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Plant-beneficial bacteria are promoted in pasture-crop rotations in the Uruguayan Pampa, contributing to soil health and crop performance

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Introduction: Understanding how pasture-crop rotation design influences long-term soil health and microbial dynamics is crucial for sustainable agroecosystems. Pasture-crop rotations may alleviate soil degradation, but their long-term effects or legacy on soil and rhizosphere microbiomes, including potential recruitment of plant-beneficial microorganisms, and their link to crop yield need to be better understood. This study examines how land use intensity and grazed pasture legacy influence soil and rhizosphere prokaryotic diversity, composition, functionality, and crop productivity.

Methods: A gradient of land use intensities ranging from continuous cropping (CCG), short (SR) and long (LR) pasture-crop rotations, permanent improved pasture (PIP), and natural grassland (NGL) were sampled in a long-term field experiment established in 1995 in the Uruguayan Pampa. Moreover, two stages of the rotation, one year after pasture sown with sorghum (SRS, LRS) and two years after pasture with soybean (SRG, LRG), were studied to assess the persistence of pasture-derived legacies. Soil physicochemical and biological properties were measured to evaluate soil health along with crop productivity. Bulk soil, soybean, and sorghum rhizosphere prokaryotic communities were analyzed across land use intensities using *16S rRNA* gene amplicon sequencing and functional assays on rhizosphere bacterial isolates.

Results: Results showed a partial retention of soil health assessed through selected soil properties, i.e. soil organic C, total N, and soil extractable protein content were higher in LRG compared to CCG, while an intermediate response was observed in SRG. LR preserved the pasture legacy, maintaining bulk soil prokaryotic community composition similar to PIP and distinct from CCG, while SR converged to CCG and diverged from PIP. Soybean rhizosphere prokaryotic diversity and composition was strongly shaped by crop type and by soybean inoculation with *Bradyrhizobium elkanii*, overriding the effects of intensification and pasture legacy. Key soil taxa (*Streptomyces, Solibacillus, Sphingomonas* and *Bradyrhizobium*) were linked with improved soil functionality. Linking *16S rRNA* gene sequencing data of rhizosphere taxa with rhizobacterial isolates showed that *Pseudomonas*, *Bacillus*, and *Microbacterium*, all exhibiting multiple plant-beneficial activities *in vitro*, were enriched in pasture rotations.

Discussion: This study highlights that pasture-crop rotation design, particularly pasture duration and plant composition, influences prokaryotic services and soil health, contributing towards the development of resilient agroecosystems.

1 Introduction

Soil provides essential ecosystem services such as nutrient cycling, carbon (C) sequestration, water regulation, and biodiversity conservation (Karlen et al., 2019; Lehmann et al., 2020). Soil health, defined as the capacity of soil to function as a living ecosystem, integrates biological, chemical and physical properties that are crucial for sustainable agriculture and food security (Lehmann et al., 2020; Banerjee and van der Heijden, 2023). Traditional physicochemical assessments are insufficient to capture soil health, making microbial parameters, such as community structure, function, abundance, and diversity, important bioindicators due to the role of microorganisms in nutrient cycling, organic matter decomposition, and disease suppression (Lehman et al., 2015; Verdenelli et al., 2019; Hermans et al., 2020; Lehmann et al., 2020; Norris et al., 2020; Fierer et al., 2021; Banerjee and van der Heijden, 2023). Intensive agricultural practices, characterized by short-term economic benefits and simplified management supported by an intensive use of synthetic inputs (fuel, fertilizers, pesticides, herbicides), are widespread. However, these practices pose negative impacts on soil health by increasing soil compaction, decreasing soil organic matter, accumulating agrochemicals, and consequently disrupting soil microbial communities (Sadras et al., 2005; Carbonetto et al., 2014; Pittelkow et al., 2015; Tsiafouli et al., 2015; Norris and Congreves, 2018; Lehmann et al., 2020; Cerecetto et al., 2021a). In contrast, conservation practices like reduced tillage, cover cropping, organic amendments and crop rotations have been shown to enhance soil microbial activity and diversity, and soil fertility and structure (Chaparro et al., 2012; Bender et al., 2016; Williams et al., 2023; Hartmann and Six, 2023). However, despite their proven benefits, conservation practices can result in variable yields and unbalanced plant nutrition under certain conditions, particularly with no-tillage (Pittelkow et al., 2015). Therefore, further research is needed to understand how rhizosphere biological processes influence plant performance under these conservation practices. A key concept emerging in this context is soil legacy, or soil memory, which refers to the long-term effects of past agricultural practices on soil properties and microbial communities (Lapsansky et al., 2016; Bakker et al., 2018). These legacy effects may modulate rhizosphere processes and, consequently, plant responses to current management. Understanding soil legacy is critical for designing sustainable management strategies.

The Pampa biome, spanning Uruguay, central-eastern Argentina, and southern Brazil, is one of the world's largest and most diverse grassland ecosystems (Scottá and da Fonseca, 2015). Historically managed through extensive cattle grazing on native grasslands, this biome has undergone rapid agricultural intensification, primarily through no-tillage and glyphosate-resistant soybean (*Glycine max* L.) expansion, leading to soil degradation, biodiversity loss, and reductions in ecosystem services (de Faccio Carvalho et al., 2021a; Macedo et al., 2022). To reverse these trends, the integration of crop and livestock systems through pasture-crop rotations has been proposed as a sustainable alternative to continuous cropping, improving soil structure, C sequestration, nutrient cycling, and microbial diversity (McDaniel et al., 2014; Garrett et al., 2017; Pravia et al., 2019; Macedo et al., 2022; Pereyra-Goday et al., 2022, 2024).

The positive effects of pasture-crop rotations on soil physicochemical properties have been well documented across various agroecosystems (Franzluebbers et al., 2014; Garrett et al., 2017; Ernst et al., 2018; King and Blesh, 2018; Jaurena et al., 2021; Sekaran et al., 2021; Macedo et al., 2022; Rubio et al., 2022; Santos Silva et al., 2022). Moreover, impacts of pasture-crop rotations on soil microbial community composition and diversity have been demonstrated in different regions (Walkup et al., 2020; Sekaran et al., 2021; Williams et al., 2023; dos Santos-Goulart et al., 2024). However, microbial responses are highly context-dependent, varying with soil type, climate, land use, and management practices (French et al., 2021). Therefore, results from other regions are not necessarily transferable to the Pampa biome, which features distinct environmental and management conditions. For instance, dos Santos-Goulart et al. (2024) demonstrated that soil bacteria did not respond to pasture-crop rotation when compared to a native forest in the Brazilian Cerrado biome, whereas the fungal community exhibited shifts in response to the rotation scheme. In contrast, Cerecetto et al. (2024) found that soil prokaryotic communities shifted when pasture-crop rotations were compared to a native grassland in the Uruguayan Pampa biome, and such shifts were observed only in a few of the studied pasture-crop rotations for fungal communities. These differences underscore the need for site-specific research to develop precision microbiome management tailored to local conditions with customized agricultural practices aimed at enhancing crop health and agroecosystem sustainability (Berg

et al., 2017; Lehmann et al., 2020; French et al., 2021). Although a few studies have explored how pasture-crop rotations affect soil prokaryotic communities in the Pampa biome (Frene et al., 2022; Barbero et al., 2025), they lack the grazing effects, a central component of mixed crop-livestock systems, or overlook the legacy of long-term pastures. Moreover, there is limited knowledge on how pasture duration influences the microbiome. Furthermore, few studies have investigated how grazed pastures impact rhizosphere microbial communities (Cerecetto et al., 2024; Martins Costa et al., 2024) and have linked these changes to plant-beneficial traits and their potential functional implications on crop productivity.

To address these knowledge gaps, this study expands upon earlier findings from one of the longest-running long-term field experiments (LTEs) in the Pampa region, established in 1995 in Uruguay (Cerecetto et al., 2024). Previous work in this LTE demonstrated that incorporating grazed pastures into crop rotations positively influenced subsequent grain crop cycles through physicochemical, bulk soil and rhizosphere microbial changes, preserving soil structure and nutrient availability while supporting crop performance (Cerecetto et al., 2024). However, it remained unclear whether this pasture legacy persisted over time, varied with pasture duration, or promoted plant-beneficial prokaryote recruitment in crop rhizospheres. To investigate this, the same LTE was re-sampled in a subsequent growing season to evaluate soil health indicators across five land use intensities: continuous cropping (CC), short (SR) and long (LR) pasture-crop rotations, permanent improved pasture (PIP), and natural grassland (NGL). Additionally, two rotation stages, one (SRS, LRS) and two years (SRG, LRG) after pasture, were assessed to determine the persistence of pasture-derived legacies. Moreover, SR and LR differ in pasture duration and in pasture plant species composition, which may further influence the persistence of pasture-derived legacies. Soil health was determined using physicochemical properties (bulk density, soil nutrients), biological proxies (heterotrophic soil respiration, soil potentially oxidizable C, soil extractable protein content), and microbial indicators (diversity and composition of bulk soil and rhizosphere prokaryotic communities via *16S rRNA* gene amplicon sequencing). Potential rhizosphere functions were inferred by linking sequencing data to *16S rRNA* gene sequences of rhizobacterial isolates with plant-beneficial traits. Crop performance was evaluated through biomass and nutrient content, serving as an indirect proxy for soil health and enabling the exploration of connections between soil properties, prokaryotic communities, and plant growth. It was hypothesized that introducing grazed pastures into crop rotations in the Uruguayan Pampa biome preserves soil health by maintaining soil properties, shaping soil and rhizosphere prokaryotic communities, and recruiting plant-beneficial prokaryotes, which collectively enhance crop performance. Furthermore, it was hypothesized that the legacy effects of grazed pastures persist longer in LR than in SR, due to extended exposure to and stabilization of soil-microbiome feedback mechanisms. Differences in pasture plant species composition between LR and SR may contribute to these effects, although this was not directly assessed in the present study.

2 Materials and methods

2.1 Field experiment

This study was carried out in a long-term field experiment (LTE) at the National Institute of Agricultural Research (INIA) Palo a Pique Research Unit (33°15'54.4" S 54°29'28.1" W, elevation 60 m), Treinta y Tres, Uruguay. The LTE was installed in 1995 in a slightly degraded area after a short history of soybean cropping with conventional tillage followed by a sown pasture (Pravia et al., 2019). The dominant soil type is Typic Argiudol with a clay loam texture, and high to moderate erosion risk due to the gently sloping hills of modest altitude dominating the landscape. The region has a temperate sub-humid climate (Rovira et al., 2020).

Five land use intensities were evaluated under no-tillage. From highest to lowest land use intensity, the treatments are continuous grain crop rotation without pasture (CC), short pasture-crop rotation (SR, two years of sown pasture, two years of grain crops), long pasture-crop rotation (LR, four years of sown pasture, two years of grain crops), permanent improved pasture (PIP, permanent sown pasture) and natural grassland (NGL; Table 1). Pasture plant species composition in SR and LR differs, in SR, the pasture is composed of red clover (*Trifolium pratense* L.) and wheat (*Triticum aestivum* L.), and in LR of tall fescue (*Festuca arundinacea* L.), white clover (*Trifolium repens* L.) and bird's-foot trefoil (*Lotus corniculatus* L.) (Rovira et al., 2020; Table 1). Cattle of the same age and body weight graze the pastures in SR, LR, PIP and NGL, mimicking a commercial farm. Grazing was excluded from SR and LR plots at the grain cropping phase. NGL was chosen as a reference for soil with minimum disturbance since only grazing cattle was permitted, while neither fertilization nor herbicides have been used.

Sorghum (*Sorghum bicolor* L. cv ACA 558) was sampled one year after pasture, after the first winter crop [oat (*Avena sativa* L.)], while soybean (cv DM 60i62 IPRO) was sampled two years after pasture, after the second winter crop (oat; Table 1). This cropping sequence reflects different pasture legacy durations, specifically one and two years after pasture, respectively. Soybean was sampled in CC, SR, and LR plots further on referred to CCG, SRG, and LRG (with "G" indicating *Glycine max*), and sorghum was sampled additionally in SR and LR plots further on referred to SRS and LRS (Table 1).

Management practices, including machinery operations, fertilization, and agrochemical applications were similar among CC, SR, and LR, following standard commercial farm practices (Terra et al., 2006). Soybean seeds sown in CCG, SRG, and LRG were inoculated with two commercially recommended strains of *Bradyrhizobium elkanii*, U1301 (syn. SEMIA 587) and U1302 (syn. SEMIA 5019), in accordance with national agricultural recommendations (Cerecetto et al., 2021b). The inoculant was applied at a rate of 300 mL per 50 kg of seeds, resulting in a concentration of 1.2×10^{10} to 6×10^{9} colony-forming units (CFU) per kg of seeds, following manufacturer's instructions. Details on fertilization and herbicide applications are provided in the Supplementary Material (Supplementary Table S1). Further

TABLE 1 Crop sequences of the studied land use intensities in the long-term field experiment established in 1995.

Land use intensities	Year											
	1st		2nd		3rd		4th		5th		6th	
	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer
CCG	wheat	sorghum	oat	soybean	wheat	sorghum	oat	soybean	wheat	sorghum	oat	**soybean** *
SRG	wheat, red clover				oat	sorghum	oat	**soybean** *	wheat, red clover			
LRG	wheat, tall fescue, white clover, bird's-foot trefoil								oat	sorghum	oat	**soybean** *
SRS	wheat, red clover				oat	soybean	oat	**sorghum** *	wheat, red clover			
LRS	wheat, tall fescue, white clover, bird's-foot trefoil								oat	soybean	oat	**sorghum** *
PIP	white clover, bird's-foot trefoil, annual ryegrass											
NGL	80-90% Cynodon dactylon and 10-20% of a mix of Paspalum dilatatum, Bothriochloa alaguroides, Sporobolus indicus, Paspalum notatum, Schizachyrium spicatum											

Land use intensity abbreviations: CCG (continuous cropping where soybean is grown), SRG (short pasture-crop rotation with sorghum grown one year after pasture), LRG (long pasture-crop rotation with soybean grown two years after pasture), LRS (long pasture-crop rotation with sorghum grown two years after pasture), SRS (short pasture-crop rotation with soybean grown one year after pasture), NGL (natural grassland), PIP (permanent improved pasture). Crops grown in the long-term field experiment: oat (*Avena sativa* L.), wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* L.), soybean (*Glycine max* L.), red clover (*Trifolium pratense* L.), tall fescue (*Festuca arundinacea* L.), white clover (*Trifolium repens* L.), bird's-foot trefoil (*Lotus corniculatus* L.), annual ryegrass (*Lolium multiflorum* L.). Bold and * indicate the sampled stage of the rotation in February 2019.

information on the LTE and grazing management can be found in Terra et al. (2006), Rovira et al. (2020), Pereyra-Goday et al. (2022), and Cerecetto et al. (2024).

2.2 Soil and plant sampling

The sampling was carried out in the summer of 2019 (February). Soil and plant samples were collected when plants were mostly at their full flowering growth stage, with plants ranging from beginning to end of flowering (BBCH stages 61-69; Meier, 2018). Six independent samples (replicates) assigned along a slope gradient were sampled within a 3-ha plot for each land use intensity (CCG, SRG, SRS, LRG, LRS and PIP) as well as within the NGL located next to the LTE (Table 1).

Bulk soil samples were taken from CCG, SRG, LRG, SRS, LRS, PIP and NGL. A composite sample of 15–20 soil cores per replicate was randomly collected between plants with a 2 cm soil core auger at 0–15 cm depth, and mixed and homogenized by sieving with a 2 mm mesh size to remove roots and macrofauna. A fraction of these bulk soil samples were kept at –20°C until DNA extraction. Two samples per replicate were taken from the 0–5 cm soil layer to determine bulk density (BD) using a soil core sampler (5 cm diameter x 5 cm depth).

Shoot biomass and rhizosphere samples were taken from CCG, SRG, and LRG (all soybean) as well as from SRS and LRS (both sorghum). In detail, plants growing within one linear meter were collected per replicate to estimate shoot biomass and nutrient content. Soybean and sorghum grains were harvested with a commercial harvester at harvesting time to estimate grain yield and grain nutrient content. The root system of three plants was sampled per replicate, briefly washed with distilled water to remove loosely adhering soil and pooled into a composite sample. The rhizosphere, defined as soil closely attached to roots, was retrieved from 5 g of roots resuspended in a total of 45 mL of 0.3% (w/v) saline solution, applied in three successive 15 mL extractions. Each extraction was followed by 1 min of Stomacher treatment according to Schreiter et al. (2014). One ml of the supernatant was used for serial dilutions to isolate rhizobacteria (see section 2.8), and the remaining supernatant was centrifuged (Schreiter et al. 2014) to harvest rhizosphere pellets, which were kept at –20°C until DNA extraction.

2.3 Soil properties

Bulk soil samples were first dried at room temperature and sent to INIA Soil and Plant Laboratory to determine soil chemical properties. pH was measured by potentiometric determination in water (Beretta et al., 2014). Soil total nitrogen (N) was analyzed by combustion at 900°C and subsequent N_2 thermal conductivity detection. Plant available phosphorus (P) was measured by the Bray-I method (Bray and Kurtz, 1945). Plant-available potassium (K) was evaluated by ammonium acetate (pH 7) extraction followed by atomic emission (Jackson, 1964). Soil organic C (SOC) was quantified by 900°C combustion and subsequent CO_2 infrared detection (Wright and Bailey, 2001).

Heterotrophic soil respiration was measured as an indicator of microbial activity using an incubation-alkaline absorption method (Moebius-Clune et al., 2016). The CO_2 trapped in a 0.5 M KOH solution incubated together with 20 g of air-dried soil was used to estimate heterotrophic respiration as mg CO_2 g^{-1} dry soil day^{-1}. Potentially oxidizable C (PoxC), an indicator of easily available C for soil heterotrophic community, was assessed by oxidation of a solution of 0.2 M $KMnO_4$ in 1 M $CaCl_2$ (pH 7.2) (Weil et al., 2003). The soil autoclaved citrate extractable (ACE) protein content, an indicator of N-bound of soil organic matter that can be mineralized by microorganisms, was determined according to Moebius-Clune et al. (2016). Briefly, proteins were extracted from air-dried soil with sodium citrate buffer (20 mM, pH 7.0), autoclaved, and determined using the bicinchoninic acid (BCA) assay.

BD was calculated after drying the soil at 105°C for 24 hours (BD = soil core dry weight/soil core volume; Lienhard et al., 2013).

2.4 Plant properties

Shoot biomass was oven-dried at 55°C and weighed afterwards to determine the dry weight. All grains harvested in each plot were weighed at 13% humidity to obtain grain yield. Shoot and grain dry samples were sent to INIA Soil and Plant Laboratory to determine shoot nutrients content. Shoot organic C was determined by 900°C combustion and subsequent CO_2 infrared detection technique. Shoot and grain N were analyzed by combustion at 900°C and subsequent N_2 thermal conductivity detection. Shoot and grain P were measured by sulfuric digestion and vanadomolybdate colorimetry. Shoot and grain K were evaluated by dry digestion and atomic emission.

2.5 DNA extraction and sequencing

DNA was extracted from 0.5 g of frozen bulk soil or frozen rhizosphere pellet (wet weight) using a FastPrep-24 bead-beating system and the FastDNA Spin Kit for Soil (MP Biomedicals, USA), following the manufacturer's protocol. DNA quality was checked by agarose gel electrophoresis and stored at -20°C.

Four out of six replicates per land use intensity were randomly selected for sequencing to achieve sufficient sequencing depth per sample within budget. Bulk soil and rhizosphere prokaryotic communities were characterized by sequencing the V3-V4 region of the *16S rRNA* gene, amplified with primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'; Sundberg et al., 2013), targeting both bacterial and archaeal domains. Library construction and high-throughput amplicon sequencing were performed by Novogene (UK) on an Illumina NovaSeq PE250 platform (Illumina, USA).

2.6 Processing of amplicon sequencing reads

Cutadapt version 2.3 (Martin, 2011) was used to remove primer sequences of the first PCR. Primer-trimmed sequence reads were error-corrected and merged using a minimum overlap of five base pairs. Amplicon sequence variants (ASVs, = 100% sequence similarity) were identified using DADA2 version 1.10.0 (Callahan et al., 2016) plugin for QIIME2 (Bolyen et al., 2018). Each ASV sequence was given a taxonomic annotation (80% confidence threshold) using a q2-feature-classifier classify-sklearn module trained with the SILVA v. 138 database (Quast et al., 2013). Sequences affiliated to chloroplasts, mitochondria, or unclassified at the domain level and ASVs with less than ten reads were discarded. Furthermore, spurious ASVs with a length < 380 bp or > 420 bp were also discarded based on the expected amplicon length range for the 341F/806R primers. Raw sequences are available at NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA1051053.

Sequencing of *16S rRNA* gene amplicons generated 5,919,482 high-quality sequences (between 55,576 and 113,187 reads per sample) and 18,002 ASVs. Rarefaction curves reached saturation (Supplementary Figure S1).

2.7 Amplicon sequencing data analyses

Amplicon sequencing data analyses were done with R v. 4.2.2 (https://www.r-project.org/) using packages phyloseq v. 1.42.0 (McMurdie and Holmes, 2013), vegan v. 2.6-2 (Oksanen et al., 2022) and ANCOM-BC v. 2.0.1 (Lin and Peddada, 2020; Lin et al., 2022). Graphics were prepared with the R packages ggplot2 v. 3.4.0 (Wickham, 2016), pheatmap v. 1.0.12 (Kolde, 2019) and corrplot v. 0.92 (Wei and Simko, 2021).

Data analyses were performed separately for each soil compartment. Repeated rarefaction was performed for alpha-diversity analysis by subsampling the ASV table 999 times to a standardized sequencing depth equal to the minimum library size per compartment after quality filtering, in order to retain all samples (53,833 reads for bulk soil and 68,293 reads for rhizosphere samples). Alpha-diversity indices, including richness, Shannon diversity and Pielou's evenness, were calculated and averaged across these iterations to obtain robust diversity estimates and minimize biases introduced by differences in sequencing depth. For beta-diversity, repeated rarefaction was applied using the avgdist function from the vegan package. The ASV table was subsampled 999 times at the same sequencing depth as used for alpha-diversity (53,833 for bulk soil and 68,293 for rhizosphere samples). An averaged Bray-Curtis distance matrix was then generated to reduce the impact of sequencing depth variation, ensuring a stable estimation of community dissimilarities for downstream multivariate analyses. A rank-based approach of land use intensity was used to perform a non-parametric multivariate analysis of variance (PERMANOVA; Anderson, 2001) based on Bray-Curtis dissimilarities to test the effect of land use intensity on the prokaryotic communities. NGL was ranked as no intensity (0), PIP as lowest intensity (1), LR and SR as medium intensity (2 and 3, respectively, based on the proportion of time with sown pasture within the rotation), and CC as highest intensity (4). Afterwards, pairwise PERMANOVA analyses were performed using a Benjamini-Hochberg correction. Non-metric multidimensional scaling (NMDS) analyses based on the Bray-Curtis distance were used to visualize community dissimilarities.

The heteroscedasticity of community assemblages using Bray–Curtis distance was tested using permutational analysis of multivariate dispersions (PERMDISP; Anderson, 2006).

Distance-based redundancy analyses (db-RDA) based on Bray-Curtis dissimilarities were performed to assess the contribution of land use intensity and soil properties to the variation in bulk soil prokaryotic communities. Relevant soil parameters were selected using backward and forward selection (ordiR2step function from the vegan package).

The relative abundances of the 25 most abundant prokaryotic genera in bulk soil and rhizosphere were visualized in heatmaps to compare their abundance distributions. Partial 16S rRNA gene sequences of the commercial soybean inoculant strains (B. elkanii U1301, B. elkanii U1302) were mapped to ASV sequences obtained from 16S rRNA gene amplicon sequences to assess the presence of the inoculant in the sequencing data. The mapping was performed using the NCBI BLAST+ blastn megablast tool (Camacho et al., 2009; Cock et al., 2015) on the local Galaxy server of the Julius Kühn Institute (Germany).

An analysis of the composition of microbiomes with bias correction (ANCOM-BC; Lin and Peddada, 2020; Lin et al., 2022) was performed at the genus level for bulk soil samples to identify genera whose relative abundances consistently varied among land use intensities. Afterwards, logistic regression models were applied to each discriminant genus to assess its association to each land use intensity. Only models with adjusted p-values < 0.05, after applying the Benjamini-Hochberg correction, were considered. Only the taxa that were significantly and positively associated with a specific land use intensity were considered in this study and termed unique responders, as previously applied in Cerecetto et al. (2024). Additional ANCOM-BC were performed at the ASV level for rhizosphere samples to identify differentially abundant ASVs among CCG, SRG, and LRG and between SRS and LRS.

Spearman correlations were performed between the relative abundance of the most abundant prokaryotic genera in the bulk soil and soil properties, and between bulk soil unique responders and soil properties to explore the relationship between the prokaryotic bulk soil community and soil properties.

2.8 Cultivation-dependent isolation of soybean and sorghum rhizobacteria

Dilution series up to 10^{-8} of each supernatant were prepared and plated on R2A supplemented with 100 µg mL^{-1} cycloheximide to reduce fungal growth. The plates were incubated at 28°C, and CFU were determined after 24 h, 48 h, and seven days. In total, one hundred bacterial colonies were picked randomly and further cultivated on R2A to obtain purified isolates.

2.9 Identification of soybean and sorghum rhizobacterial isolates

Genomic DNA of bacterial isolates was extracted using the Genomic DNA Extraction Kit (Qiagen, Germany) and the Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Isolates partial 16S rRNA

gene sequences were amplified according to Heuer et al. (2009) and sequenced in Macrogen Europe (The Netherlands). The obtained forward and reverse sequences were trimmed and assembled using CLC MainWorkbench v. 20.0.3 (Qiagen, Denmark). The resulting consensus sequences were blasted to the RDP database.

Isolates' 16S rRNA gene consensus sequences were mapped to ASV sequences, obtained by 16S rRNA gene amplicon sequencing, on the local Galaxy server of the Julius Kühn Institute, employing NCBI BLAST + blastn megablast tool (Camacho et al., 2009; Cock et al., 2015) and using a 98% of sequence similarity as threshold, commonly used to approximate species-level delineation for partial 16S rRNA gene sequences while accounting for sequencing and PCR errors (Nishioka and Tamaki, 2022).

A maximum likelihood phylogenetic tree was constructed using MEGA v. 11.0.13. This tree was based on the 16S rRNA gene sequences of ASVs that were found to be differentially abundant in sorghum and soybean rhizospheres (as identified by ANCOM-BC), as well as isolates that shared more than 98% similarity with these ASVs, and relevant NCBI reference strains.

2.10 Screening of rhizobacterial isolates for potential plant-beneficial traits

Protease, β-1,3-glucanase, and cellulase activity were tested according to Weinert et al. (2010), and chitinolytic activity was determined after Berg et al. (2001) to characterize biocontrol potential of isolates. These enzymes degrade proteins and polysaccharides present e.g. in fungal cell walls, thereby suppressing fungal pathogens (Elsayed et al., 2021). Phosphate solubilization and secretion of siderophores were assessed according to Nautiyal (1999) and Schwyn and Neilands (1987), respectively. Phosphate solubilization enhances phosphate availability for plant growth and development (Alori et al., 2017), while siderophores chelate iron, facilitating its uptake by plants and restricting its access to pathogens (Ansari et al., 2017). The production of phytohormone indole-3-acetic acid (IAA) and the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity were measured based on Koo et al. (2010). IAA promotes root elongation and branching, enhancing water and nutrient uptake (Lebrazi et al., 2020), while ACC deaminase alleviates plant stress by lowering ethylene levels under salinity or drought (Orozco-Mosqueda et al., 2018). The production of N-Acyl homoserine lactones (AHLs) was detected in a cross-streak assay using Chromobacterium violaceum cv026 for short-chain detection of C4-C8 AHLs and C. violaceum VIr07 for long-chain detection of C10-C16 AHLs (Durán et al., 2016). The ability to produce AHLs indicates quorum sensing activity, which influences bacterial communication, biofilm formation, and plant responses, with short-chain AHLs promoting growth and long-chain AHLs enhancing resistance (Shrestha and Schikora, 2020).

2.11 Univariate statistical analyses

Univariate statistical analyses and graphics were done with R v. 4.2.2 using packages agricolae v. 1.3-5 (de Mendiburu, 2020) and ggplot2 v. 3.4.0 (Wickham, 2016).

Plant and soil properties, and prokaryotic alpha-diversity indices were evaluated as follows: if normality or homogeneity of variance were violated, overall differences were assessed with Kruskal-Wallis tests and pairwise differences with Wilcoxon rank sum tests with Benjamini-Hochberg corrections. If assumptions were not violated, overall differences were evaluated with one-way ANOVAs and pairwise differences with Tukey's HSD tests. All statistical tests were performed separately for each soil compartment.

3 Results

3.1 Land use intensification negatively affected soil properties

The measured soil nutrients were affected by land use intensity at 0–15 cm depth. Differences among land use intensities were assessed based on ANOVA-Tukey´s HSD or Kruskal-Wallis-pairwise Wilcoxon tests ($p < 0.05$) when normality or homogeneity of variance assumptions were violated. Soil organic C (SOC) and total N were lowest in continuous cropping with soybean (CCG), with the highest values of SOC in long pasture-crop rotation with soybean grown two years after pasture (LRG, $p = 0.022$) and total N in LRG, short pasture-crop rotation with soybean grown two years after pasture (SRG) and permanent improved pasture (PIP, $p = 0.041$, Figures 1a, b). PIP showed the highest levels of potentially oxidizable C (PoxC), and CCG and natural grassland (NGL) the lowest ($p = 0.002$, Figure 1c). Available P was highest in CCG and lowest in NGL ($p = 0.030$, Figure 1d). Contrary to available P, K levels were higher in NGL compared to the rest of the land use intensities, except for SRG and PIP ($p = 0.016$, Figure 1e). The pH was also significantly different among land use intensities at 0–15 cm, and was higher in PIP than in pasture-crop rotations ($p = 0.026$, Figure 1f).

Intensity affected soil bulk density (BD), with the highest values observed in CCG and the lowest in PIP ($p = 0.019$, Figure 1g). Pasture-crop rotations showed BD values comparable to PIP and to CCG (Figure 1g).

Furthermore, heterotrophic soil respiration and soil autoclaved citrate extractable (ACE) protein content were affected by land use intensity (Figures 1h, i). Heterotrophic soil respiration was significantly lower only in CCG compared to NGL ($p = 0.024$), while all pasture-crop rotations and PIP did not differ from either CCG or NGL (Figure 1h). Moreover, soil ACE protein content was higher in LRG compared to CCG, long pasture-crop rotation with sorghum grown one year after pasture (LRS), PIP and NGL ($p < 0.001$, Figure 1i).

3.2 Bulk soil prokaryotic communities shaped by land use intensity

Shannon diversity indices of bulk soil communities were highest in LRG, SRG and short pasture-crop rotation with sorghum grown

one year after pasture (SRS) and lowest in NGL ($p = 0.011$, Figure 2a). Species richness followed the same pattern, with the highest values observed in SRG, LRG, and PIP, and the lowest in NGL ($p < 0.001$, Supplementary Figure S2a). However, Pielou's evenness index did not show significant differences among land use intensities ($p = 0.070$, Supplementary Figure S2b).

PERMANOVA, NMDS, and db-RDA revealed that bulk soil prokaryotic community composition was significantly affected by land use intensity ($p = 0.001$, Table 2, Figure 2b; Supplementary Figure S3). Pairwise PERMANOVA tests showed that LRG and LRS communities were significantly different from those in CCG ($p = 0.004$, $p = 0.048$) but similar to those in PIP ($p = 0.060$, $p = 0.095$), whereas SRG and SRS communities were similar to CCG ($p = 0.500$, $p = 0.054$) and significantly different from PIP ($p = 0.020$, $p = 0.029$, Figure 2b; Supplementary Table S2). Notably, all bulk soil land use intensities communities differed significantly from NGL ($p < 0.05$, Figure 2b; Supplementary Table S2). Additionally, PERMDISP tests were non-significant ($p > 0.05$), indicating that community differences were driven by land use intensity rather than dispersion effects (Supplementary Tables S3, S4).

Moreover, a db-RDA was conducted to assess the contribution of land use intensity, soil physicochemical properties, and biological proxies to the differentiation of bulk soil prokaryotic communities. The results indicated that intensity, along with PoxC and soil ACE protein content, were the primary drivers of bulk soil prokaryotic communities (Figure 2b). Land use intensity and soil ACE protein content were positively correlated with bulk soil prokaryotic communities in CCG, SRG, LRG, and SRS and negatively with LRS, PIP, and NGL, contributing to their separation along axis 1 (Figure 2b). Contrarily, PoxC was positively correlated with LRS and PIP and negatively with CCG, SRG, LRG, SRS, and NGL, contributing to their separation along axes 1 and 2 (Figure 2b).

3.3 Key taxa as indicators for soil health

Spearman's correlations were employed to explore the correlation of the 25 most abundant bulk soil prokaryotic genera with measured soil physicochemical properties and biological proxies (Figures 3a, b). *Bacillus* (Firmicutes), unclassified *Nitrososphaeraceae* (Thaumarchaeota) and Acidobacteriales (Acidobacteria) members, *Candidatus Udaeobacter* (Verrucomicrobia), unclassified Gaiellales (Actinobacteria) members, *Candidatus Nitrocosmicus* (Thaumarchaeota) and *Acidothermus* (Actinobacteria) were among the dominant genera (Figure 3a). For a further description of the taxonomic composition of prokaryotic bulk soil community, please see the Supplementary Material (Supplementary Figure S4a).

This initial correlation analysis of the top 25 most abundant genera revealed significant relationships between prokaryotic composition and soil properties. Sixteen genera showed significant correlations ($p < 0.05$) with heterotrophic soil respiration, BD, PoxC, K, available P, SOC, and pH. Among them, 11 had correlation coefficients greater than 0.5 ($|r| > 0.5$)
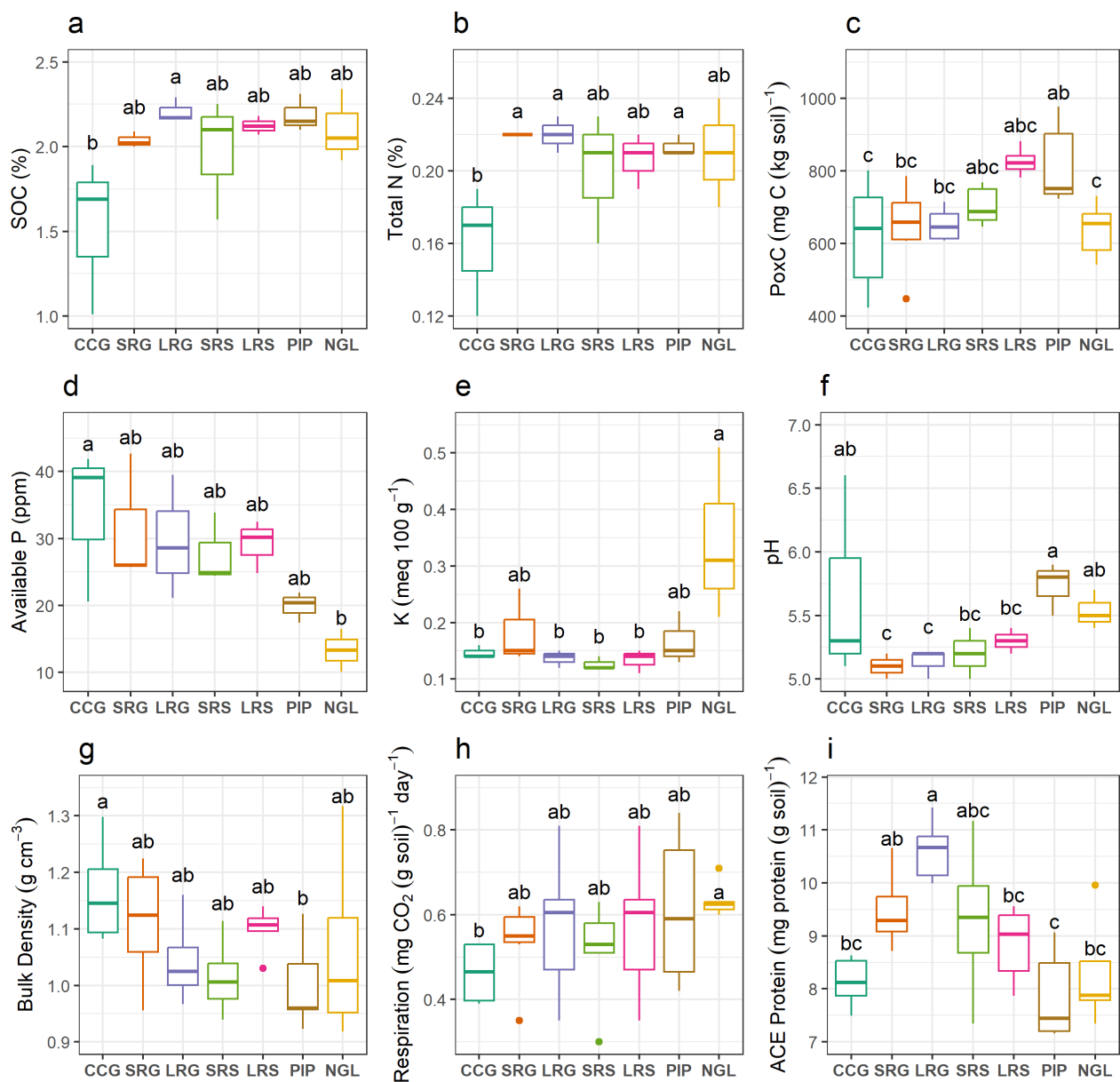
FIGURE 1
Measured soil physicochemical properties and biological proxies: **(a)** Soil organic C (SOC); **(b)** Total N; **(c)** Potentially oxidizable C (PoxC); **(d)** Available P; **(e)** K; **(f)** pH; **(g)** Bulk density; **(h)** heterotrophic soil respiration; **(i)** Soil autoclaved citrate extractable (ACE) protein content. Data represents mean and standard deviation of six replicates. Significant differences among land use intensities are indicated by different letters, according to ANOVA-Tukey´s HSD ($p < 0.05$). Land use intensity abbreviations: CCG (continuous cropping where soybean is grown), SRG (short pasture-crop rotation with soybean grown two years after pasture), LRG (long pasture-crop rotation with soybean grown two years after pasture), SRS (short pasture-crop rotation with sorghum grown one year after pasture), LRS (long pasture-crop rotation with sorghum grown one year after pasture), PIP (permanent improved pasture), NGL (natural grassland).

(Figure 3b). The genera that positively correlated with BD [i.e. Acidobacteriales, *Candidatus Nitrocosmicus*, *Bryobacter* (Acidobacteria), *FCPS473* (Chloroflexi), *Candidatus Koribacter* (Acidobacteria)] were also positively correlated with available P and exhibited highest relative abundances in CCG, SRG, and LRG (Figures 3a, b). Contrarily, the genera that positively correlated with PoxC, heterotrophic soil respiration and pH [*Sphingomonas* (Proteobacteria) with PoxC, *Streptomyces* (Actinobacteria) with

heterotrophic soil respiration and pH, and *Candidatus Udaeobacter* and *RB41* (Acidobacteria) with pH] exhibited highest relative abundances in LRS, PIP, and NGL (Figures 3a, b).

Using ANCOM-BC and multiple logistic regressions we identified unique responders to land use intensities (Figure 3c), however, none of them belonged to the dominant genera (Figure 3a), with the exception of *Sphingomonas*, which was a unique responder in NGL (Figure 3c). A Spearman's correlation
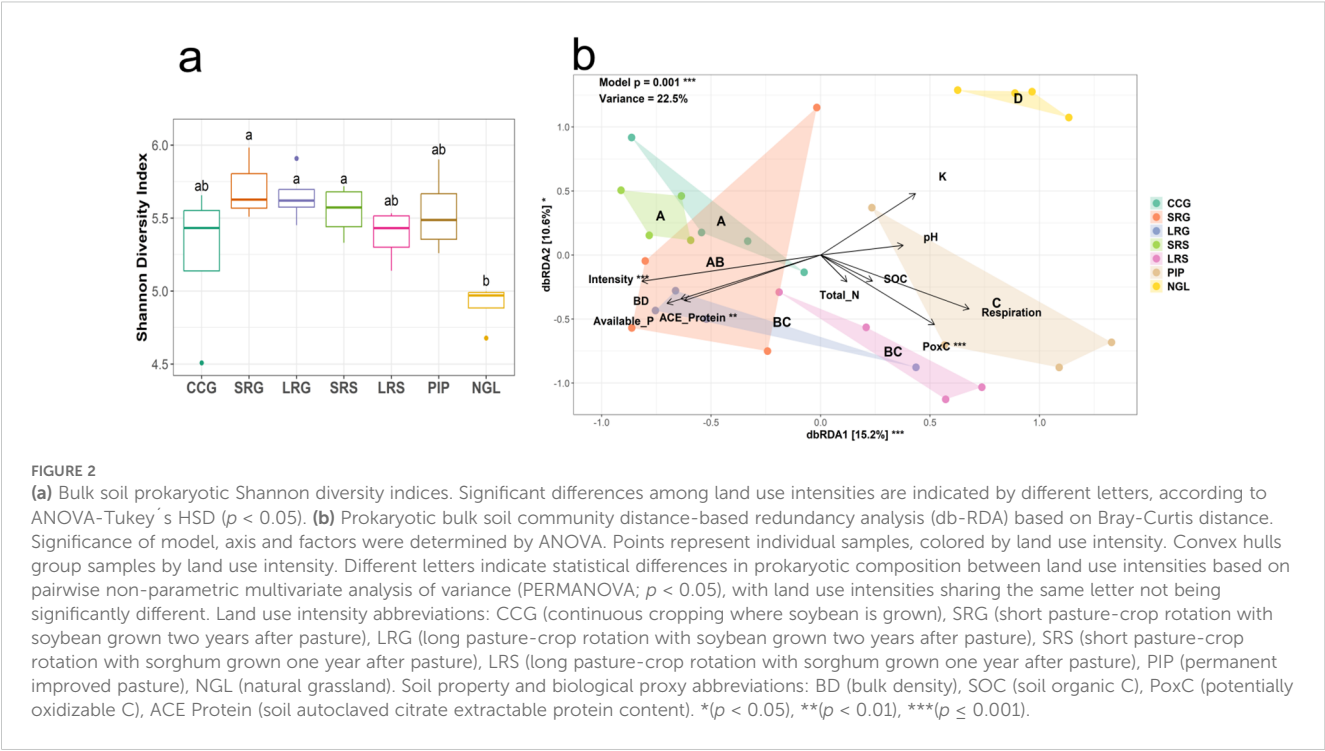
FIGURE 2
(a) Bulk soil prokaryotic Shannon diversity indices. Significant differences among land use intensities are indicated by different letters, according to ANOVA-Tukey´s HSD ($p < 0.05$). **(b)** Prokaryotic bulk soil community distance-based redundancy analysis (db-RDA) based on Bray-Curtis distance. Significance of model, axis and factors were determined by ANOVA. Points represent individual samples, colored by land use intensity. Convex hulls group samples by land use intensity. Different letters indicate statistical differences in prokaryotic composition between land use intensities based on pairwise non-parametric multivariate analysis of variance (PERMANOVA; $p < 0.05$), with land use intensities sharing the same letter not being significantly different. Land use intensity abbreviations: CCG (continuous cropping where soybean is grown), SRG (short pasture-crop rotation with soybean grown two years after pasture), LRG (long pasture-crop rotation with soybean grown two years after pasture), SRS (short pasture-crop rotation with sorghum grown one year after pasture), LRS (long pasture-crop rotation with sorghum grown one year after pasture), PIP (permanent improved pasture), NGL (natural grassland). Soil property and biological proxy abbreviations: BD (bulk density), SOC (soil organic C), PoxC (potentially oxidizable C), ACE Protein (soil autoclaved citrate extractable protein content). *($p < 0.05$), **($p < 0.01$), ***($p \leq 0.001$).

TABLE 2 Land use intensity effect on bulk soil and soybean and sorghum rhizosphere prokaryotic communities tested by non-parametric multivariate analysis of variance (PERMANOVA).

Explanatory variable	Bulk Soil		Rhizosphere			
			Soybean		Sorghum	
	Explained variance (%)	*p*-value	Explained variance (%)	*p*-value	Explained variance (%)	*p*-value
Land use Intensity	40.21	**0.001 *****	27.18	0.075	20.32	**0.033 ***

Bold and * indicate significant differences (* = $p < 0.05$, *** = $p \leq 0.001$).

analysis was conducted to explore the relationships between unique responders and soil physicochemical properties and biological proxies. *Bradyrhizobium* (Proteobacteria) showed significant positive correlations with heterotrophic soil respiration and K, and significant negative correlations with BD and available P, while *Solibacillus* (Firmicutes) was significantly positively correlated with heterotrophic soil respiration and significantly negatively with BD (Figure 3d). Additionally, unclassified members of *Planococcaceae* (Firmicutes) were significantly negatively correlated with BD and available P, and members of Subgroup 5 (Acidobacteria) were significantly negatively correlated with BD (Figure 3d). Moreover, *Sphingomonas*, *Bradyrhizobium*, *Solibacillus*, and unclassified members of *Planococcaceae* were identified as unique responders in NGL, while Subgroup 5 was a unique responder in PIP (Figure 3c). Furthermore, it is worth noting that at the genus level, pasture-crop rotations did not have any unique responders as identified by ANCOM-BC and multiple logistic regressions. Instead, the main compositional differences in terms of differentially abundant genera were observed among the intensity extremes, i.e., CCG, PIP, and NGL (Figure 3c).

3.4 Rhizosphere prokaryotic communities mainly shaped by crop type and, to a lesser extent, by land use intensity

Shannon diversity and Pielou's evenness indices significantly differed between crops and were higher in sorghum than in soybean ($p < 0.001$). Within each crop type, no differences were observed among land use intensities ($p > 0.05$, Figure 4a; Supplementary Figure S2b). Moreover, species richness did not significantly differ among crops and/or intensities ($p > 0.05$, Supplementary Figure S2a).

PERMANOVA tests and NMDS indicated that the effects of land use intensity were detected only in the sorghum rhizosphere ($p = 0.033$), not in the soybean rhizosphere ($p = 0.075$, Table 2, Figures 4b–d; Supplementary Table S2). Prokaryotic community composition differed significantly between short and long pasture-crop rotations with sorghum (SRS and LRS), but not among CCG, short, and long pasture-crop rotations with soybean (SRG, and LRG, Figures 4c, d, Table 2; Supplementary Table S2). Moreover, pairwise PERMANOVA tests comparing SRS *vs.* SRG ($p = 0.027$) and LRS *vs.*
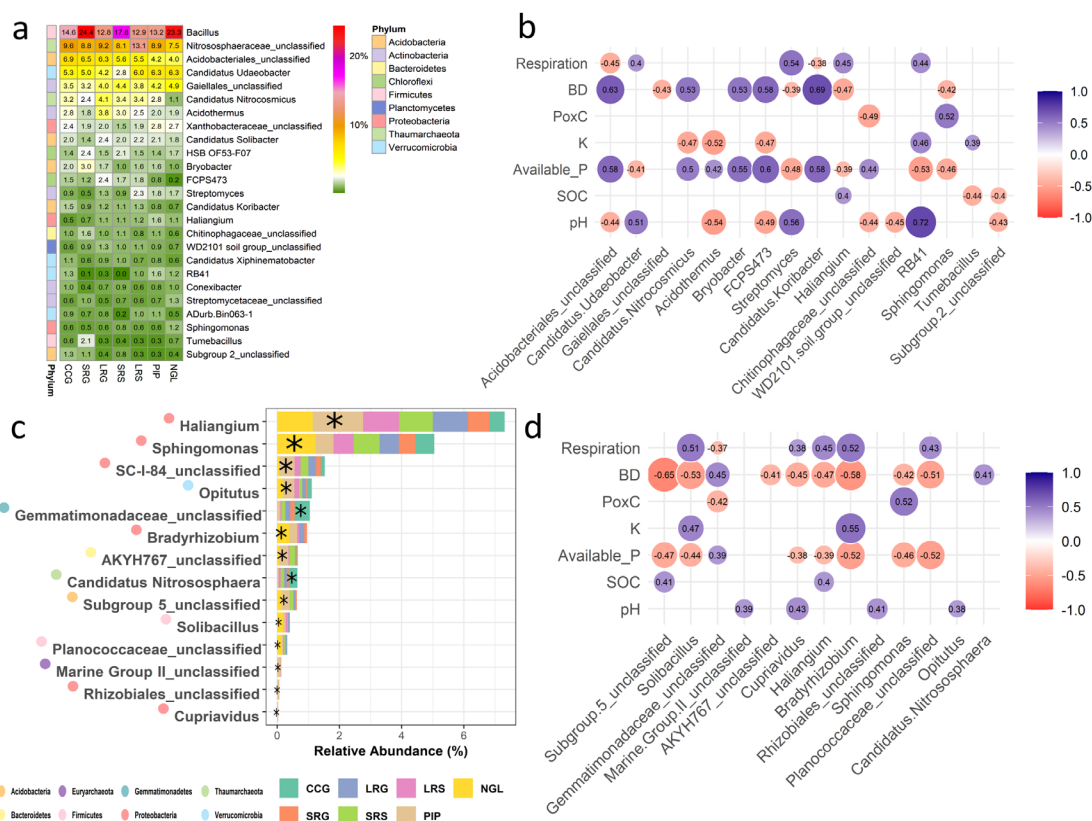
FIGURE 3
(a) Relative abundance of the 25 most abundant prokaryotic taxa in bulk soil, based on ASVs assigned to the genus level. Colors and numbers indicate mean relative abundance per taxon (n = 4). (b) Spearman correlations between the relative abundance of the 25 most abundant prokaryotic genera in bulk soil and measured soil physicochemical properties and biological proxies. (c) Prokaryotic unique responders to each land use intensity in bulk soil determined by analysis of the composition of microbiomes with bias correction (ANCOM-BC) at the genus level and logistic regression models applied to each discriminant genus. Data represents the mean relative abundance of each taxon (n = 4) in each land use intensity. * Indicates the land use intensity for which the taxa are unique responders. (d) Spearman correlations between the relative abundance of the bulk soil prokaryotic unique responders to land use intensities and measured soil physicochemical properties and biological proxies. Only significant correlations are shown (p < 0.05). Colors and numbers indicate correlation coefficients. Land use intensity abbreviations: CCG (continuous cropping where soybean is grown), SRG (short pasture-crop rotation with soybean grown two years after pasture), LRG (long pasture-crop rotation with soybean grown two years after pasture), SRS (short pasture-crop rotation with sorghum grown one year after pasture), LRS (long pasture-crop rotation with sorghum grown one year after pasture), PIP (permanent improved pasture), NGL (natural grassland). Soil property and biological proxy abbreviations: BD (bulk density), SOC (soil organic C), PoxC (potentially oxidizable C).

LRG (p = 0.033) showed significant differences (Supplementary Table S2). Additionally, PERMDISP tests were non-significant (p > 0.05), indicating that community differences were driven by land use intensity, crop type or pasture legacy duration rather than dispersion effects (Supplementary Tables S3, S4).

3.5 Rhizosphere isolates linked with ASVs increased in pasture-crop rotations expressed a diverse set of plant-beneficial traits

When examining the top 25 most abundant genera in the soybean rhizosphere, *Bradyrhizobium* (Proteobacteria) and unclassified members of *Xanthobacteraceae* (Proteobacteria) were dominant across CCG, SRG, and LRG (Figure 5a). Additionally, a total of 100 ASVs, obtained from amplicon sequencing and assigned to these two taxa, matched the *16S rRNA* gene sequences of the

commercial soybean inoculant strains (*B. elkanii* U1301, *B. elkanii* U1302) with > 99.74% sequence identity. However, one ASV matched both strains with 100% sequence identity, but could only be reliably classified within the *Xanthobacteraceae* family, likely due to the limited resolution of the *16S rRNA* gene V3-V4 region V3-V4 region for differentiating closely related genera within this family (Supplementary Table S5). Moreover, the relative abundance of all ASVs assigned to unclassified members of *Xanthobacteraceae*, that matched the inoculated strains, was significantly higher than that of *Bradyrhizobium*, with the highest values observed in SRG and LRG (Supplementary Table S6).

An ANCOM-BC was conducted to assess whether specific ASVs differed in abundance among CCG, SRG, and LRG. This analysis revealed that a total of 339 ASVs were differentially abundant among CCG, SRG, and LRG (p < 0.05, Figure 5b). Furthermore, an additional ANCOM-BC was conducted to detect significant differences between SRS and LRS at ASV level, revealing a total of 315 differentially abundant ASVs (p < 0.05, Figure 5b).
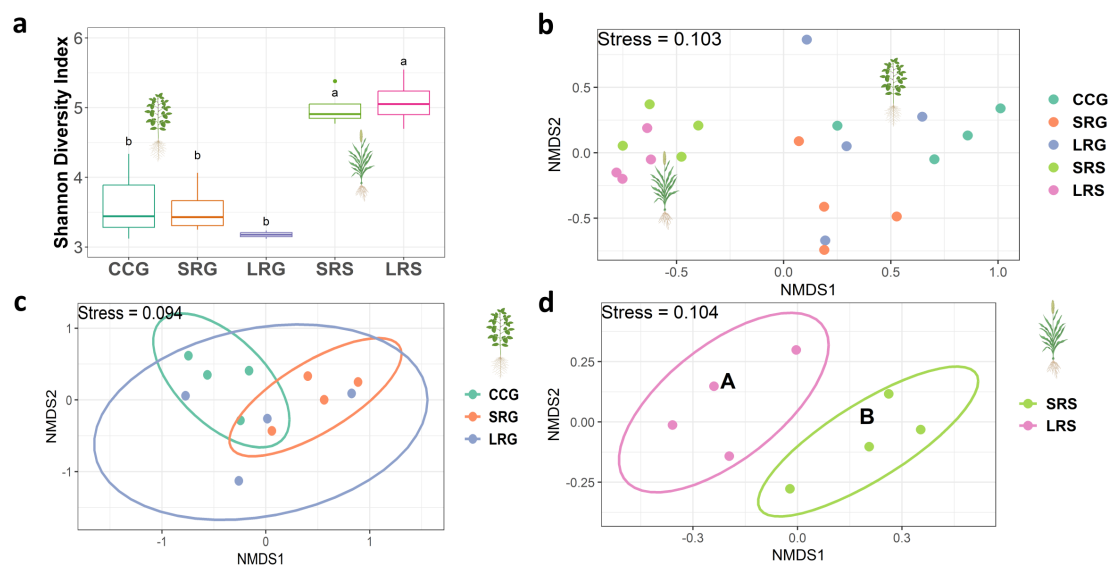
FIGURE 4

(a) Soybean and sorghum rhizosphere prokaryotic Shannon diversity indices. Significant differences between crop type are indicated by different letters, according to ANOVA-Tukey´s HSD ($p < 0.05$). No significant differences among land use intensities were found ($p > 0.05$). **(b)** Soybean and sorghum rhizosphere prokaryotic communities non-metric multi-dimensional scaling (NMDS) based on Bray-Curtis distance. Each point represents an individual sample, colored by land use intensity. **(c)** Soybean rhizosphere prokaryotic community NMDS based on Bray-Curtis distance. Each point represents an individual sample, colored by land use intensity. Ellipses represent 95% confidence intervals around the group centroids, assuming a multivariate normal distribution. Missing letters indicate no significant differences in prokaryotic composition among land use intensities based on pairwise non-parametric multivariate analysis of variance (PERMANOVA; $p > 0.05$). **(d)** Sorghum rhizosphere prokaryotic community NMDS based on Bray-Curtis distance. Each point represents an individual sample, colored by land use intensity. Ellipses represent 95% confidence intervals around the group centroids, assuming a multivariate normal distribution. Different letters indicate statistical differences in prokaryotic composition between land use intensities based on pairwise PERMANOVA ($p < 0.05$). Land use intensity abbreviations: CCG (continuous cropping where soybean is grown), SRG (short pasture-crop rotation with soybean grown two years after pasture), LRG (long pasture-crop rotation with soybean grown two years after pasture), SRS (short pasture-crop rotation with sorghum grown one year after pasture), LRS (long pasture-crop rotation with sorghum grown one year after pasture). Plant figures created with BioRender.

Across both comparisons, twelve of these differentially abundant ASVs shared more than 98% sequence similarity with 32 of the 100 bacterial rhizosphere isolates obtained in this study (Figure 5b). Most of these isolates belonged to the genera *Bacillus* (Firmicutes) and *Pseudomonas* (Proteobacteria), and a few to *Microbacterium* (Actinobacteria), *Delftia* (Proteobacteria) and *Stenotrophomonas* (Proteobacteria) (Figures 5b, c, Supplementary Table S7, Supplementary Figure S5). Additionally, most isolates showed a wide range of plant-beneficial traits according to *in vitro* tests and were mapped to ASVs significantly increased in pasture-crop rotations compared to CCG (Figure 5c). *Stenotrophomonas maltophilia* 8.3.2 and *Delftia acidovorans* 9.1.1 were the only two isolates which were linked to ASVs increased in CCG and showed the least plant-beneficial traits. *S. maltophilia* 8.3.2 was only able to produce siderophores and protease, and *D. acidovarans* 9.1.1 only produced siderophores (Figure 5c). It is worth noting that the ASV mapped to *D. acidovorans* 9.1.1 (ASV416) was also increased in LRG, apart from CCG, when compared to SRG, and in LRS when compared to SRS. Yet the ASV mapped to *S. maltophilia* 8.3.2 (ASV290) was only increased in CCG when compared to SRG and LRG (Figure 5c).

Moreover, it should be noted that *Pseudomonas* and *Bacillus* were among the top 25 genera in the sorghum rhizosphere, while in the soybean rhizosphere, they were the most abundant after *Bradyrhizobium* and unclassified members of *Xanthobacteraceae*.

Although, *Microbacterium*, *Delftia*, and *Stenotrophomonas* were not among the most abundant genera (Figure 5a), they were successfully isolated. For a further description of the taxonomic composition of sorghum and soybean rhizosphere prokaryotic communities, please see the Supplementary Material (Supplementary Figure S4b).

3.6 Pasture legacy in commercial grain yield

Soybean shoot biomass did not differ significantly between plants cultivated two years after pasture in SRG and LRG, and those cultivated in CCG ($p = 0.083$); however, a trend towards higher shoot biomass in LRG and SRG than CCG was observed (Table 3). This pattern was also reflected in commercial grain yield, where LRG and SRG produced twice the grain yield of CCG (Table 3). It is worth noting that these commercial grain yields were obtained for the whole plot without replicates. Additionally, the majority of measured shoot and grain nutrient contents did not differ significantly among CCG, SRG, and LRG ($p > 0.05$), except for shoot K, which was highest in CCG and LRG ($p = 0.002$, Supplementary Tables S8, S9).

For sorghum, grown one year after pasture in SRS and LRS, shoot biomass differed significantly ($p < 0.001$), with LRS exhibiting
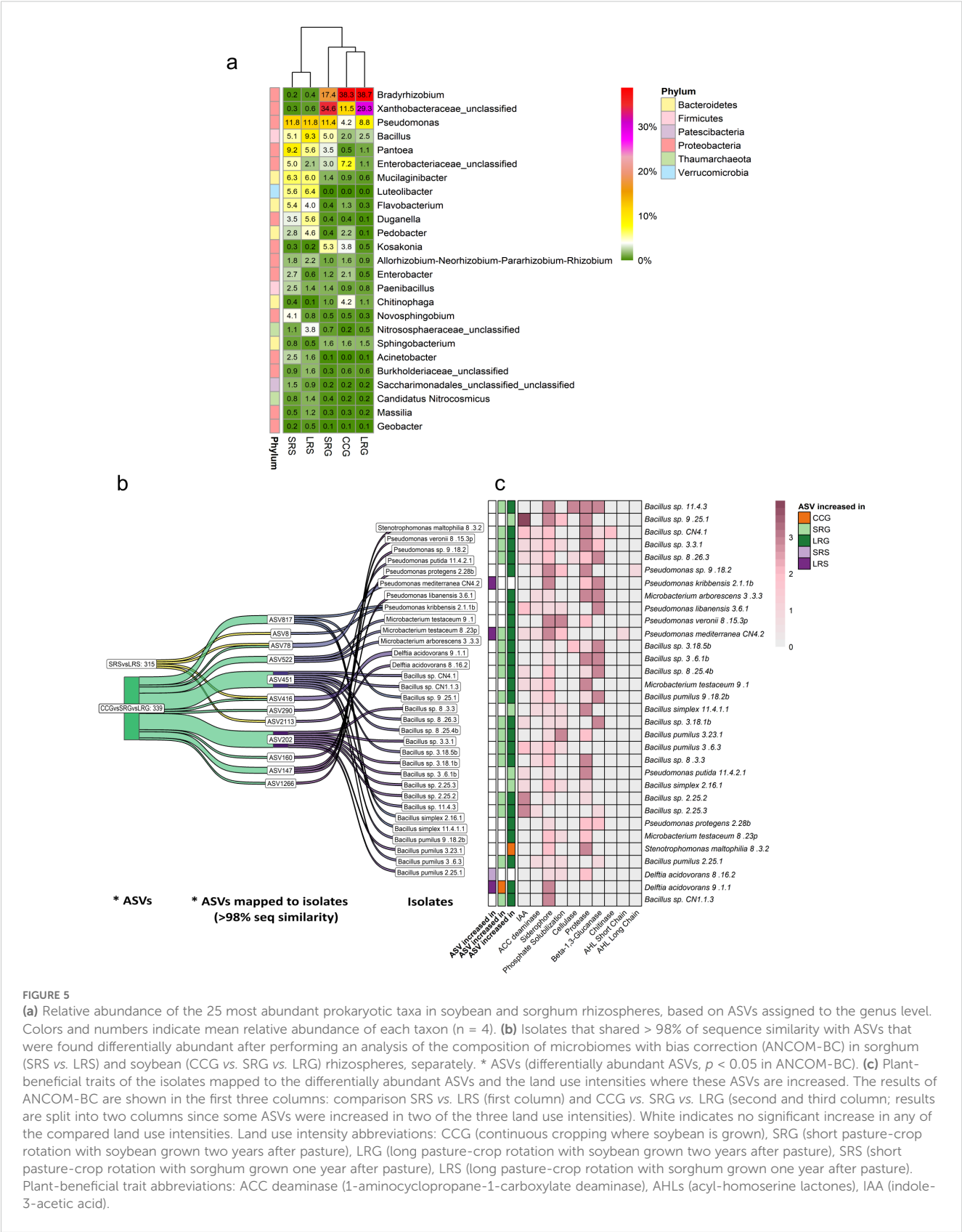
FIGURE 5
(a) Relative abundance of the 25 most abundant prokaryotic taxa in soybean and sorghum rhizospheres, based on ASVs assigned to the genus level. Colors and numbers indicate mean relative abundance of each taxon (n = 4). **(b)** Isolates that shared > 98% of sequence similarity with ASVs that were found differentially abundant after performing an analysis of the composition of microbiomes with bias correction (ANCOM-BC) in sorghum (SRS *vs.* LRS) and soybean (CCG *vs.* SRG *vs.* LRG) rhizospheres, separately. * ASVs (differentially abundant ASVs, *p* < 0.05 in ANCOM-BC). **(c)** Plant-beneficial traits of the isolates mapped to the differentially abundant ASVs and the land use intensities where these ASVs are increased. The results of ANCOM-BC are shown in the first three columns: comparison SRS *vs.* LRS (first column) and CCG *vs.* SRG *vs.* LRG (second and third column; results are split into two columns since some ASVs were increased in two of the three land use intensities). White indicates no significant increase in any of the compared land use intensities. Land use intensity abbreviations: CCG (continuous cropping where soybean is grown), SRG (short pasture-crop rotation with soybean grown two years after pasture), LRG (long pasture-crop rotation with soybean grown two years after pasture), SRS (short pasture-crop rotation with sorghum grown one year after pasture), LRS (long pasture-crop rotation with sorghum grown one year after pasture). Plant-beneficial trait abbreviations: ACC deaminase (1-aminocyclopropane-1-carboxylate deaminase), AHLs (acyl-homoserine lactones), IAA (indole-3-acetic acid).

TABLE 3 Soybean and sorghum shoot biomass and commercial grain yield, expressed as dry weight of shoot and grain per hectare, respectively.

Crop	Land use Intensity	Shoot Biomass (t ha^{-1})	Commercial Grain Yield (t ha^{-1})
Soybean	CCG	4.28 ± 1.29 **A**	2.45
	SRG	6.31 ± 1.91 **A**	3.04
	LRG	6.25 ± 1.75 **A**	2.93
Sorghum	SRS	4.25 ± 2.20 **b**	3.72
	LRS	7.55 ± 3.64 **a**	5.04

In the case of shoot biomass, data represents mean and standard deviation of six replicates. Bold capital letters and bold lower cases indicate significant differences among land use intensities within soybean or sorghum, respectively, according to ANOVA-Tukey's HSD ($p <$ 0.05). Commercial grain yield represents the yield of the sown grain in each 3ha-plot. Land use intensity abbreviations: CCG (continuous cropping where soybean is grown), SRG (short pasture-crop rotation with soybean grown two years after pasture), LRG (long pasture-crop rotation with soybean grown two years after pasture), SRS (short pasture-crop rotation with sorghum grown one year after pasture), LRS (long pasture-crop rotation with sorghum grown one year after pasture).

a higher shoot biomass and commercial grain yield (Table 3). Nevertheless, no significant differences were found in their shoot and grain nutrient contents ($p >$ 0.05, Supplementary Tables S8, S9).

4 Discussion

Understanding the role of grazed pastures in shaping bulk soil and rhizosphere microbiomes is essential for designing sustainable rotation strategies that maintain soil health and enhance crop performance. Therefore, in a LTE in the Uruguayan Pampa, the effects of land use intensification and pasture legacy on soil properties, bulk soil and rhizosphere prokaryotic communities and crop performance were evaluated. The first hypothesis was partially supported: while introducing grazed pastures into crop rotations preserved certain soil health indicators in comparison to continuous cropping (CC), the enhancement was limited to certain soil properties (e.g., SOC, total N, soil ACE protein content) (Figure 1). In addition, shifts in bulk soil prokaryotic community composition were associated with increased values of key soil health indicators, such as PoxC, heterotrophic soil respiration, and soil ACE protein content, and with decreased values of bulk density (Figures 2, 3). Moreover, pasture-crop rotations enriched rhizosphere prokaryotic communities with potentially plant-beneficial bacteria, and overall, they led to improved grain yield (Figure 5, Table 3). The second hypothesis was partially supported: while the pasture legacy persisted in the long pasture-crop rotation (LR), shaping prokaryotic communities and influencing sorghum performance (Figures 2–5, Table 3), the lack of significant differences between LR and short pasture-crop rotation (SR) in most soil physicochemical properties and biological proxies (Figure 1) suggests that pasture duration and plant species composition had only a minor effect on these conventional indicators of soil health. However, differences in prokaryotic composition and sorghum performance between LR and SR (Figures 2–5, Table 3) point to the biological relevance of more

subtle shifts in soil conditions, emphasizing the role of prokaryotic dynamics in mediating long-term management effects.

4.1 Impact of intensification and pasture legacy on soil properties

The intensification of agricultural practices significantly influenced soil physicochemical properties and biological proxies. Less intensive systems, such as pasture-crop rotation, permanent improved pasture (PIP) and natural grassland (NGL), significantly improved soil properties in seven out of nine comparisons relative to continuous cropping with soybean (CCG), highlighting the degradative effect of CCG. Particularly, pasture-crop rotations showed significant differences from CCG in only a subset of parameters, even two years after pasture. Nonetheless, the pasture legacy effect persisted, with higher SOC, total N, and soil ACE protein content observed relative to CCG. For the remaining soil properties, pasture-crop rotations occupied an intermediate position between CCG and the less intensive systems (PIP and NGL), as they did not show significant differences with either CCG, PIP, or NGL, which suggests a gradual but incomplete decline in soil health benefits. This reflects a residual pasture legacy effect, although less pronounced than that reported by Cerecetto et al. (2024), where measurements conducted immediately after the pasture phase showed stronger benefits for soil health.

The persistence of elevated SOC in long pasture-crop rotations two years after pasture (LRG) compared to CCG, along with similar levels to NGL and PIP, supports findings by Cerecetto et al. (2024), who observed higher SOC under LR and NGL at 0–15 cm depth. Furthermore, long-term monitoring at this LTE corroborates the observed trends, with CC leading to a 17% reduction in SOC compared to systems with a high proportion of pastures (Rovira et al., 2020). These benefits are attributed to increased organic matter inputs through plant residues and cattle manure (Souza et al., 2008; de Moraes et al., 2014). Moreover, among SOC fractions, PoxC, a proxy of readily available C for soil microorganisms (Moebius-Clune et al., 2016), was highest under PIP and pasture-crop rotations one year after the pasture (SRS and LRS), while NGL and CCG exhibited the lowest levels. The observed lower PoxC levels in NGL and CCG may reflect limited inputs of fresh and diverse organic matter (Wooliver et al., 2022), though for different underlying reasons. In NGL, despite having high SOC, the absence of recent inputs from plant residues or amendments, typical of more intensively managed systems like pasture-crop rotations, may result in a greater proportion of recalcitrant C and a lower labile fraction (Cotrufo et al., 2013). In contrast, CCG showed the lowest SOC values, likely due to continuous biomass removal, low stubble levels, reduced crop diversity, and long-term soil degradation, all of which limit the overall buildup of SOC, including the labile pool measured by PoxC (Lal, 2004). This, in turn, negatively affected heterotrophic soil respiration, a proxy for microbial activity (Błońska et al., 2017). Unexpectedly, while NGL had low PoxC, it exhibited the highest heterotrophic soil respiration rates, suggesting an active microbial community capable of

efficiently metabolizing recalcitrant organic matter. Similar findings have been reported in other studies, suggesting that agricultural practices can decrease total microbial activity (Kumar et al., 2023). In a study performed in the Argentinian Pampas, the highest microbial activity values were recorded in a pristine environment, while the lowest were observed under crop monoculture (Barbero et al., 2025). Moreover, Rubio et al. (2022) reported SOC and PoxC depletion and diminished microbial activity under CC in comparable LTEs in the Uruguayan Pampa, consistent with findings from the present study.

Soil compaction, estimated by bulk density, followed similar trends, with CCG exhibiting the highest bulk density, while PIP the lowest. Intermediate bulk density levels under pasture-crop rotations, especially SRS and LRG, suggest partial maintenance of soil porosity probably due to root effect. Yet, unlike Cerecetto et al. (2024), where significantly lower bulk density was maintained in the same LTE under pasture-crop rotations compared to CC, our findings suggest the structural benefits of pasture diminished with time, possibly due to increasing machinery traffic and decreased plant-cover time (Alvarez et al., 2014). This time-dependent decay of pasture-derived structural benefits was similarly reported by Franzluebbers et al. (2014) and Ernst et al. (2018).

Nutrient availability, particularly N, also differed among land use intensities. As reported in Cerecetto et al. (2024), total N remained higher in pasture-crop rotations and PIP than in CCG even two years after pasture, indicating long-term fertility benefits. This persistence can be attributed to N-fixing symbioses of legumes, cattle manure inputs, and root turnover (Ramirez et al., 2012; McDaniel et al., 2014; Pereyra-Goday et al., 2022). Microbial biomass and necromass can also act as reservoirs of slowly mineralizable N, contributing to long-term nutrient retention (Liang et al., 2017). Furthermore, microbial transformation of plant-derived N into microbial products may promote its stabilization within soil aggregates (Cotrufo et al., 2013), especially under systems with regular organic matter additions, such as grazed pasture-crop rotations. Elevated soil ACE protein content, a proxy of organic N available for microbial mineralization, in short and long pasture-crop rotations two years after the pasture (SRG and LRG) further supports the role of grazed pasture in enhancing N storage over the long term. However, this improvement in soil N was not reflected in soybean shoot N content, likely due to effective symbiotic N fixation (SNF) across all land uses with soybean. This can be attributed to the routine inoculation of soybean crops with *Bradyrhizobium elkanii* N fixing strains (Cerecetto et al., 2021b) that ensures effective SNF regardless of land use. Similar findings have been reported in other studies in South America, where inoculation consistently sustains SNF and minimizes the influence of background soil fertility on shoot N content (review by Hungria et al., 2005).

Overall, the lack of significant differences between LR and SR for most soil physicochemical properties and biological proxies suggests that pasture duration and differences in pasture plant species composition have a minor effect on these type of soil health indicators. However, when prokaryotic communities and crop performance were considered, LR and SR differed,

emphasizing the impact of long-term management practices on prokaryotic assemblages and their interactions with soil properties. Although these properties did not differ significantly on their own, the observed shifts in prokaryotic communities and sorghum performance suggest that subtle changes in soil conditions may be biologically meaningful. This indicates that soil health cannot be fully captured by physicochemical and biological proxies alone, as prokaryotic dynamics, shaped by legacy effects of management, may mediate key processes that ultimately influence plant performance.

4.2 Effects of intensification and pasture legacy on bulk soil prokaryotic community diversity and composition

Land use intensification significantly shaped bulk soil prokaryotic diversity and composition, consistent with previous reports in the literature (Walkup et al., 2020; Sekaran et al., 2021; Frene et al., 2022; Williams et al., 2023; dos Santos-Goulart et al., 2024; Barbero et al., 2025). Interestingly, alpha-diversity was lowest in NGL and highest in CCG, with pasture-crop rotations in between. The dominance of the invasive plant species *Cynodon dactylon* in NGL led to more homogeneous vegetation, limiting root exudate diversity and reducing microbial habitat heterogeneity, hence likely resulting in a reduced prokaryotic alpha-diversity. This pattern reflects the Intermediate Disturbance Hypothesis, which posits that moderate disturbance, such as no-tillage CCG, can increase microbial alpha-diversity, while more stable conditions, such as NGL, may support lower alpha-diversity levels (Mendes et al., 2015). Despite no-tillage, CCG is the most intensive system due to high agrochemical use and machinery traffic over time. Similar trends were observed by Mendes et al. (2015) and Tomazelli et al. (2023), who found increased microbial diversity in managed systems compared to undisturbed soils. However, as noted by Bansal et al. (2024) and Barbero et al. (2025), greater microbial diversity does not necessarily reflect improved soil functionality. In low-disturbance systems, microbial diversity and functions may even be decoupled (Bansal et al., 2024; Barbero et al., 2025). Likewise, a review by Wooliver et al. (2022) highlighted that SOC responses to crop diversification are more closely linked to changes in microbial community composition than to shifts in alpha-diversity. This supports the idea that community composition and the presence of key functional groups may be more relevant indicators of soil health than alpha-diversity alone (Bender et al., 2016). Therefore, although alpha-diversity has been widely proposed as an indicator of soil health (Bender et al., 2016), the results of this study suggest that in relatively stable no-tillage systems, low alpha-diversity indices do not necessarily indicate loss of soil functions and health. In fact, NGL, PIP, and pasture-crop rotations, despite showing lower alpha-diversity than CCG, often exhibited parameters that indicated better soil health. This underscores that alpha-diversity alone is not a reliable indicator of soil health in this context and should be interpreted along with functional and physicochemical properties.

Shifts in community composition from NGL to CCG were observed, with land use intensity as one of the main drivers of bulk soil prokaryotic community structure, underscoring the profound impact of long-term management practices, including the legacy effects of grazed pasture, as reported in similar studies in the Cerrado and Pampa biomes (Frene et al., 2022; Cerecetto et al., 2024; dos Santos-Goulart et al., 2024). These shifts are related to cumulative effects of grazed pasture legacy, mediated through root exudates, rhizodepositions, plant residues, and cattle manure, that regulate microbial biomass, activity and shape microbial assemblages (Acosta-Martínez et al., 2010; Schmidt et al., 2019; Frene et al., 2022). As reviewed by Wooliver et al. (2022), crop rotation influences beta-diversity more than alpha-diversity, by driving shifts in the relative abundance of microbial taxa in response to variations in organic matter quality, root exudates, and nutrient availability due to different plant-derived inputs and management practices. These shifts often involve functionally important microbial groups, especially those involved in C cycling, without necessarily affecting overall richness. This further supports the idea that changes in prokaryotic community composition, rather than alpha-diversity alone, are more informative indicators of soil health, particularly in systems under reduced disturbance, such as no-tillage. Therefore, assessing beta-diversity may offer a more reliable perspective on prokaryotic responses to management, as it captures shifts in community structure that are closely linked to soil functions and health, beyond what alpha-diversity indices can reveal.

Furthermore, it was observed that prokaryotic communities in LR differed from CCG and closely resembled those in PIP, while SR was indistinguishable from CCG, suggesting that pasture legacy persists in LR but is lost in SR. This partially aligns with the findings of Cerecetto et al. (2024), who reported that the pasture legacy effect was strongest in SR immediately after the pasture phase but disappeared after one year. Moreover, Giongo et al. (2024) and Kaloterakis et al. (2024) demonstrated that the rotational position of crops significantly shapes microbial community composition driven by the distinct soil legacy effects of the preceding crop. In SR, the shorter pasture duration and simpler plant composition of the pasture may explain the rapid loss of legacy, while in LR, the more complex plant composition and extended pasture duration likely contributed to support more stable prokaryotic assemblages. Garland et al. (2021) and Wooliver et al. (2022) emphasize that time under cover crops and plant diversity are key drivers of microbial structure and soil functions. Therefore, strategies that promote lasting pasture phases with grazing cattle, as in LR, have a stronger impact on prokaryotic community composition.

4.3 Impacts of prokaryotic community shifts on soil health

Besides being closely linked to the intensification of the agricultural system, shifts in bulk soil prokaryotic community composition were also associated with key soil properties, such as PoxC and soil ACE protein content, as revealed by the db-RDA

analysis, highlighting their functional relevance. Less intensive systems, LRS and PIP, were closely associated with higher PoxC levels, supporting the use of PoxC as a sensitive indicator of soil health in pasture-crop rotations. Also, SRG and LRG were associated with increased soil ACE protein content, likely due to the effect of legume-based pastures and strengthened by soybean inoculation with N-fixing bacteria. Recent evidence showed that legume rhizodeposition, including the release of specific metabolites such as flavonoids and coumarins, can enhance biological N fixation by stimulating both symbiotic and free-living N-fixing bacteria in diversified cropping systems (Qiao et al., 2024). In summary, the interaction between plant inputs, plant functional types (e.g., legumes vs. grasses), and the soil microbiome plays a key role in C and nutrient cycling and can drive both compositional shifts and functional changes in microbial communities (Villarino et al., 2021; Sokol et al., 2022). Overall, these results highlight the dynamic nature of bulk soil prokaryotic communities in response to land use intensity and reinforce the role of pasture in maintaining soil health, even under increasing cropping intensity. Moreover, they support the notion that prokaryotic community composition, rather than alpha-diversity alone, offers a more sensitive indicator of soil health, especially under low-disturbance systems. This distinction is crucial for interpreting prokaryotic indicators in systems where disturbance is minimized and supports a shift toward composition-based assessments of soil health.

Further insights were provided by ANCOM-BC and multiple logistic regressions, which identified unique responders associated with healthier soils in pasture-based land use intensities, such as NGL and PIP. Unclassified members of *Planococcaceae* (NGL), *Solibacillus* (NGL), *Bradyrhizobium* (NGL), *Sphingomonas* (NGL), and Subgroup 5 (PIP) showed significant positive correlations with heterotrophic soil respiration and PoxC and significant negative correlations with bulk density and available P, suggesting their potential as positive bioindicators of soil health. These key taxa are known for key functional roles, including exopolysaccharide (EPS) production, phosphate solubilization, and organic matter decomposition that contributes to the pool of labile C (PoxC) and the cycling of C and other nutrients, thereby supporting microbial biomass and respiration (Shivaji et al., 2014; Cerecetto et al., 2021b; Chaddad et al., 2024; Sorouri et al., 2024). EPS and microbial activity promote soil aggregation, enhancing soil structure and reducing compaction, thereby improving aeration, water infiltration, and root penetration (Zethof et al., 2020; Bettermann et al., 2021; de Faccio Carvalho et al., 2021b; O'Callaghan et al., 2022).

Interestingly, no specific unique responders, were detected for pasture-crop rotations, which may reflect functional redundancy, where soil health is maintained through a broader network of taxa rather than a few dominant indicators (Delgado-Baquerizo et al., 2016), or subtle differences undetected across broader comparisons. Although not identified as a unique responder based on ANCOM-BC and multiple logistic regressions, the genus *Streptomyces* was significantly positively correlated with heterotrophic soil respiration and had a higher relative abundance in the less intensive land uses, i.e. LR, PIP and NGL. This genus is known for degrading complex

organic compounds that release C and N substrates, which can be utilized by other microorganisms (Moreno-Espíndola et al., 2018), further supporting its potential as a positive functional bio-indicator. Conversely, *Candidatus Nitrososphaera* was identified as a unique responder in the most intensive land use, CCG, and was associated with higher bulk density, supporting the idea that taxonomic indicators are more likely to emerge under intensity-driven microbial selection (Shade et al., 2012; Banerjee et al., 2019). It is important to note that while CCG exhibited higher alpha-diversity, this does not contradict the identification of key taxa adapted to agricultural intensification in this system. Alpha-diversity metrics do not necessarily reflect the community composition and abundance distribution patterns or functional dynamics of microbial communities. In CCG, elevated diversity may coexist with the enrichment of a few disturbance-tolerant taxa, such as *Candidatus Nitrososphaera*, which are favored under intensive management. These findings underscore the need to consider broader ecological interactions when evaluating soil prokaryotic communities. Land use intensification, plant diversity, and prokaryotic communities are tightly interconnected and co-regulate key soil processes (Frene et al., 2022; Cerecetto et al., 2024). Therefore, it is relevant to consider both community composition and potential functionality along with alpha-diversity metrics and soil properties when assessing soil health through soil prokaryotes.

4.4 Effects of intensification and pasture legacy on rhizosphere prokaryotic community diversity and composition

In contrast to bulk soil, crop type and inoculation with N-fixing bacteria emerged as dominant factors shaping the diversity and composition of rhizosphere prokaryotic communities. The observed differences between sorghum and soybean were primarily attributed to plant species, particularly through their influence on root exudates, other rhizodeposits and root morphology (Berg and Smalla, 2009). The impact of land use intensification was significant only in the rhizosphere of sorghum under SRS and LRS, similar to previous studies (Banerjee et al., 2019; Zhou et al., 2023; Cerecetto et al., 2024; Domeignoz-Horta et al., 2024; Martins Costa et al., 2024). The contrasting responses between SR and LR may result from their differences in pasture duration and plant species composition, which likely shaped distinct legacy effects and influenced rhizosphere microbial sensitivity to subsequent intensification. However, the pasture legacy previously detected in this LTE in the oat rhizosphere communities (Cerecetto et al., 2024) was no longer observed for soybean, likely due to the overriding effect of soybean inoculation with *Bradyrhizobium elkanii*. *Bradyrhizobium* was the dominant genus across all soybean-based land use intensities, and BLAST analysis confirmed the presence of both inoculated strains (*B. elkanii* U1301, U1302) in the soybean rhizosphere. Similar observations have been made in other cropping systems, where inoculation significantly shaped rhizosphere microbial communities regardless of agricultural management (Deng et al.,

2019; Behr et al., 2023). Interestingly, Martins Costa et al. (2024) reported a significant pasture legacy effect on plant-growth-promoting bacteria in the soybean rhizosphere in a mixed crop-livestock system where no inoculation was applied, allowing the pasture legacy to become evident. These findings highlight that beyond plant species, both anthropogenic factors, such as inoculation, and biological factors, such as plant diversity, play central roles in structuring rhizosphere microbial communities (Zhou et al., 2023; Domeignoz-Horta et al., 2024).

4.5 The role of pasture legacy on shoot biomass and grain yield

The effect of inoculation also appeared to override the influence of both land use intensity and pasture legacy on soybean shoot biomass and shoot nutrient content. This suggests that inoculation of soybean with *B. elkanii* may buffer or standardize plant nutrient uptake and growth across different land use intensities. In contrast, in the absence of inoculation, as in sorghum, biomass differences reflected the underlying effects of land use intensity and pasture legacy persistence, reinforcing the idea that inoculation can modulate or mask soil-driven variability in plant responses. Global meta-analyses have shown that longer cover crop durations (Peng et al., 2024) and legume-based mixtures (Chahal and Van Eerd, 2023) enhance subsequent crop yields. These findings suggest that the longer and more legume-rich pasture phase in LR may have contributed to improved productivity through soil legacy effects. In line with this, long-term field experiments and modeling studies in Uruguay have shown that increasing the frequency and duration of grass–legume pastures in rotations improves subsequent crop yields under no-tillage (Baethgen et al., 2021).

Despite the masking effect of inoculation on shoot biomass and shoot nutrient content, pasture-based rotations still resulted in 20% higher commercial grain yields compared to CCG. This outcome may be attributed to two key factors. First, the pasture legacy improved soil physicochemical properties and functionality through the soil microbiome, enhancing conditions for crop establishment and growth. Second, it enriched beneficial rhizobacteria, potentially supporting crop performance beyond soil properties alone. The presence of bacterial taxa with multiple plant-beneficial traits under pasture-crop rotations supports this interpretation, highlighting the role of microbial legacy in sustaining crop productivity. In the present study, several isolates belonging to genera such as *Bacillus, Pseudomonas, Microbacterium*, and *Delftia* were also enriched in pasture-crop rotations according to sequencing data, and exhibited diverse plant-beneficial traits, including IAA production, ACC deaminase activity, siderophore production, phosphate solubilization, protease, and glucanase activity. The enrichment of these taxa in the rhizosphere suggests that pasture-crop rotations promote rhizosphere prokaryotic communities with high functional potential. Furthermore, by promoting root development, nutrient availability, and stress tolerance, these traits not only support plant growth but also create positive feedback loops that improve soil health

through increased organic inputs and microbial activity (Rubio et al., 2022). In contrast, the *Stenotrophomonas maltophilia* strain linked to ASVs enriched in CCG exhibited fewer beneficial traits, indicating a less supportive prokaryotic environment for crop productivity in CCG. These patterns further reinforce the importance of legacy effects in shaping rhizosphere microbiome functionality.

As in the present study, Martins Costa et al. (2024) reported an enrichment of *Pseudomonas* and other taxa in the soybean rhizosphere under integrated crop-livestock systems compared to CC. Moreover, another study found that *Pseudomonas* enhances soybean's resilience to abiotic stress and promotes plant growth (Zheng et al., 2024). Furthermore, Martins Costa et al. (2024) identified *Bacillus* as a keystone taxon in both CC and mixed crop-livestock systems. Chang et al. (2022) also found a high abundance and diversity of *Bacillus* species in the soybean rhizosphere under intercropping systems, suggesting its potential role in plant growth promotion. It is well documented that many *Bacillus* members contribute to pathogen suppression in the soybean rhizosphere (Liu et al., 2021) and to N fixation (Ding et al., 2005; Senger et al., 2022). Additionally, *Microbacterium* emerged as another rhizosphere key taxon associated with pasture legacy. This genus is known to exhibit a wide range of plant growth-promoting traits, such as phosphate solubilization, siderophore and IAA production, ACC deaminase activity, N fixation, biocontrol potential, and to enhance crop performance across various plant species (Barnett et al., 2006; Pereira et al., 2007; Sheng et al., 2009; Madhaiyan et al., 2010; Bhattacharyya and Jha, 2012; Lin et al., 2012; Rashid et al., 2012; Zhang et al., 2017; Freitas et al., 2019; Tsavkelova et al., 2024). In a previous study in the same LTE, *Microbacterium* was also identified as a key taxon linked to increased oat biomass under pasture legacy effects (Cerecetto et al., 2024). Although, in the present study, *Microbacterium* was not among the 25 most abundant genera in the soybean and sorghum rhizosphere based on sequencing data, it was still successfully isolated on R2A, underscoring the persistence of the pasture legacy. This highlights the value of integrating both approaches to gain a more comprehensive understanding of prokaryotic function and legacy persistence, providing insights into the role of microbiomes in sustaining soil health and crop productivity. Moreover, different strains of the same species with identical *16S rRNA* gene sequences varied in the presence or expression of traits *in vitro*, underscoring the necessity of complementary genotypic and phenotypic analyses. The functional potential cannot be captured by *16S rRNA* gene sequencing data alone, and gene presence does not guarantee expression under all conditions. Identifying key microbial taxa associated with improved crop performance is essential for developing microbiome-based agricultural strategies (Bender et al., 2016; Bano et al., 2021). In this context, *Microbacterium* emerges as a promising candidate due to its functional diversity and persistence across multiple crop species (oat, sorghum, soybean) and rotation phases.

5 Conclusions

Pasture-crop rotations improved certain soil properties, such as SOC, total N, and soil ACE protein content, compared to continuous cropping (CC), while for other properties, an intermediate response was observed, suggesting a partial and residual pasture legacy effect. Bulk soil prokaryotic communities were shifted by land use intensity and pasture legacy, with composition rather than diversity more strongly associated with soil functionality. Although the inoculation with *B. elkanii* masked the effect of land use intensity and pasture legacy on soybean rhizosphere prokaryotic communities, pasture-crop rotations still supported taxa with potential plant-beneficial traits, which may have contributed to the higher grain yields. Finally, when considering prokaryotic community composition and sorghum performance, the pasture legacy effect on soil health persisted in long pasture-crop rotation (LR), while it seemed to fade within a year in short pasture-crop rotation (SR), likely due to its shorter pasture duration and differences in plant species composition inherent to the experimental design. Together, the findings highlight that both pasture duration and plant species composition may play a role in sustaining prokaryote-mediated ecosystem functions, and reinforce the value of pasture-based rotations, especially those with longer, legume-rich pastures, in maintaining soil functionality and crop productivity under no-tillage conditions. Future studies should further disentangle the functional contributions of microbial communities to crop performance in grazed pasture-crop rotations. In particular, the recurrent detection of *Microbacterium* across different rotation phases highlights its potential as a key taxon for plant–bacteria interactions in this particular LTE in the Uruguayan Pampa biome. The isolated strains could provide valuable insights into their functional role in pasture-based agroecosystems.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA1051053.

Author contributions

VC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. KS: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Validation, Writing – review & editing. DB: Conceptualization, Investigation, Validation, Writing – review & editing. CL: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbrio.2025.1582787/full#supplementary-material

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