



The effect of sheep grazing abandonment on soil bacterial communities in productive mountain grasslands

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ABSTRACT

Livestock grazing abandonment entails important shifts on the overall ecosystem function, but the effects of this land-use change on specific bacterial taxa remain poorly understood in mountain grasslands. Moreover, we currently lack knowledge about the feedbacks between changes in ecosystem functions affected by livestock abandonment in mountain grasslands and the soil bacterial communities. Here, we evaluated the behavior of bacterial communities' structure and composition at taxa level as a function of short (1-year) and long-term (15-years) grazing abandonment in a mountain grassland. We also linked the observed responses in the bacterial communities to changes in several ecosystem functions (i.e. primary production, plant species biodiversity, carbon stocks and soil fertility). The alpha diversity of the bacterial communities did not show a significant response as a consequence of grazing abandonment. However, we identified significant changes on the overall composition of soil bacterial communities between the long-term abandoned grassland areas and grazed or abandoned areas in the short term. We also evidenced a balance between the number of operational taxonomic units (OTUs) whose relative abundance is favored by livestock grazing (19.51 %) and those with higher relative abundances in long-term grazing exclusion areas (20.23 %) that could behave as indicators of grazing abandonment. Structural Equation Modeling analyses proved that several bacterial taxa associated with relevant ecosystem functions, such as Rhodospirillales order within Alphaproteobacteria phylum, featured significant changes in their relative abundance between grazing treatments. The direct and indirect effects of grazing exclusion on woody species encroachment and soil organic carbon were strongly linked to the changes in the abundance of bacterial taxa indicators. The assessment of the bacterial community response to livestock abandonment in mountain grasslands may thus provide early warning signs before subtle changes in ecosystem functions occur.

1. Introduction

Extensive livestock grazing encompasses about 50 % of the Earth's land surface, particularly in Europe, Asia and Africa (Oggioni et al., 2020). In fact, the complex socio-ecological systems created by this traditional land use (Ingty, 2021) help to support millions of households globally (Dong et al., 2011). Semi-natural grasslands cover 30 % of the agricultural surfaces in the European Union and are the main source of fodder for extensive livestock herds (Lomba et al., 2014; Bengtsson et al., 2019). These ecosystems also provide other valuable services and associated ecosystem functions for people's livelihoods around the globe. These services include, among others: global warming mitigation (Chen et al., 2022a), plant pollination (Orford et al., 2016), nutrient cycling (Evans et al., 2019) or wildlife habitat (Faria et al., 2012). Re-

markably, the provision of ecosystem services in semi-natural grasslands have evolved through centuries of extensive pastoral activities with low livestock grazing intensities (Zhao et al., 2020; Fernández-Guisuraga et al., 2022).

Currently, bidirectional land-use changes involving livestock intensification (Chen et al., 2014) and abandonment (Jarque-Bascuñana et al., 2022) are threatening the ability of semi-natural grasslands to deliver multiple ecosystem functions or services (Fernández-Guisuraga et al., 2022; Yang et al., 2022). Extensive livestock abandonment is particularly important in less-favored mountain regions of Europe since the mid-20th century (Lasanta et al., 2006; Stoate et al., 2009). This is a consequence of the low extensive livestock benefits in comparison with intensive systems in lowland farmlands (Lasanta et al., 2006) and the little support of the Common Agricultural Policy (CAP) of the European

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Union to extensive systems (Lomba et al., 2014; García-Llamas et al., 2019). This decline is particularly relevant in the case of traditional livestock transhumance system between different regions that involves the summer use of mountain pastures by sheep flocks with low stocking rates (Román-Trufero et al., 2019; Fernández-Guisuraga et al., 2022).

The abandonment of traditional systems entails direct effects on the vegetation and soils of mountain grasslands, resulting from the absence of processes directly driven by grazing such as defoliation, dunging and trampling (Aldezabal et al., 2015; Oggioni et al., 2020). Aboveground biomass consumption by livestock leads to less litter inputs to soils (Yang et al., 2013), changes in plant species diversity (Odriozola et al., 2017), increases in plant root exudation (Aldezabal et al., 2015) and modification of soil temperature and moisture (Eldridge et al., 2017). Meanwhile, livestock urine-feces deposition to soils, together with changes in litter quality and C—N inputs to the soil, accelerates nutrient cycling through enhanced mineralization rates (Semmartin et al., 2004; Medina-Roldán et al., 2012; Bi et al., 2018). Similarly, livestock trampling involves negative changes in soil structure by means of soil compaction (Gao and DeLuca, 2022), affecting soil infiltration rate (Döbert et al., 2021), plant root development (Hempson et al., 2015) and soil porosity (Aldezabal et al., 2015). Ultimately, traditional livestock grazing abandonment undermines the capability of productive grasslands to sustain ecosystem multifunctionality (Fernández-Guisuraga et al., 2022). Altogether, changes in these driving processes of vegetation and soils in mountain grasslands could involve important shifts in the abundance, composition, structure and activity of soil microbial communities (Butenschoen et al., 2011; Anderson et al., 2014; Sauvadet et al., 2016). Soil microorganisms play a critical role in the maintenance of grassland ecosystems' functioning (Prober et al., 2015; Eldridge et al., 2017) and, in particular, bacterial communities are essential in driving nutrient biogeochemistry cycles and primary production (e.g. Van Der Heijden et al., 2008; Schimel and Schaeffer, 2012; Zeng et al., 2017). Most research to date on the effect of grazing abandonment on soil bacteria worldwide (e.g. Aldezabal et al., 2015; Eldridge et al., 2017; Ren et al., 2018; Oggioni et al., 2020) have been focused on microbial biomass, activity (i.e. function) or communities' structure. Few studies have evaluated the different behavior among bacterial communities in grazed and abandoned areas at the operational taxonomic units (OTUs) and taxa level, but this approach has only been implemented in arid or semiarid grasslands (Zeng et al., 2017; Zhang et al., 2018). However, the behavior of soil microbial communities in response to livestock abandonment is expected to be completely different in productive mountain grasslands than in dry environments, where grazing processes on soil microbial communities would be stronger (Eldridge et al., 2017). Similarly, we currently lack knowledge about the feedbacks between changes in ecosystem functions affected by livestock abandonment in productive mountain grasslands (Fernández-Guisuraga et al., 2022) and the soil bacterial community. Filling these knowledge gaps would improve the understanding of the sustainability of traditional livestock farming practices with respect to the functionality of mountain grassland ecosystems in order to better guide management policies in the context of land-use changes.

We aimed to bridge these gaps by examining the effects of livestock abandonment on the bacterial communities in a mountain grassland of the Cantabrian Mountains range. Specifically, we evaluated the behavior of the bacterial communities' structure and composition at taxa level as a function of short and long-term grazing abandonment, linking the observed responses in the bacterial communities to changes in several ecosystem functions (i.e. vegetation biodiversity, nutrient cycling and carbon storage). We hypothesized that: (i) grazing exclusion will hinder bacterial community diversity, especially in long-term (>15-years) abandonment areas (Zhou et al., 2010) via changes in plant diversity and ecosystem nutrient cycling (Fernández-Guisuraga et al., 2022); (ii) shifts in bacterial communities composition as a consequence of competitive exclusion mediated by changes in ecosystem functions

(Eldridge et al., 2017) would allow the identification of bacterial taxa that could serve as indicators of livestock abandonment.

2. Material and methods

2.1. Site description and experimental design

The experimental site was located within the western sector of the Cantabrian Mountains, the Europe's westernmost mountain range, which extends along the northern border of Spain. The site, known as Las Pintas mountain pass (León province), covers an area of 389.39 ha (42°57'18" N and 5°07'10" W). The landscape configuration of Las Pintas, with high cultural value, is the result of transhumance sheep flocks' management since the last centuries (Morán-Ordóñez et al., 2013). Transhumant grazing by Spanish merino sheep breed was the dominant anthropic use up to the early 2000s, whereas no land use was recorded at any location in the study site between 2005 and 2015. Las Pintas reintroduced in 2015 the transhumant Spanish merino grazing activity in the grasslands with moderate stocking rates (0.2–0.4 livestock units/ha) per summer grazing season (July to September; Fernández-Guisuraga et al., 2022). Large wildlife herbivores present in the study site are Pyrenean chamois (*Rupicapra pyrenaica*) and European roe deer (*Capreolus capreolus*).

The altitude of the site ranges between 1060 and 1989 m, featuring an abrupt topography. Soils are classified as Humic Cambisols (CMu) and Dystric Leptosols (LPd) (ITACyL, 2022), originated over limestone and sandstone lithologies (GEODE, 2022). The site registered for a period of 50-years a mean annual temperature and rainfall of 6.5 °C and 1350 mm, respectively (Ninyerola et al., 2005). The land cover of Las Pintas mountain pass is dominated by rock outcrops, grasslands with high pastoral value species such as *Festuca rubra* L., *Sanguisorba minor* Scop. and *Trifolium repens* L., *Fagus sylvatica* L. forests, as well as *Juniperus communis* L. subsp. *alpina* (Suter) Čelak. and *Genista hispanica* subsp. *occidentalis* Rouy shrublands.

Three sampling sites were established in June 2020 within the grasslands of Las Pintas mountain pass: (i) valley-bottom site (12.9 ha), (ii) north slope site (13.8 ha) and (iii) south slope site (17.6 ha). The mean altitude of the three sites showed little variability (1564–1612 m). The grazing exclusion experiment comprised a randomized complete block design in order to reduce the heterogeneity in abiotic environmental conditions, with five blocks of 50 m × 50 m per sampling site, located within each site to include grazed and abandoned areas. Three levels of grazing treatment were encompassed within each block: (i) allowed sheep grazing (G), (ii) short-term grazing exclusion (1-year; ST), and (iii) long-term grazing exclusion (between 15/20-years; LT). We had five replicates per sampling site, featuring 45 experimental plots with a size of 1 m × 1 m for soil and vegetation sampling. Based on field knowledge and observations, the grassland communities composition was similar at each sampling site. ST plots were randomly established within each block and fenced from June 2020 to June 2021 to prevent sheep grazing. Extensive sheep flocks were allowed to graze during the summer season (July to September) in G plots within the blocks, whereas LT plots were established within each block in areas still abandoned between 15/20-years based on personal interviews with local shepherds and technicians of the city hall with high field knowledge, as performed by previous grazing exclusion experiments in the site (Fernández-Guisuraga et al., 2022) and worldwide (De Bello et al., 2005; Sebastià et al., 2008; Aynekulu et al., 2017).

2.2. Soil sampling, DNA extraction, sequencing and bioinformatics analyses

A composite soil sample from the top 0–10 cm was collected with a trowel in the cardinal points of each field plot (Fernández-Guisuraga et al., 2022) to obtain a representative sample of the soil during the field survey in June 2021. Soil samples were homogenized and mesh-sieved

(2 mm) in the field. Soil subsamples of 50 ml were transferred to falcon tubes while in the field and frozen at -20°C before being delivered to the laboratory.

Soil DNA was extracted from 0.35 g of each sample using the DNeasy Power Soil Pro kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. A Nanodrop instrument (Thermo Fisher Scientific Inc., Waltham, USA) and a Qubit 2.0 fluorometer (Invitrogen Inc., Carlsbad, USA) were used to determine spectrophotometrically the DNA concentration and quality. Subsequently, DNA integrity was confirmed by means of agarose gel electrophoresis. Bacterial V3-V4 region of the 16S SSU rRNA was amplified using the 341F (CCTACGGGNG-GCWGAG) and 805R (GACTACHVGGGTATCTAATCC) primers (Herlemann et al., 2011). Barcoded PCR libraries from each sample were quantified by real-time PCR in a LightCycler 480 (Roche, Basel, Switzerland) and sequenced using Illumina Miseq (Illumina Inc., San Diego, USA) 2×250 bp paired-end sequencing.

Primers and barcodes were removed using *seqtk* (<https://github.com/lh3/seqtk>). Overlapping paired-end sequences were merged into a unique fused read (hereinafter referred to as read) and screened to discard low-quality reads and reads that differ from the expected size (460 to 480 bp) using *vsearch* (Rognes et al., 2016). Reads were clustered in OTUs at 97 % sequence similarity using *vsearch*. Taxonomic assignment of each OTU was performed by means of the naïve Bayesian classifier (Wang et al., 2007) using RDP-Classifer tool from the Ribosomal Database Project (RDP) web (Cole et al., 2014). Centroids of each OTU determined with *vsearch* were aligned using Infernal (Nawrocki et al., 2009) in the RDP. FastTree (Price et al., 2010) was used to construct a maximum-likelihood phylogenetic tree based in the *gtr* evolutionary model. The frequencies of each OTU and the phylogenetic tree were used to estimate alpha and beta diversities in the *vegan* (Oksanen et al., 2020) and *GUniFrac* (Chen et al., 2022b) packages in R (R Core Team, 2021). We computed the following alpha diversity metrics: (i) rarefied richness (S_{rare}) using random subsamples with a size equal to minimum sample size, (ii) Shannon's diversity index (H_{bac}), and (iii) Simpson's dominance index (S_{bac}). Two beta diversity indices were computed: (i) Bray-Curtis index of dissimilarity, and (ii) weighted UniFrac phylogenetic metric (Lozupone and Knight, 2005). A principal coordinate analysis (PCoA) was used to explore graphically the distances between communities based on beta diversity indices among the grazing treatment levels.

2.3. Quantification of ecosystem functions

We considered four key grassland ecosystem functions (Ren et al., 2018; Garland et al., 2021; Fernández-Guisuraga et al., 2022) that could drive the responses of the bacterial communities (Lamb et al., 2011; Haichar et al., 2014; Sauvadet et al., 2016) as a consequence of livestock abandonment: (i) herbaceous species productivity, (ii) woody species productivity, (iii) soil organic carbon (SOC) stocks, and (iv) soil fertility.

During the field survey in June 2021, vegetation cover by plant species was visually estimated within each plot in steps of 5 % (Calvo et al., 2008). We determined the relative cover of herbaceous and woody species to be used as a proxy for species productivity in grassland ecosystems (Maestre et al., 2012). SOC involves substantial feedbacks to climate regulation (Davidson and Janssens, 2007) and constitutes the largest terrestrial pool in grassland ecosystems (Oñativia et al., 2015). Moreover, SOC controls many biogeochemical processes and nutrient availability in terrestrial ecosystems, as well as plant and microbial productivity (Maestre et al., 2012). Soil fertility function was derived from ammonium (NH_4^+ -N), nitrate (NO_3^- -N), available phosphorous (P) and potassium (K) content in the soil samples, which are considered key nutrients for biogeochemical activities of soil microorganisms (Van Der Heijden et al., 2008). See Fernández-Guisuraga et al. (2022) for the methodology of SOC and nutrients determination. Nutrients' content

was normalized and standardized by means of a square root transformation and a z-score transformation, respectively (Bradford et al., 2014), and then grouped into the soil fertility function through an averaging approach (Maestre et al., 2012). The individual proxies/indicators for the other ecosystem functions (i.e. herbaceous species productivity, woody species productivity and SOC stocks) were also normalized and standardized using the averaging approach.

2.4. Data analyses

The effect of grazing exclusion on the alpha diversity (S_{rare} , H_{bac} and S_{bac}) of the bacterial communities was examined through generalized linear mixed models (GLMMs) using the *glmmPQL* function of the MASS package (Venables and Ripley, 2002) in R. A quasi-Poisson error distribution (log link function) and a quasi-binomial error distribution (logit link function) were used to fit S_{rare} and S_{bac} models, respectively, in order to account for overdispersion, whereas a Gaussian error distribution (identity link function) was used to fit H_{bac} model. Grazing treatment (allowed sheep grazing -G-, short-term grazing exclusion -ST-, and long-term grazing exclusion -LT-) was included as a fixed factor in the models. We included in the models the identity of the blocks nested within each sampling site (valley-bottom, north slope and south slope) as a nested random factor, removing the associated uncertainty of the environmental variability (Fernández-Guisuraga et al., 2022). Tukey's HSD test was implemented to evaluate the significance of the differences in the response variables between the levels of grazing treatment. Statistical significance was determined at the 0.05 level.

Prior to following analyses, unique read counts of the bacterial communities were normalized to 100,000 per sample. Differences in the frequencies of bacterial OTUs and taxa between the levels of grazing treatment were examined through GLMMs assuming a Gaussian error distribution with an identity link function, and subsequent Tukey's HSD tests. Model structure regarding fixed and random effects were the same than for alpha diversity analyses.

Piecewise Structural Equation Modeling (pSEM; Shipley, 2009) was used to disentangle the direct and indirect effects of livestock abandonment on soil bacterial communities mediated by changes of the considered ecosystem functions. We first parametrized a conceptual *priori* model based on the suspected interactions between (i) grazing treatment, (ii) ecosystem functions, and (iii) the abundance of the most important bacterial taxa detected in previous analyses that could behave as indicators of grazing abandonment. The mixed model set of the path diagram was constructed using the *glmmPQL* function. Grazing treatment was treated as an exogenous categorical variable in pSEM and thus we used a marginal means approach to explain the effect of all treatment levels on each path. The fit of individual model pieces in pSEM was evaluated through χ^2 likelihood ratio tests (Shipley, 2013). Then, we reported standardized path coefficients. pSEM was constructed using *piecewiseSEM* package (Lefcheck, 2016) in R.

3. Results and discussion

3.1. Sequencing of the bacterial communities

We obtained a total of 4,896,943 usable reads (83.44 % of the baseline sequences) after controlling for quality by discarding unpaired and too short (< 417 bp) reads, chimeric reads and those from chloroplasts, featuring an average next to 10^5 reads per sample (minimum and maximum values between 98,054 and 117,752, respectively). A total of 216,057 OTUs at 97 % sequence similarity were identified, but rare OTUs (< 100 reads among all soil samples) were also discarded. Finally, we retained 4,067,718 reads distributed among 4255 OTUs with > 100 reads, featuring each OTU 90,394 reads on average. These data are relatively similar to other studies involving bacterial sequencing in

grasslands (Wang et al., 2021; Kang et al., 2022), indicating that our sequencing could precisely reflect the actual soil bacterial community.

Total reads were clustered to 20 bacterial phyla. *Proteobacteria* was the most frequent bacterial phylum, accounting for 32.97 % of all reads across sampling sites, followed by *Acidobacteria* (23.50 %), *Verrucomicrobia* (14.87 %), *Actinobacteria* (12.86 %) and *Bacteroidetes* (8.23 %). Among the *Proteobacteria* phylum, the most frequent classes are *Alphaproteobacteria* (49.95 %) and *Betaproteobacteria* (31.07 %). These relative abundances are consistent with those evidenced for the firsts soil centimeters in other mountain grasslands worldwide (e.g. Zhang et al., 2014; Praeg et al., 2019; Mayerhofer et al., 2021; Wang et al., 2021; Kang et al., 2022; Li et al., 2022), and correspond to intermediate values of the bacterial phylotypes' ecological clusters of low and high pH in the *global atlas of the dominant bacteria found in soil* (Delgado-Baquerizo et al., 2018). Indeed, intermediate soil pH values (6.54 ± 0.89) were observed in the sampling sites within the grasslands, the soil pH being the major driver of changes in bacterial communities' composition in terrestrial ecosystems (Lauber et al., 2009; Fierer et al., 2012).

3.2. Effect of grazing abandonment on bacterial diversity

The alpha diversity metrics of bacterial communities (rarefied richness, Shannon's diversity and Simpson's dominance) showed a large variability between sampling sites with different aspect in the grasslands (Fig. 1). Rarefied richness and Shannon's diversity metrics significantly increased on northern and southern slopes, and were significantly

lower in valley bottom areas, whereas Simpson's dominance metric followed the opposite pattern (Supplementary Material S1). Indeed, slope aspect and topographic position may involve major impacts on bacterial community diversity via shifts in soil moisture content and pH (Chu et al., 2016). First, increased moisture content in valley bottom grasslands may lead to direct responses of bacterial communities' composition mediated by competition processes (Castro et al., 2010). Second, lower soil pH values observed in valley bottom site (mean \pm standard deviation: 5.62 ± 0.50), compared to sites located in hillslope areas (7.00 ± 0.65), may impact microbial intracellular homeostasis and soil nutrient availability (Zhalnina et al., 2015; Deng et al., 2022), reducing bacterial richness and diversity as has been observed worldwide (Fierer and Jackson, 2006). Notwithstanding, all the considered diversity metrics showed no clear pattern of variation (i.e. the same behavior) as a function of grazing treatment within each sampling site (Fig. 1). This evidence supported the inclusion of sampling site as a random factor in mixed models for eliminating the associated variability in abiotic environmental conditions.

When fitting mixed models to isolate the effect of grazing treatment on the diversity of bacterial communities, we found that there were no significant differences (p -values > 0.05) for any of the considered diversity indices between treatment levels, except for the rarefied richness, which was only marginally lower (p -value = 0.063) in short-term grazing exclusions (ST) as compared to allowed sheep grazing areas (G) (Supplementary Material S1 and Fig. 2).

We expected that changes in litter quantity and quality, rhizosphere exudation patterns, nutrient cycling and soil structure as a consequence

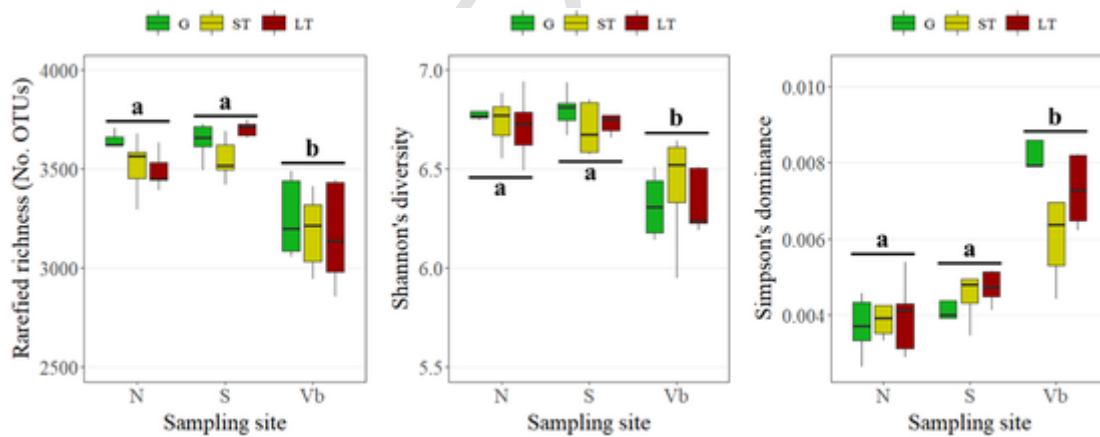


Fig. 1. Boxplots showing the variation in the alpha diversity of bacterial communities between the levels of grazing treatment (allowed sheep grazing -G-, short-term -ST- and long-term -LT- grazing exclusion), grouped by sampling site (north slope site —N—, south slope site —S—, and valley-bottom site -Vb-). Lowercase letters denote significant differences at the 0.05 level between sampling sites (see Supplementary Material S1).

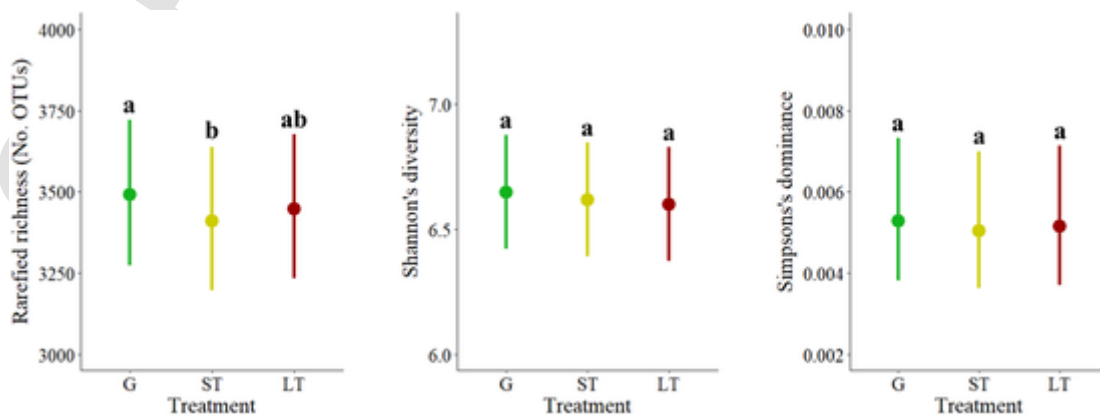


Fig. 2. Generalized linear mixed model (GLMM) predicted mean \pm 95 % confidence intervals of grazing treatment (allowed sheep grazing -G-, short-term -ST- and long-term -LT- grazing exclusion) on the alpha diversity of bacterial. Lowercase letters denote significant differences at the 0.05 level between treatment levels (see Supplementary Material S1).

of livestock abandonment in mountain grasslands (Berg and Smalla, 2009; Yang et al., 2013; Gao and DeLuca, 2022) could exert a direct or indirect influence on bacterial community structure. However, no clear pattern emerged in the line with the results obtained by Aldezabal et al. (2015) in productive mountain grasslands under moderate-to-high stocking rates. The authors evidenced negative effects of grazing abandonment on microbial activity, but not accompanied by a significant response of the bacterial community structure. In productive grasslands, increased soil resource availability and richer plant communities as compared to semiarid grasslands (Brun et al., 2019; Sun et al., 2020) could support a greater range of resources and microhabitats for bacterial communities (Lamb et al., 2011; Parker and Schimel, 2011). We speculate that resource heterogeneity in productive mountain grasslands may produce a non-competitive diversity pattern of soil bacterial communities (Zhou et al., 2002) in the presence of vegetation and soil changes mediated by livestock abandonment, promoting a stable structure of bacterial communities. These results contrasted with the findings of previous research in semiarid mountain grasslands (Cheng et al., 2016; Zhang et al., 2018; Wang et al., 2019), in which secondary succession after livestock abandonment induced significant shifts on the diversity of bacterial communities. In these areas, grazing could modulate the diversity of soil bacterial communities through a reduction in the competitive intensity of dominant bacterial taxa (Eldridge et al., 2017).

Although the alpha diversity of the bacterial communities did not show a clear significant response between grazing treatment levels, PCoA of beta diversity indices (Bray-Curtis and weighted UniFrac) identified changes in the community composition among them. Considering all the sampling sites together (global PCoA; Fig. 3), soil samples in G and ST areas clustered together for weighted UniFrac index, indicating similar bacterial community compositions, and separated from LT areas. Grazing treatment effect was found in the third axis as confirmed by the significant linear mixed model of the samples' projection on the axis (p -value = 3.46×10^{-6}), featuring significant differences between LT and G (p -value = 6.63×10^{-6}), as well as LT and ST (p -value = 1.32×10^{-5}) areas. The effect of sampling site was significant in the first axis (p -value = 2.07×10^{-14}). For Bray-Curtis index, soil samples of the three grazing treatment areas clustered together in the third axis (p -value = 0.26), being sampling site effect found in the first axis (p -value = 7.62×10^{-16}). When beta diversity analyses are stratified by sampling site (north, south and valley-bottom PCoA; Fig. 3), samples corresponding to LT grazing treatment were clearly separated from G and ST treatments in the second axis (p -values < 5×10^{-12}), both in terms of Bray-Curtis and Weighted UniFrac indices (Supplementary Material S1).

These results confirmed that livestock grazing abandonment had relevant effects on the overall composition of soil bacterial communities between mountain grassland areas abandoned by livestock in the long term, and grazed or abandoned areas in the short term. The different behavior observed for both beta diversity indices suggest that individual samples did not have many specific OTUs with quite different abundances, but those OTUs were distant in the phylogenetic tree (Sáenz de Miera et al., 2020), and would belong to very different taxonomic groups with distinct functions and environmental adaptations (Lozupone and Knight, 2005). These assumptions support the previously evidenced absence of clear changes in bacterial communities' diversity among grazing treatment levels.

3.3. Effect of grazing abandonment on bacterial taxa

When assessing changes in the composition of bacterial communities at the OTUs and taxa level following livestock abandonment in the short and long term, 1460 of 4255 OTUs showed significant differences in their abundance between grazing treatment levels (Fig. 4). There is a balance between the number of OTUs whose relative abundance of the

reads is favored by livestock grazing (827; relative abundance of 19.51 %) and those with higher relative abundances in grazing exclusion areas (633; relative abundance of 20.23 %). In both cases, the most frequent scenario corresponds to significant changes in the relative abundance of the OTUs in LT areas. In this sense, 212 + 294 OTUs featured a decrease in abundance in LT treatment, contributing to $8.35 + 5.36$ % of relative abundance, whereas for 334 + 131 OTUs ($14.63 + 3.75$ %), their abundance increased in LT. Only a single OTU with negligible relative abundance (<0.01 %) showed a progressive decrease or increase as a consequence of livestock abandonment. The relative abundance of OTUs with a significantly higher frequency in ST compared to G and LT areas was markedly low (<1 %).

These findings are consistent with the results of our alpha and beta diversity analyses. Although there are a relatively large number of OTUs that can serve as microbiological indicators of livestock abandonment due to the distinct behavior between grazing treatment scenarios, their relative changes in abundance are not high enough to produce clear shifts in the alpha diversity of bacterial communities. Conversely, the important variation in the number and abundance at the OTU level in LT treatment may explain the observed changes in the bacterial community composition through the Weighted UniFrac index.

Within Alphaproteobacteria class, Rhodospirillales and Rhizobiales orders, involved in atmospheric nitrogen fixation in the rhizosphere, especially when soil nitrogen availability is low (Friel and Friesen, 2019), accounted respectively for 2.39 % and 7.99 % of the mean relative abundance in the grasslands, and featured a significant increase in LT areas. The contribution to this behavior of *Bradyrhizobium* genus within Rhizobiales stands out, with a mean relative abundance of 2.10 %. Sphingomonadales (relative abundance of 3.27 %), mainly represented by *Sphingomonas* genus, showed an opposite trend compared to the previous orders. These changes did not translate into significant variations in the abundance of Alphaproteobacteria class (Fig. 5). Betaproteobacteria class exhibited no significant changes between grazing treatment levels (Fig. 5). However, within this class, Nitrosomonadales order (relative abundance of 0.22 %), an important taxon because of its role as ammonia oxidizers in grasslands' nitrogen cycle (Nelson et al., 2015; Duff et al., 2022), doubled its abundance in LT as compared to G and ST samples. The relative abundance of Gammaproteobacteria class featured a little but significant increase in LT areas (2.30 % with respect to 1.77 % both in G and ST) (Fig. 5), due to the higher abundance of *Acidibacter* genus in LT soils. These changes did not lead to a clear trend in the relative abundance of the Proteobacteria phylum, with no significant differences between G and both ST and LT areas (Fig. 5). The minor changes in the abundance of the dominant Proteobacteria phylum, along with the absence of differences in Acidobacteria (Fig. 5), the two most ubiquitous and common bacterial taxa in soils (Zhang et al., 2016b), may corroborate the stability of bacterial communities under land-use changes in mountain grassland ecosystems (Zhang et al., 2016a), particularly livestock abandonment in the short term.

Livestock abandonment in the mountain grasslands produced a significant decrease in the relative abundance of Actinobacteria phylum, from 11.15 % and 10.87 % in G and ST, respectively, to 7.67 % in LT areas (Fig. 5). These differences resulted primarily from the lower abundance of the orders Gaiellales (represented mainly by the genus *Gaiella*) and Solirubrobacterales (genus *Solirubrobacter*) within the Thermoleophilia class in LT areas. That behavior agrees with other studies evidencing that Actinobacteria are less abundant across later successional stages with turnovers in soil nutrients (Lozano et al., 2014; Zhang et al., 2016a), which could correspond to shrub encroachment scenarios in areas subject to long-term grazing abandonment in mountain grasslands (Fernández-Guisuraga et al., 2022). This result contrasts with those obtained by Eldridge et al. (2017), who observed a reduction in the relative abundance of Actinobacteria phylum linked to grazing in semiarid grasslands, probably because of the impact of aridity on soil carbon and nutrients availability (Maestre et al., 2015), and the copi-

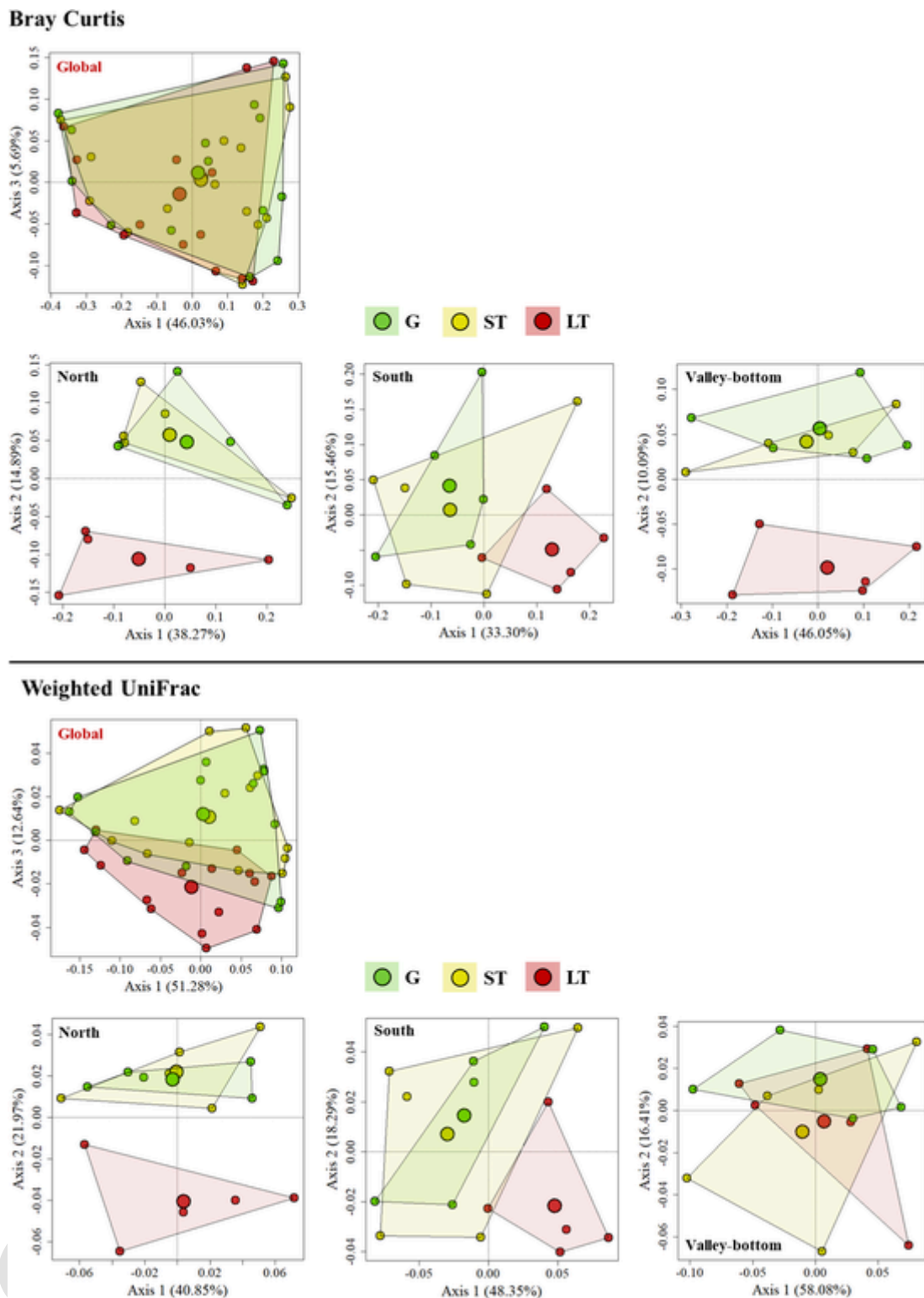


Fig. 3. Ordination of the bacterial communities along grazing treatment areas (allowed sheep grazing -G-, short-term -ST- and long-term -LT- grazing exclusion) using principal coordinates analysis (PCoA), considering all the sampling sites together (global PCoA) and stratified by sampling site (north, south and valley-bottom PCoA). Large points indicate the centroids of each treatment.

otrophic strategy of Actinobacteria (Leff et al., 2015). The reported relative abundances were determined from the most frequent bacterial taxa in the grasslands, presented in Fig. 5. See Supplementary Material S2 for the relative abundances of all bacterial taxa. The drivers of the evidenced changes at the bacterial OTUs and taxa level, related to ecosystem functions, are analyzed and discussed in the next section.

3.4. Functions associated with the shifts in bacterial communities

The direct effects of grazing exclusion on the productivity of woody species, together with the indirect effect on SOC mediated via changes in the productivity of herbaceous species and soil fertility, provided the key mechanisms responsible of the main changes in the abundance of

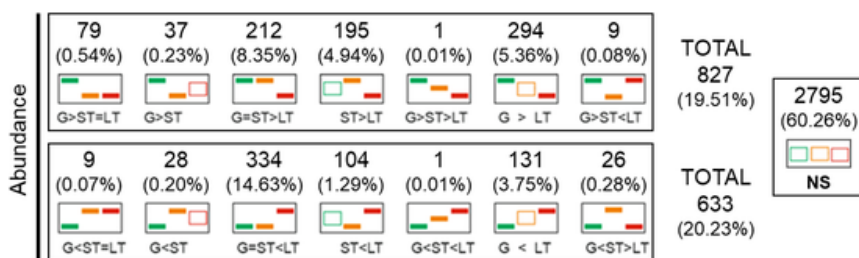


Fig. 4. Band schemes of the number of OTUs that show significant differences in their abundance between grazing treatment areas (allowed sheep grazing -G-, short-term -ST- and long-term -LT- grazing exclusion) (Supplementary Material S1). We indicate in parentheses the relative abundance of the reads for each scenario.

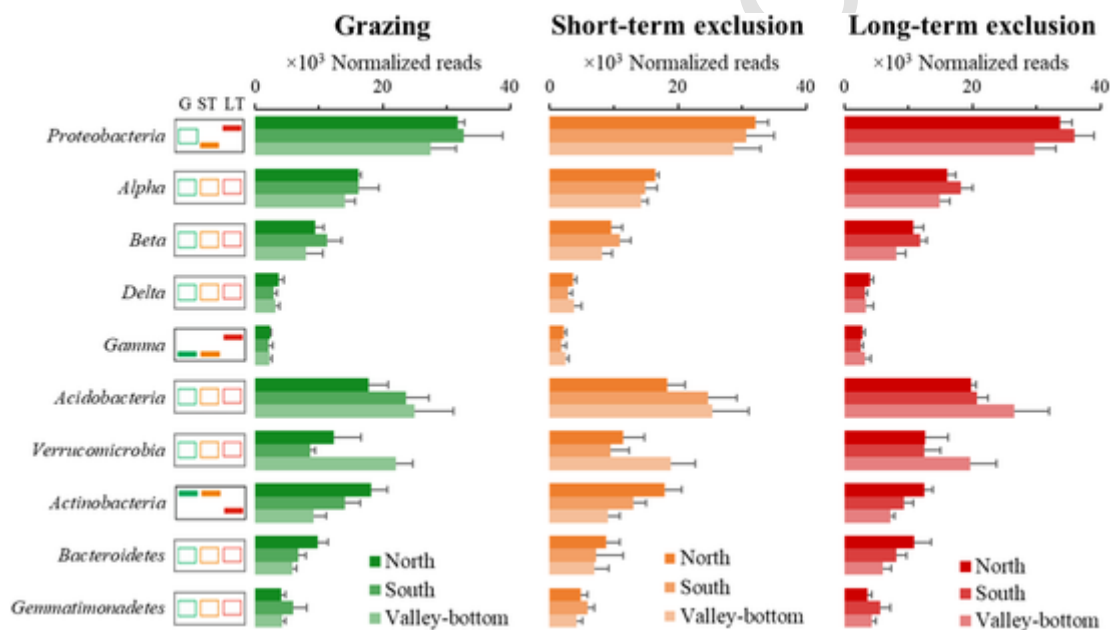


Fig. 5. Normalized reads (mean and standard deviation) of the dominant bacteria phyla between grazing treatment areas. Band schemes denote significant differences in taxa relative abundance (Supplementary Material S2) as shown in Fig. 4.

several bacterial taxa. These mechanisms were evidenced using piecewise Structural Equation Modeling (Fig. 6). In addition, the expected modulation in the soil physical characteristics (e.g. temperature and moisture, although not evaluated) by plant community succession (Odrizola et al., 2014) could also be responsible for the observed shifts in bacterial taxa abundance. Grazing exclusion leveraged the positive effect of encroaching shrub species and SOC (Fernández-Guisuraga et al., 2022) on the abundance of Rhodospirillales and Nitrosomonadales orders, and Gammaproteobacteria class. In grassland ecosystem across Europe, Szoboszlai et al. (2017) found that land-use conversion to forests promoted an increase in relative abundance of Rhodospirillales probably due to the increased litter inputs and labile carbon to the soil, as well as changes in litter quality. Increased vegetation biomass has been also shown to promote the relative abundance of taxa within ammonia oxidizing community (Mushinski et al., 2017), which may explain the increase of Nitrosomonadales order abundance in LT areas. In the same way, Gammaproteobacteria class have recently been considered to exhibit copiotrophic traits associated with high SOC availability (Männistö et al., 2016; Kurm et al., 2017), in line with our findings. Conversely, grazing exclusion and woody species encroachment diminished the relative abundance of Gaiellales and Solirubrobacterales orders affiliated with Actinobacteria phylum, as reported by Lozano et al. (2014) in late stages of the secondary succession in shrublands of south-east Spain. This behavior may be related to the quality of different organic matter compounds associated with distinct vegetation types, instead of organic matter quantity (Chan et al., 2008), as observed in

alpine grasslands in northwestern China for different Actinobacteria taxa (Zhang et al., 2019).

4. Conclusions

This study provided novel evidence on the behavior of soil bacterial communities in response to grazing abandonment in productive mountain grasslands, improving the understanding of the role of traditional livestock farming practices with respect to the ecosystem multifunctionality. Our data suggest that several bacterial taxa with an important role in the ecosystem functioning can serve as microbiological indicators of land-use changes in mountain grasslands, particularly traditional sheep grazing abandonment in the long-term (< 15-years). However, the relative changes in their abundance were not high enough to affect the stability of bacterial communities' structure. As a result of the grazing exclusion effects in the long-term, the evidenced shifts in vegetation productivity and soil organic carbon content were strongly linked to the abundance of bacterial taxa indicators. Assessment of the bacterial community response to livestock abandonment in mountain grasslands may thus provide insights before subtle changes in ecosystem functions occur. Further research is needed based on longer-term experiments to better understand the sustainability of traditional livestock grazing for preserving the stability of soil bacterial communities in mountain grasslands.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2022.158398>.

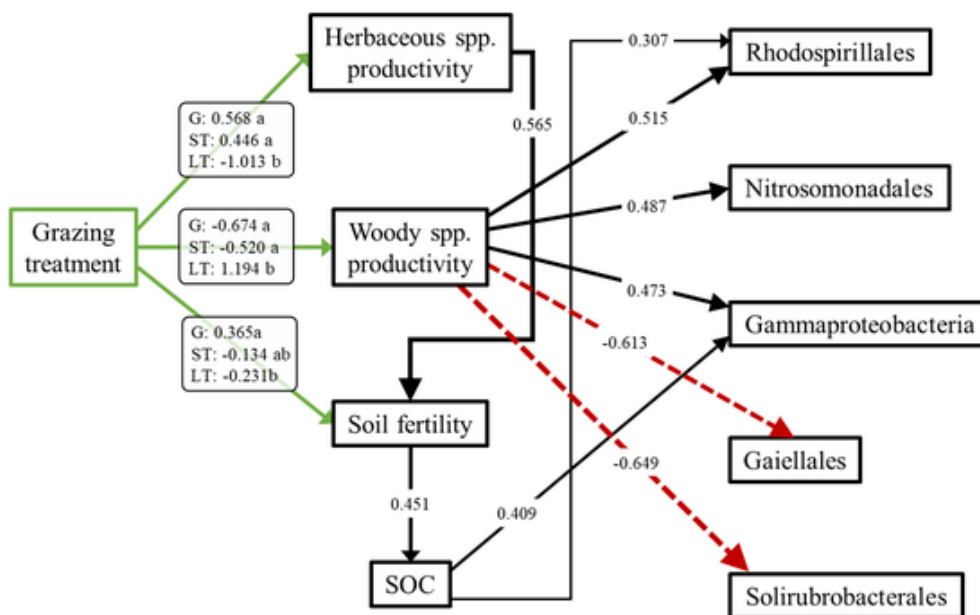


Fig. 6. Piecemeal Structural Equation Modeling diagram. Grazing treatment (allowed sheep grazing -G-, short-term -ST- and long-term -LT- grazing exclusion) is an exogenous categorical variable used to explain the effect of all levels on each path (green arrows with estimated marginal means and post-hoc letters). Black solid and red dashed arrows represent paths with positive and negative relationships, respectively. The arrow width is proportional to the magnitude of the effect, represented by significant standardized path coefficients (p -values < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CRedit authorship contribution statement

José Manuel Fernández-Guisuraga : Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Leonor Calvo** : Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Gemma Ansola** : Conceptualization, Writing – review & editing. **Rayo Pinto** : Conceptualization, Writing – review & editing. **Luis E. Sáenz de Miera** : Conceptualization, Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

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