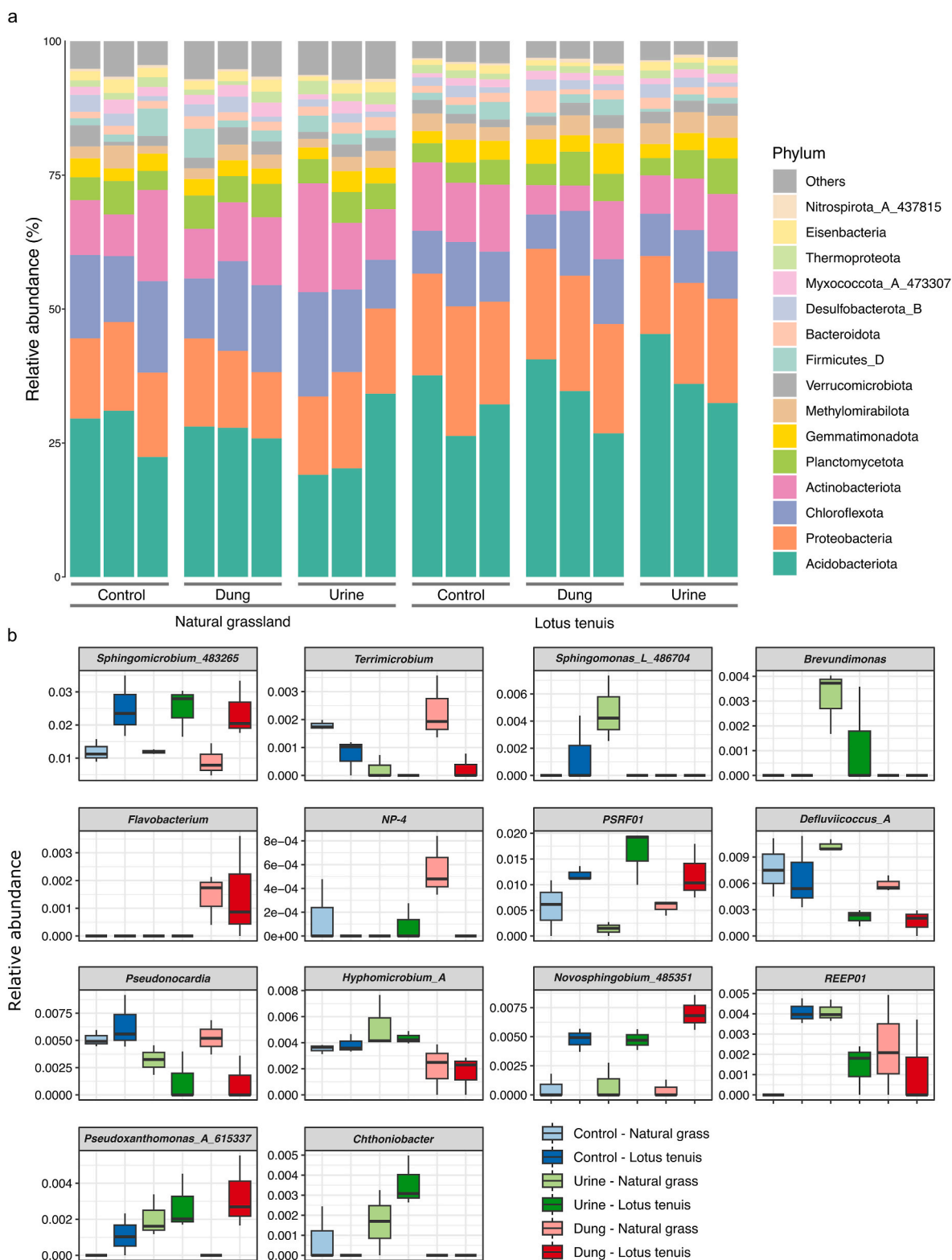


Fig. 3. Relative abundance of ASV obtained by 16 S rRNA gene sequencing of soils of the systems *L. tenuis* or Natural grassland treated with water (Control), urine and dung from the abovementioned systems. a) ASV grouped at the *phylum* level according to the *sklearn* classifier and *Greengenes* database. b) Genera that revealed significant differences according to Kruskal-Wallis test ($p < 0.05$). P-value adjusted by False Discovery Rate (FDR).

N₂O emissions (Fig. 5). However, the soils of *L. tenuis* subjected to this excreta treatment showed lower N₂O emission levels compared to those of natural grassland. Meanwhile, the control and dung treatment showed similar N₂O emissions across both grassland systems.

Correlations between soil bacteria abundance and N₂O content were

analysed using standardised N₂O data and rarefied abundance of ASV at the genus level (Table 1). The Spearman correlation coefficients indicated significant positive correlations between the N₂O emissions and several bacterial classes including *Alphaproteobacteria* (*Hyphomicrobium_A* and *Brevundimonas*), *Thermoanaerobaculia* (*JAAAYLR01*),

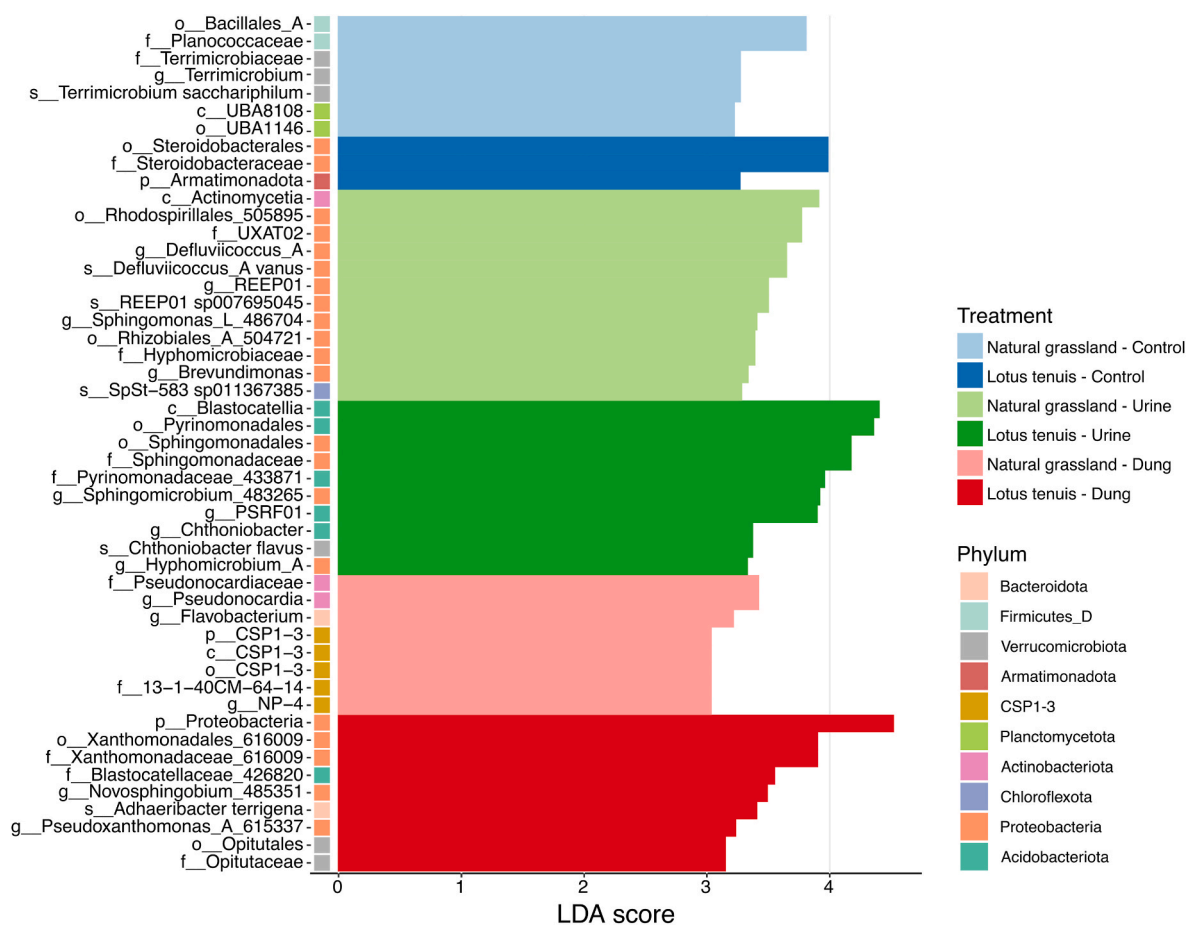


Fig. 4. Linear Discriminant Analysis (LDA) scores of differential relative abundances of ASV grouped at all taxonomic ranks.

Verrucomicrobiae (*Chthoniobacter* and *ER46*), *Phycisphaerae* (*JAAYXS01*), *Actinomycetia* (*Isopetricola*), as well as the *Archaea Nitrosocosmicus*. Conversely, negative correlations were observed for *Myxococcota*, *Planctomycetota* and certain members of the phylum *Acidobacteriota*.

4. Discussion

The soil microorganisms play crucial roles in pasture systems due to their involvement in nutrient cycling, soil structure, and the dynamics of water and organic matter. In our study, the diversity of the soil bacterial community was evaluated under the effect of simulated cattle depositions and two different pasture systems. By applying specific amounts of urine and dung, we replicated the expected deposition events of cattle on soils. This experimental system demonstrated the importance of the integrated pasture system, which encompasses the arrangement of vegetation type, the soil microbiome, and N_2O emissions, in shaping the soil microbial system.

Previous studies performed in the Flooding Pampa by Perez et al. [25] revealed the increase of N_2O emissions in grasslands composed of *Festuca*, *Agropyron*, *Trifolium* and *Lotus* after urine application. Although the results presented in this research revealed the same tendency in the urine application, the soils under the *L. tenuis* system displayed lower levels of N_2O emissions. This reduction may be due to the mitigating effect of legumes on N_2O emissions. These results are in line with previous studies showing comparable effects of legumes on N_2O emissions [26–28]. Although, legumes can increase mineral nitrogen availability and thus potentially increase emissions, they also contribute to the immobilisation of inorganic nitrogen, stabilise soil organic matter, and promote carbon sequestration.

4.1. Soil bacterial diversity decreased under dung depositions of *Lotus tenuis*

The alpha diversity analysis indicated similar trends for all the indices evaluated. Our results suggest that dung depositions have a significant impact on soil bacterial diversity in the *L. tenuis*-based system. The decrease in the alpha diversity of soils affected by dung depositions indicated significant differences between the *L. tenuis* system and the natural grassland cover. This finding contradicts previous studies suggesting that cow manure increases soil bacterial diversity [29–31]. On the other hand, research has determined that diet composition is a decisive factor in faecal protein content [32,33]. This evidence could explain the modification of the alpha diversity after the dung incorporation in the *L. tenuis* system and the nitrogen addition in soils mediated by the legume. The incorporation of dung may introduce rumen-derived bacteria into the soil, which could maintain a distinct microbial community composition, characteristic of this organic material and partially explain the observed differences in diversity. This trend may indicate the long-term stabilisation of the microbial community in this condition. Our previous research on these grassland systems revealed differences in physical soil properties in soils with *L. tenuis* [8]. It suggests that urine may drain easily and that its effects on soil bacterial diversity were lower than expected due to the short time of exposure. The Faith's phylogenetic index shows lower values for samples treated with excreta in *L. tenuis* than in natural grassland, indicating reduced phylogenetic diversity and fewer represented lineages. These findings align with the presence of taxa that are highly specialised and associated with dung environments.

The differences in beta diversity revealed differential effects caused by the implementation of *L. tenuis* in the Flooding Pampa, considering

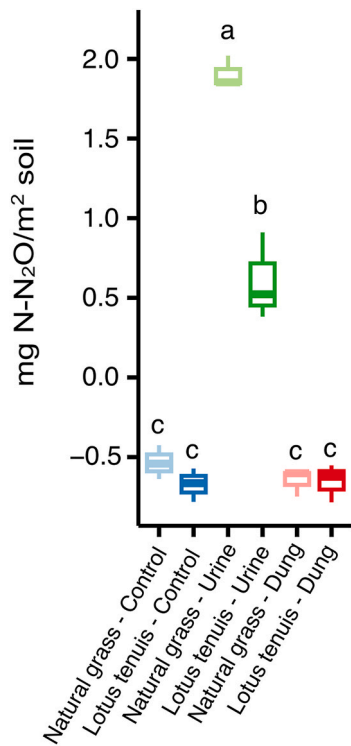


Fig. 5. Standardized mean of cumulative nitrous oxide (N_2O) emissions measured on the soil mesocosms after 10 days of water (control), urine, and dung application. Different letters indicate significant differences for Duncan's Test ($p < 0.05$).

the cattle depositions as part of the management system. Our previous studies in the same experimental area suggested *L. tenuis* did not modify the soil bacterial community [8]. This trend was evaluated by comparing the control conditions (without excreta) between *L. tenuis* and natural grassland, confirming the absence of differences in beta diversity. This result may indicate that the main contributions to modifying the soil microbiome are driven by the combination of animal depositions and plant diversity.

The *Acidobacteriota* was the most abundant phylum. This finding aligns with previous studies, which indicate that these microorganisms are characteristic of soils and grassland environments [34,35]. Similar relative abundances of the dominant phyla (*Proteobacteria*, *Actinobacteria* and *Chloroflexota*) were also observed in grassland systems [36]. The relative abundance of *Proteobacteria* and *Actinobacteria* in soils of pasture legumes may indicate the relation between these phyla and the presence of nitrogen-fixing bacteria [37]. The abovementioned phyla are typical soil and grassland microorganisms that have been widely reported, and they may be related to a combination of grassland systems, legume, and soil conditions.

Although similar trends were observed in the distribution of ASV abundance at the phylum level, some ASV groups at the genus level increased significantly their relative abundance after excreta application.

LEfSe analysis identified distinct microbial biomarkers across pasture systems and excreta treatments. Under control conditions, only three indicators were identified for *L. tenuis* system, underscoring the influence of excreta depositions on microbial marker selection. In urine-treated natural grasslands, biomarkers were primarily *Proteobacteria* and *Actinomycetia*, reflecting their roles in the nitrogen cycle. Conversely, *L. tenuis* soils treated with urine also exhibited indicators from the *Acidobacteriota* and *Verrucomicrobiota* phyla, suggesting higher functional diversification.

In soils affected by dung, biomarkers such as *Pseudonocardia* and

Table 1

Spearman correlation analysis between rarefied ASV abundance and standardised N_2O content ($p < 0.05$). P-value adjusted by False Discovery Rate (FDR).

Taxa	Correlation coefficient	P-value	Adj. P-value
<i>d_Bacteria p_Proteobacteria </i>	0.648	0.003	0.003
<i>c_Alphaproteobacteria o_Caulobacteriales </i>			
<i>f_Caulobacteraceae g_Brevundimonas</i>	0.623	0.005	0.005
<i>d_Bacteria p_Proteobacteria </i>			
<i>c_Alphaproteobacteria </i>			
<i>o_Rhizobiales_A_504721 </i>			
<i>f_Hyphomicrobiaceae g_Hyphomicrobium_A</i>	0.595	0.009	0.009
<i>d_Archaea p_Thermoproteota </i>			
<i>c_Nitrososphaeria_A o_Nitrososphaerales </i>			
<i>f_Nitrososphaeraeae g_Nitrosococcus</i>	0.589	0.010	0.010
<i>d_Bacteria p_Acidobacteriota </i>			
<i>c_Thermoanaerobaculia o_UBA5704 </i>			
<i>f_UBA5704 g_JAAYLR01</i>	0.564	0.014	0.014
<i>d_Bacteria p_Verrucomicrobiota </i>			
<i>c_Verrucomicrobiae o_Opitutales </i>			
<i>f_Opitutaceae g_ER46</i>	0.534	0.022	0.022
<i>d_Bacteria p_Verrucomicrobiota </i>			
<i>c_Verrucomicrobiae o_Chthoniobacteriales </i>			
<i>f_Chthoniobacteraceae g_Chthoniobacter</i>	0.513	0.029	0.029
<i>d_Bacteria p_Planctomycetota </i>			
<i>c_Phycisphaerae o_UBA1845 f_Fen-1342 </i>			
<i>g_JAAYXS01</i>	0.478	0.044	0.044
<i>d_Bacteria p_Actinobacteriota </i>			
<i>c_Actinomycetia o_Actinomycetales </i>			
<i>f_Cellulomonadaceae g_Isopterocola_389305</i>	-0.474	0.046	0.046
<i>d_Bacteria p_Myxococcota_A_473307 </i>			
<i>c_UBA9160 o_SZUA-336 f_SZUA-336 </i>			
<i>g_SMWZ01</i>	-0.481	0.042	0.042
<i>d_Bacteria p_Planctomycetota c_UBA1135 </i>			
<i>o_UBA1135 f_GCA-002686595 g_Pla86</i>	-0.486	0.042	0.042
<i>d_Bacteria p_Acidobacteriota </i>			
<i>c_Blastocatellia o_Pyrinomonadales </i>			
<i>f_Pyrinomonadaceae_433912 g_QHXM01</i>	-0.495	0.036	0.036
<i>d_Bacteria p_Acidobacteriota c_HRBIN11 </i>			
<i>o_HRBIN11 f_HRBIN11 g_HRBIN11</i>	-0.513	0.029	0.029
<i>d_Bacteria p_Chloroflexota c_Chloroflexia </i>			
<i>o_Chloroflexales_407288 </i>			
<i>f_Chloroflexaceae g_Oscillochloris</i>	-0.535	0.021	0.021
<i>d_Bacteria p_Proteobacteria </i>			
<i>c_Alphaproteobacteria o_Dongiales </i>			
<i>f_Dongiaceae g_SYSU-D60009</i>			

Flavobacterium were detected within the *Actinobacteriota* phylum. Both genera have been reported in agricultural soils [38,39]. In the case of *L. tenuis*, the dung incorporation into the soil surface displayed indicators of the phylum *Proteobacteria* (*Xanthomonas*, *Pseudoxanthomonas* and *Novosphingobium*) and *Bacteroidota* (*Adhaeribacter*).

4.2. N_2O emissions fluctuated among the excreta and pasture system and correlated with the bacterial relative abundance of nitrogen-related taxa

The high levels of nitrous oxide gas emissions observed in urine-treated soils were expected due to the introduction of a nitrogen-rich substrate like urine. The impairment in the N_2O emissions in soils linked to the *L. tenuis* system suggests a more efficient utilisation of nitrogen associated with the legume-based diet. This result is consistent with research on the condensed tannins present in *Lotus* species, which prevent cattle bloating by inhibiting the degradation of plant protein [2, 40–42].

The correlation between the relative abundance of ASV and N_2O content revealed significant positive and negative correlations associated with the nitrogen cycle for both *Bacteria* and *Archaea*. We identified ASV linked to denitrification activity, like *Hyphomicrobium* [43,44] and *Brevundimonas* [45], which displayed the highest correlation coefficients. The relative abundance of *Brevundimonas* was significantly elevated in soils with urine treatment, indicating the influence of this excreta on natural grassland soils. This finding reinforces the relation

between nitrogen sources and the denitrification process. However, *Hyphomicrobium* was an indicator of the same treatment in the *L. tenuis* system, suggesting that different taxonomic groups may play roles in driving the denitrification process depending on the specific grassland system.

The *Archaea Nitrosocosmicus* exhibited a positively correlation with N_2O levels. However, it was not identified as a microbial indicator and did not show significant variations in relative abundance across the treatments and grassland system combinations. Previous studies have reported it as ammonia-oxidising organisms in soils with elevated ammonia content [46–48], contributing to the production of N_2O in the soil environment. Some studies showed that nitrous oxide emissions are influenced by abiotic factors such as moisture and nitrate levels, as well as by microbial communities involved in nitrification and denitrification [49,50].

In pasture systems, the interaction between specialised microorganisms including ammonia-oxidising archaea and denitrifying bacteria, significantly impacts on N_2O emissions. Furthermore, management practices that influence microbial composition include plant species and grazing intensity [51]. Our results suggest that systems based on *L. tenuis* may promote microbial communities associated with lower N_2O production, in contrast to systems dominated by grasses.

Recent findings underscore the critical role of microbial management in reducing N_2O emissions [52]. These studies highlight the significance of plant–soil interactions and animal management in shaping microbial communities for N_2O mitigation, aligning with our observations in *L. tenuis*-based systems. The correlation analysis also displayed significant correlations for ASV previously isolated from restricted environments or agricultural soils subjected to recurrent flooding, such as those found in the Flooding Pampa region. This includes *Isopterocola* isolated from saline sediments [53,54] and *ER46* identified in paddy soils [55]. This trend suggests that the conditions influencing emissions may also be shaped by the soil type and moisture level [56].

5. Conclusion

This study identified the biological effects of integrating plant and animal components in cattle production systems based on legumes or natural grasslands. The incorporation of *L. tenuis* as a predominant legume in these grasslands, along with its use as cattle feed assists a distinctive soil microbial response under dung application. Furthermore, N_2O emissions were attenuated in *L. tenuis* grasslands, even with the addition of urine, suggesting that *L. tenuis*-based grassland systems play a role in mitigating emissions and promoting nitrogen immobilisation. Our findings support the hypothesis that *L. tenuis*-based grassland systems influence the microbiome composition and reduce N_2O emissions. Incorporating legumes into pasture management may promote more sustainable livestock practices by modulating the soil microbiome and reducing N_2O emissions.

CRedit authorship contribution statement

Amira Susana del V. Nieva: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Mónica G. Pérez:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Cristian J. Antonelli:** Methodology, Investigation, Formal analysis. **Alejandro O. Costantini:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Oscar A. Ruiz:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejsobi.2026.103805>.

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