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RESEARCH ARTICLE

Long-term grazing influences root-associated fungal communities in a common Mediterranean grass

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Abstract

Background and aims Ungulates are integral to Mediterranean ecosystems, influencing vegetation dynamics and soil microbial communities. While previous studies have documented the effects of herbivory on the soil microbiome (e.g. nitrogen-fixation groups), the impacts on root-associated fungal communities and their role in plant responses to herbivory remain poorly understood. These interactions are vital for ecosystem functioning and deserve further investigation.

Methods To explore these interactions, we used *Agrostis* spp. as species model, a common grass genus, highly consumed by ungulates worldwide. Roots were sampled from areas with high ungulate activity (deer density of 25–40 ind/km^2) and from adjacent 14-year ungulate exclosures to assess long-term changes in root-associated fungal communities. Additionally, *Agrostis* plants were grown in soils from these areas under greenhouse conditions to evaluate their regrowth capacity after simulated herbivory.

Results The fungal communities of the roots differed significantly between plants grown inside and outside the ungulate exclosures. Despite these differences, the regrowth capacity after simulated herbivory was not significantly affected by the soil origin (areas with and without herbivory). Plants grown in sterilized soils with recolonized fungal communities exhibited enhanced growth compared to those in untreated soils.

Conclusions The study demonstrates that ungulate herbivory alters root-associated fungal communities in *Agrostis* spp., yet plant growth remains unaffected, suggesting resilient plant-fungal interactions. Our findings show that microbial diversity alone may not predict ecosystem function, emphasizing the need to consider both taxonomic and functional dimensions of soil communities in conservation strategies for Mediterranean ecosystems.

Keywords *Agrostis* · Red deer exclusion · Fungal community · Fungal traits · Ungulate overabundance

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Abbreviation
AMF Arbuscular Mycorrhizal Fungi

Introduction

The influence of human activities, particularly land-use change, on the structure and functioning of ecosystems has been extensively studied in recent decades (Acevedo et al. 2011; Bajocco et al. 2012; Zheng et al. 2022). This impact, closely linked to climate change and biodiversity loss,have been assessed from multiple perspectives, with growing attention to socio-oeconomic changes (Valbuena-Carabaña et al. 2010). One significant consequence of the shift toward technology-based economies has been widespread land abandonment and a decline in agricultural and livestock practices (Peco et al. 2005). This shift has triggered significant ecological alterations, especially in human-managed landscapes, including landscape homogenization (Gámez-Virués et al. 2015), reduced functional diversity (Abadie et al. 2011) and overpopulation of unmanaged wild ungulates (Acevedo et al. 2011; Carpio et al. 2021). Animal overpopulation is a pressing issue in the current management of natural areas in Mediterranean regions (Carpio et al. 2017), particularly wild herbivores, which can severely inhibit plant survival, reproduction, and regeneration (Tremblay et al. 2007; Perea and Gil 2014). Studies show that the overpopulation of herbivores, especially wild ungulates such as deer (*Cervidae*), has led to a substantial reduction in palatable species (Milne-Rostkowska et al. 2020). These unmanaged ungulates are particularly relevant due to their strong and selective browsing pressure, which directly affects plant community composition and forest regeneration dynamics in Mediterranean landscapes (Gómez-Aparicio et al. 2005; Capó et al. 2021b).

Studies investigating plant responses to herbivore-dominated environments have mainly focused on above-ground adaptations to avoid predation (i.e. resistance; Strauss and Agrawal 1999), quickly recovering after predation (i.e. tolerance; Bailey and Schweitzer 2010; Barton 2016; Capó et al. 2021a), or adapting to conditions with diminished herbivore interactions (i.e., escape; Parachnowitsch et al. 2012). Despite substantial progress in understanding plant–herbivore interactions, less is known about the consequences for below-ground plant traits (Eldridge and Myers 2001) or their impact on plant-microbiome dynamics (Pulido-Suárez et al. 2021). Yet, this below-ground dimension is critical, as ungulates not only affect the physical structure of soils—promoting erosion and compaction (Ayres et al. 2007; van Klink et al. 2015)—but also profoundly influence nutrient cycling and availability, shaping the productivity and composition of entire plant and soil communities (Carpio et al. 2017; Wang et al. 2019). These effects arise largely from their capacity to modify both the quantity and quality of organic inputs through their impact on plant biomass, root growth, and nutrient-rich dung and urine (Carline and Bardgett 2005; Gordon and Prins 2008; Sitters and Andriuzzi 2019; Barbero-Palacios et al. 2020). Given their sensitivity to shifts in soil structure, resource quality, and plant traits, fungi likely play a pivotal but underappreciated role in mediating plant responses to herbivory (Hannula et al. 2019; Bahram et al. 2020).

Herbivores can directly influence soil microbial community composition by modifying nutrient inputs through dung and urine deposition (Bardgett et al. 1998), altering plant community structure and litter quality (Wang et al. 2019), and disrupting soil physical properties through trampling and foraging behaviours (Sitters and Andriuzzi 2019). However, responses vary depending on the taxa and functional group; in some cases, microbiomes have benefited from herbivory (Yang et al. 2013), while at a local scale, herbivory may increase, decrease, or have no effect on the microbiome (Wardle et al. 2001). Shu et al. (2024), in a recent meta-analysis, emphasized that the effects of grazing exclusion (GE) on soil microbial diversity—particularly fungal and bacterial richness—depend on climate, grassland type, and the duration of exclusion. They found that GE generally enhances microbial diversity in arid to semi-humid regions, semidesert and alpine grasslands, and under long-term exclusion (> 20 years), whereas it may reduce diversity in humid areas and shows limited effects in temperate grasslands. Other studies have revealed great variation in some important microbial groups such as those responsible for nitrogen fixation, like rhizobia (Capó et al. 2021b) symbiotic organisms such as ectomycorrhizae (Markkola et al. 2004), or arbuscular mycorrhizal fungi (Faghihinia et al. 2020). Ruotsalainen and Eskelinen (2011) investigated the impact of mammalian herbivory on the colonization of arbuscular mycorrhizal fungi (AMF) through a

field experiment in which *Solidago virgaurea* plantlets were transplanted into contrasting mountain tundra habitats—acidic and non-acidic—and exposed to multiple treatments, including herbivore exclusion. Their findings revealed that the presence of mammalian herbivores enhanced AMF root colonization in fertile, non-acidic sites characterized by high availability of compatible host species, whereas in acidic, nutrient-poor environments, where such hosts were limited, herbivory reduced colonization levels. Moreover, Fan et al. (2023) reported that ungulate grazing on the Tibetan Plateau did not alter AMF diversity or community structure. Together, these findings suggest that the impact of herbivory on soil and root-associated fungal communities is highly context-dependent and influenced by plant identity, herbivore type, and ecosystem characteristics. However, the effects of herbivores on root-associated fungi and their impact on plant responses to herbivory remain understudied (Kaštovská et al. 2024), despite the critical importance of these interactions for ecosystem functioning (Treseder and Lennon 2015; Wang and Sugiyama 2020).

To address this gap, we established an experiment to evaluate how plants respond to simulated herbivory, considering their interaction with the root microbiome. For this, we selected *Agrostis* spp., a common grass in Mediterranean landscapes, highly consumed by ungulate herbivores in many regions (Sherlock and Fairley 1993; Shrestha et al. 2005). To assess indirect effects, we sampled *Agrostis* roots from an area with high red deer (*Cervus elaphus*) populations (Fernández-Olalla et al. 2006; Perea et al. 2014) and from adjacent ungulate-exclusion areas over 14 years. We hypothesize that herbivore exclusion will significantly alter the composition of root-associated fungal communities in *Agrostis*, leading to a decrease in the diversity of symbiotic fungi but an increase in the phylogenetic diversity of associated fungal taxa (Frew et al. 2024). To investigate the role of the soil microbiome in modulating plant growth and herbivory responses in *Agrostis*, we conducted a controlled greenhouse experiment. We further hypothesize that soils from areas historically exposed to different intensities of natural herbivory will harbor microbial communities better adapted to herbivory-induced stress, compared to those from areas that have been excluded from ungulate herbivory for the past 14 years. These microbial assemblages may include taxa capable of forming beneficial associations with plants, thereby enhancing their performance and tolerance under herbivory pressure (Frew et al. 2024). Consequently, we predict that *Agrostis* plants grown in soils collected from herbivore-exposed sites will support distinct root-associated fungal communities and will exhibit enhanced growth and tolerance to simulated herbivory —measured as their capacity to recover aboveground biomass removed by clipping— relative to plants grown in soils from herbivore-excluded.

Materials and methods

To assess whether the fungal community associated with *Agrostis* roots differs between areas with high deer density and those excluded, we first collected root samples of *Agrostis* sp. from inside and outside two exclusion fences. Once differences in fungal communities were established (Fig. 2B), we conducted a complementary greenhouse experiment under controlled conditions. *Agrostis stolonifera* individuals were germinated and planted in pots using soils collected from the same fenced and unfenced areas. This setup aimed to evaluate the influence of the soil fungal community on the root-associated fungi and the plant's ability to resprout after simulated herbivory. The controlled conditions in the greenhouse allowed us to standardize key variables (e.g., plant age, environmental conditions, and soil physical–chemical characteristics), ensuring that any observed differences could be attributed to the microbial communities present in the soils.

In situ site and sampling

The *in-situ* study was carried out in Los Quintos de Mora National Preserve (QM), municipality of Los Yébenes, Toledo, central Spain (39º 26′48″ N, 4º 06′01″ W, 950 m a.s.l.), belonging to the Autonomous Agency of National Parks with an extension of 6864 ha. QM presents acidic and siliceous soils formed on quartzite substrates. An overpopulation of red deer (*Cervus elaphus*), with ca. 25–40 ind/km^2 has been reported for the last 30 years (Fernández-Olalla et al. 2006; Perea et al. 2014). For this reason, two exclusion fences of 342 and 559 m^2 were established in 2007 (14 years before

the experiment) in order to preserve natural woody vegetation and ensure the regeneration of the highly threatened silver birch (*Betula pendula)* (Morales-Molino et al. 2019) (Fig. 1). Currently, differences

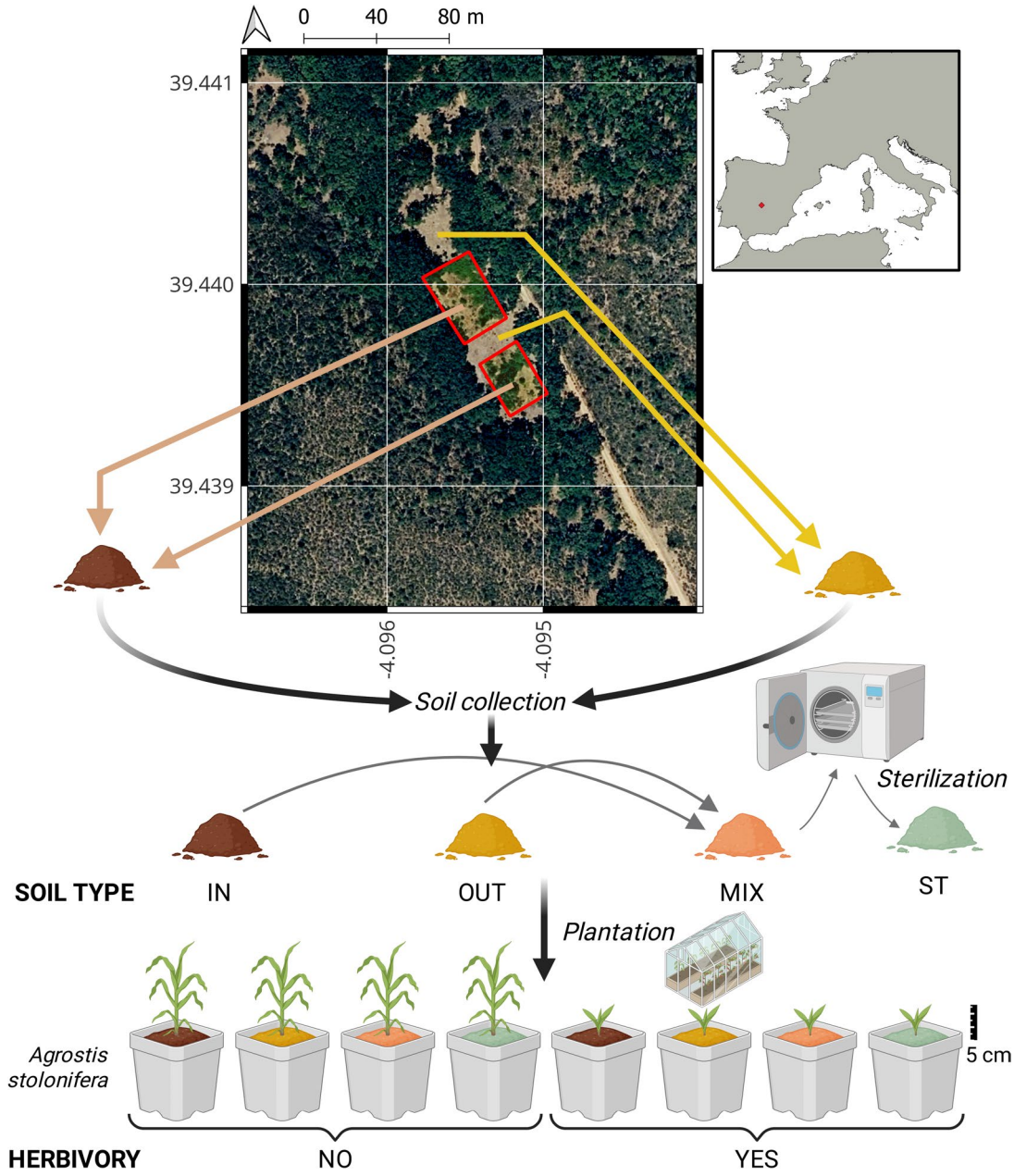

Fig. 1 Experimental design of the study. The soil and *Agrostis* sp. roots were taken from two 14-year herbivore exclosures (red rectangles) and the adjacent areas subject to deer herbivory in Quintos de Mora (Central Spain). Then, a greenhouse experiment was conducted, with three main steps: soil collection from the study site, plantation of *Agrostis stolonifera* seeds and herbivory simulation. Soils were obtained from the study area to generate 4 treatments. The IN and OUT soil types were obtained by mixing samples from 4 random points inside and outside the herbivore exclosure. MIX was a mixture of both and sterilize (ST) soil was sterilized using an autoclave. Each soil and herbivory combination had 7 replicates (n = 56 pots)

in plant coverage are evident inside and outside the exclusion (Fig. 1). In the absence of ungulates, the shrub cover was greater and taller (main genera *Cistus*, *Rubus, Daphne* and *Erica*) and three tree species were only regenerating inside ungulate exclusions: *Fraxinus angustifolia, Acer monspessulanum* and *Betula pendula* (Puyol-Castiñeira 2024). *Agrostis castellana, A. pourretii* and *A. stolonifera* are the most common *Agrostis* species in the area (Ramón et al. 2017).

Soil samples were collected from both the inside and outside of the two exclusion fences to perform physicochemical analyses (Fig. 1). Specifically, for each exclosure, three soil samples were collected at random points spaced at least 3 m apart to ensure spatial independence, both within the herbivore-excluded areas and in a comparable area outside the exclosures. Sampling outside the exclusion is carried out at least 3 m from the fence, within a rectangle with measurements similar to the fenced area. At each sampling point, five subsamples were taken within a 1-m radius. The samples were air-dried, homogenized, and sieved to <2 mm. Physicochemical analyses (total organic matter, organic carbon, total nitrogen, NO3, NH4, soil texture) were performed by Eurofins Agroambiental, SA (Lleida, Spain), following standard methods (Soil Science Division Staff, 1993). Organic carbon and oxidizable organic matter were quantified via potentiometric titration, total nitrogen by thermal conductivity, and nitrate/ammonium nitrogen by UV–Vis spectrophotometry. Soil texture was classified according to the USDA triangle using the Bouyoucos hydrometer and gravity separation methods.

Five replicate root samples of Agrostis sp. plants were collected from inside each exclusion fence, as well as five replicate samples from outside the fences. Plants were carefully excavated down to 15 cm to ensure collection of the complete root system at this depth. In all cases, sampled plants were identified as *Agrostis* based on vegetative characteristics; however, particularly outside the fenced areas, definitive species-level identification was often not possible due to the absence of inflorescences. Once the roots were collected in the field, they were stored in a cooler until they reached the laboratory, where they were cleaned with running water to remove the soil and the thinnest roots were selected to be frozen at −20 °C until DNA extraction.

The root-associated fungal community from each sample was sequenced. For this, 2 mg of roots were subsampled for DNA extraction and fungal sequencing. Total DNA was extracted using the DNeasy Plant Kit (Quiagen®). DNA from each individual sample was sequenced using the Illumina MiSeq platform, following a 2×275 PE strategy in the genomics service of the Institute of Parasitology and Biomedicine López-Neyra (CSIC; Granada, Spain). PCR amplification was performed using 1 µL of genomic DNA, 12.5 µL of EmeraldAmp® GT PCR Master Mix (TAKARA BIO Inc.), 1.5 µL of DMSO, 1 µL of each primer and 8uL of H_2O. Primers ITS4 (White et al. 1990); TCCTCCGCTTATTGATATGC) and fiTS7 (Ihrmark et al. 2012); GTGARTCATCGAATCTTTG) were used to sequence the fungal ITS2 region. We chose to amplify the ITS2 region because it provides high taxonomic resolution for fungi, performs well in environmental samples with high fungal diversity, and has a lower co-amplification rate of plant DNA compared to ITS1 (Ihrmark et al. 2012).

Greenhouse experiment

Agrostis stolonifera plants were used as a case study to evaluate the effect of soil origin (from inside or outside the ungulate exclosures) on their growth and response to herbivory. A controlled experiment was implemented in the greenhouse of the ETSI Monte (Madrid; Spain) beginning in April 2022, when the individuals were seeded, and continued until July, when harvesting and data collection took place. This covered four months of plant growth under experimental conditions, a duration chosen based on prior studies to allow sufficient time for plant establishment and regrowth after the herbivory simulation, while minimizing limitations imposed by pot volume (e.g., Poorter et al. 2012).

In situ soils were collected from two randomly selected points ($n = 4$) within each exclosure and their corresponding points ($n = 4$) outside the exclosure. The soils from each area were then mixed, creating master samples for both inside (referred to as the"In"treatment) and outside ("Out"treatment) the fenced areas. Soils were pooled prior to the greenhouse experiment to reduce within-treatment variability and focus on treatment-level effects. In equal parts these master samples ("In"and"Out") were then combined to create a third soil type,

representing a mixture of both soil sources ("Mix"). To ensure proper homogenization, weighed, and sieved portions of each soil type were placed in a tray and thoroughly mixed by hand until a uniform mixture was achieved. This process was repeated several times until the total volume of mixed soil required for the experiment was obtained. From this mixed sample, a fourth soil type was generated by sterilizing the mixed soil in an autoclave, referred to as"Sterile"(see Fig. 1).

As a result, the soil treatment had four levels: In- Soil collected from inside the exclosure, excluded to herbivory; Out- Soil collected from outside the exclosure; Mix- Soil mixed from both sites (inside and outside the exclosure) and Sterile- Soil mixed from both sites and sterilized before planting. Soil sterilization was conducted using an autoclave (Presoclave III 80). The sterilization process was performed 3 times over 3 consecutive days, at 121 °C for 30 min each time, using a porous bag (cotton cover). On the last day, the sterilization was extended for an additional 15 min, and a plastic bag was used instead of the porous one.

To account for potential nutrient release caused by soil sterilization, a specific fertilizer (Peters Professional General-Purpose Fertilizer) was applied at a concentration of 2 g/L to all treatments except for the"Sterile"treatment. This fertilizer was applied on two different occasions, in the last week of April and the first week of June, resulting in a total increase of approximately 8 mg of nitrogen (N) and 8 mg of available phosphorus (P) per pot. Pots were rotated in their greenhouse position in order to homogenize their conditions.

Each soil type was used to fill 14 pots of 1000 cm^3 containing 100 g of experimental soil, with sterile silica sand added to complete the volume. One hundred mg of seeds of *A. stolonifera* previously disinfected, were planted in each pot on 4th April 2022. Two months after the plantation (8 th June 2022), herbivory treatment were performed in 28 individuals (n = 7 for each soil type) (Fig. 1). Herbivory treatment was divided into two levels: YES, plants with simulated herbivory where the aboveground biomass was clipped up to 5 cm above ground level, and NO, plants with no clipping (Fig. 1). The biomass removed from each plant was weighed, dried in an oven at 70 ºC for 72 h and weighed again to calculate the dry biomass removed. The plants remained growing for 1.5 months until harvest (22nd July 2022).

During harvest, plants were carefully extracted from the pots and partitioned into belowground (roots) and aboveground biomass. A subsample of 2 mg of fresh root was carefully clean and frozen for DNA extraction and fungal sequencing. The samples were dried in the oven at 70ºC and then dry weight of roots and shoots were obtained. For herbivory treatment, the total dry aboveground biomass was calculated as the sum of the aboveground biomass and the biomass removed (clipped).

AMF colonization was assessed by separating 0.20 g of fresh roots from each individual and staining them following the method described by (Vierheilig et al. 1998). Briefly, the samples were submerged in boiling 10% KOH, rinsed with water, submerged again in 10% H_2O_2, rinsed again, and then submerged in 5% acetic acid. After this, the samples were treated with acetic ink (50 ml of PELIKAN 4001 ROYAL BLUE ink and 1000 ml of 5% acetic acid), rinsed with water, and submerged in 5% acetic acid. Once the roots were stained, the structures were observed using a stereomicroscope, and the abundance of mycorrhiza, including hyphae, vesicles or arbuscules, was estimated in each sample. The percentage of mycorrhization was calculated by dividing the number of roots with mycorrhiza by the total number of observed roots.

The fungal community from *A. stolonifera* roots in three pots per treatment was sequenced following the same protocol used for the *in-situ* assessment of *Agrostris* sp. roots described in the previous section.

Bioinformatic analysis

Sequence data processing and preliminary analyses were performed using QIIME2 v.2023.1 (Bolyen et al. 2019), a platform widely used in microbiome studies for its reproducibility, modular structure, and strong support for amplicon sequence variant (ASV)-based analysis, which improves taxonomic resolution compared to traditional OTU-based approaches. The QIIME2 plugin DADA2 was employed to denoise, dereplicate, and filter chimeric sequences. The first 20 nucleotides (nt) of each sequence (both forward and reverse) were trimmed and truncated to 275 nt and 257 nt, respectively, based on empirical quality scores, in order to remove low-quality base calls and ensure high-confidence downstream analyses. The result of DADA2 processing (Callahan et al.

2016) was a table of ASVs for increased taxonomic resolution (Glassman and Martiny 2018). Taxonomic classification of ASVs was performed using a Naive Bayes classifier pre-trained on the UNITE gene database v9.0 for fungi, resulting in 3,562 ASVs for both in situ and greenhouse root samples. Negative control samples were processed using the same protocol as all biological samples and were sequenced alongside them. The Decontam v1.24.0 package (Davis et al. 2018) was used to remove contaminants using a combined prevalence- and frequency-based detection method with a threshold of 0.1, effectively removing 21 ASVs more prevalent in negative controls than in true samples. The fungal ecological function of each ASV was determined using FungalTraits (Põlme et al. 2020) according to the authors instructions based on the taxonomic classification obtained from UNITE gene database (Tanunchai et al. 2023). ASVs identified as "Unknown Fungi" were filtered out, and ASVs from the same fungal species were combined.

Statistical analysis

Statistical analyses were performed using the R statistical software v4.4.1 (R Core Team 2024). To assess the effect of ungulate exclusion on soil characteristics, a Mann–Whitney–Wilcoxon test was performed using the `wilcox.test` function. To examine the effects of treatments on other response variables, we employed Linear Models (LM) and Generalized Linear Models (GLM) using the `lm` and `glm` functions of the `stats` package, respectively. Additionally, Generalized Linear Mixed Models (GLMM) were applied using the `glmer` function of the `lme4` package v1.1–35.3 (Bates et al. 2015). Specific model structures and distributions used for each response variable are detailed in the following paragraphs. Likelihood ratio tests to determine variable significance were conducted using the `Anova` function of the `car` package v3.1–2 (Fox and Weisberg 2019), and post-hoc analysis with Tukey adjustments was performed using the `emmeans` function of the `emmeans` package v1.10.2 (Lenth et al. 2024).

For the greenhouse experiment, we first modelled the dry weight of roots and shoots across different soil types (four soil types) and herbivory simulation scenarios (two scenarios). The models were fitted to a Gamma distribution with an inverse link function. AMF colonization was also modelled across soil types and herbivory simulations, with individual plants included as a random factor, as three measurements per plant were taken. This GLMM model was fitted to a binomial distribution with a logit link function.

For both the in situ and greenhouse experiments, ASV counts were transformed into a `phyloseq` object using the `qiime2R` package v0.99.13 (Bisanz 2018). Before conducting α-diversity statistics, samples were rarefied to an even depth using the `rarefy_even_depth` function form the `phyloseq` package v1.48.0 (McMurdie and Holmes 2013). The species richness, Shannon and Chao1 indices were calculated using the `estimate_richness` function of the `phyloseq`, while Faith's Phylogenetic Distance (PD) was calculated with the `picante` package v1.8.2 (Kembel et al. 2010). Species richness was modelled across soil types and herbivory simulations using a generalized linear model (GLM) fitted to a quasibinomial distribution with a logarithmic link function. Shannon, Chao1, and Faith's PD were modelled using linear models (LM).

β-diversity was assessed using Non-Metric Multidimensional Scaling (NMDS) with Bray–Curtis dissimilarity to visualize sample clustering across soil and herbivory treatments. Permutational multivariate analysis of variance based on distances (PERMANOVA) were conducted using the `adonis2` function of the `vegan` package v2.6–4 (Oksanen et al. 2024) to evaluate the effects of treatments on fungal community composition. A PERMANOVA analysis was also performed to determine whether herbivore exclusion caused significant differences in fungal functionality based on the abundance matrix classified using FungalTraits. Functional variations were illustrated through a Principal Coordinate Analysis (PCoA) plot based on the Bray–Curtis dissimilarity matrix, generated with the `cmdscale` function of the `stats` package.

We employed a Similarity Percentage (SIMPER) analysis to identify key ASVs responsible for community differences between soil treatments, using the `simper` function of the `vegan` package on the Bray–Curtis distance matrix with 9999 permutations. Finally, differences were visualized using a heatmap created with the `plot_heatmap` function of the `phyloseq` package. All statistical results and parameters are shown in Supplementary Table S1.

Results

In situ ungulate exclusion effects

Physicochemical analyses

The soil obtained from inside and outside the exclusion areas differed in physicochemical composition (Table 1). Although the soils were similar in organic-related parameters, such as total organic matter, organic carbon, and texture, significant differences were observed in nitrogen-related parameters (total nitrogen, nitric nitrogen, and ammoniacal nitrogen), with higher levels found outside the herbivory exclosure than inside (Table 1).

Root fungal community

These differences in soil composition between inside and outside the herbivory exclosure (Table 1), also influenced the fungal community associated with *Agrostis* sp. roots. The community composition varied substantially between the two sites. In terms of alpha diversity, the soils were similar based on Shannon's diversity index ($p = 0.349$, LM), but the fungal community outside the herbivory exclosure was richer ($p < 0.044$, GLM) and exhibited higher phylogenetic diversity ($p = 0.039$, GLM) than inside (Fig. 2A). Additionally, fungal community composition differed significantly between the two locations ($p = 0.007$, $R^2 = 0.5576$, PERMANOVA), clearly separating the samples from both sites in the NMDS analysis (Fig. 2B; 2D

Table 1 Physicochemical soil composition of the natural area inside and outside the herbivory exclosure. Values refer to the mean ± standard error (n = 3). Significance is indicated by asterisks: ** = p < 0.001, Ns = non-significant Wilcoxon test

Parameters	Outside (herbivory)	Inside (no herbivory)	
Total organic matter %	7.92 (± 1.15)	8.27 (± 0.40)	Ns
Organic carbon %	4.59 (± 0.27)	5.13 (± 0.66)	Ns
Total nitrogen %	0.42 (± 0.01)	0.35 (± 0.01)	***
Nitric nitrogen (NO_3) mg/Kg	41.00 (± 1)	25.67 (± 1.20)	***
Ammoniacal nitrogen (NH_4) mg/Kg	28.67 (± 0.24)	19.20 (± 0.10)	***
Clay %	14.63 (± 0.07)	11.96 (± 1.37)	Ns
Lime %	34.77 (± 0.68)	32.53 (± 0.69)	Ns
Sand %	50.57 (± 0.76)	55.50 (± 1.91)	Ns

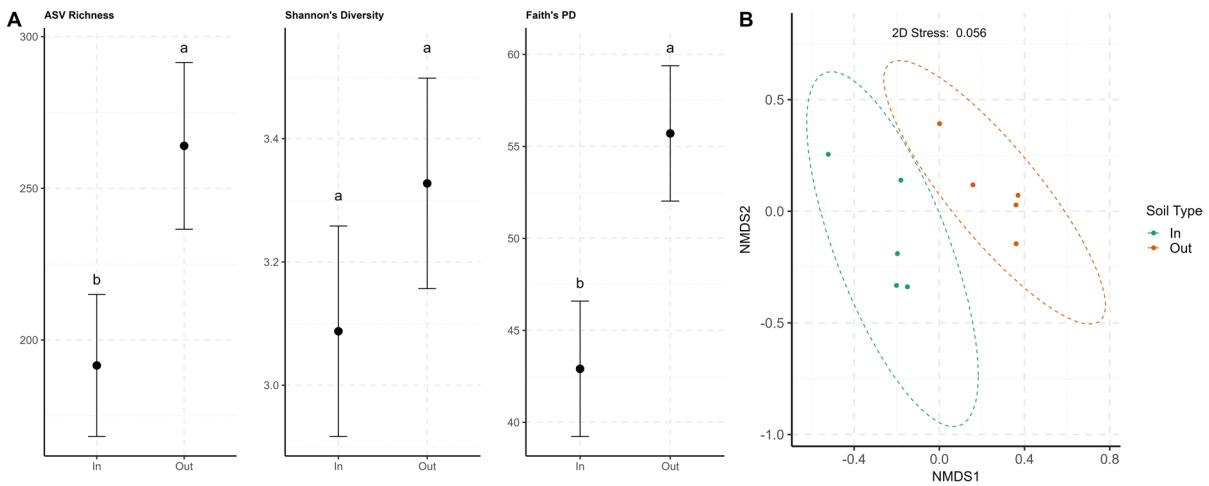

Fig. 2 In situ fungal community associated to *Agrostis* roots comparing inside and outside the herbivory exclosure. (**A**) alfa diversity indexes considering richness, Shannon diversity and faith's phylogenetic diversity and (**B**) beta diversity through NMDS organization (2D stress = 0.056)

stress = 0.056). However, no statistically significant differences in fungal functionality were detected between treatments ($R^2 = 0.08764$; $p = 0.427$).

When comparing the specific fungal composition, numerous saprotrophic and pathogenic species were found exclusively outside the herbivory exclosure (Fig. 3). Specifically, species unique to *Agrostis* roots growing outside the exclosures included saprotrophs such as *Geranomyces* spp. (saprotroph), *Malassezia* spp. (patotroph), *Sterigmatomyces* spp. (saprotroph), *Naganishia* spp. (saprotroph), *Helicodendron* spp. (saprotroph-symbiotroph), *Lachnum* spp. (saprotroph), *Plenodomus* spp. (patotroph-saprotroph), *Neoaschochyta* spp. (patotroph-saprotroph), *Sporomiella* spp. (saprotroph), *Westerndykella* spp. (saprotroph), *Microdochium* spp. (unknown). Conversely, fewer species were found exclusively within the herbivory exclosure, such as *Epicoccum nigrum* (patotroph) (Fig. 3).

Greenhouse experiment

No significant differences were observed in *Agrostis stolonifera* for the simulated herbivory treatment regarding total aboveground dry biomass ($p = 0.194$, GLM), root dry biomass ($p = 0.084$, GLM), or AMF colonization ($p = 0.458$, GLMM). However, significant differences emerged when comparing soil types ($p < 0.001$), with sterile soil treatment that stood out by producing almost twice the biomass (both roots and shoots) of other soil types, although it showed almost no AMF colonization (Fig. 4). The response to total aboveground biomass also varied significantly by soil treatment (soil type × herbivory interaction, $p = 0.02$, GLM). Plants grown in sterile soil showed lower final aboveground biomass under simulated herbivory, while plants in other soil types exhibited greater biomass under simulated herbivory compared to control plants (Fig. 4A).

The diversity indices of the root-associated fungal community (richness, Shannon diversity, and

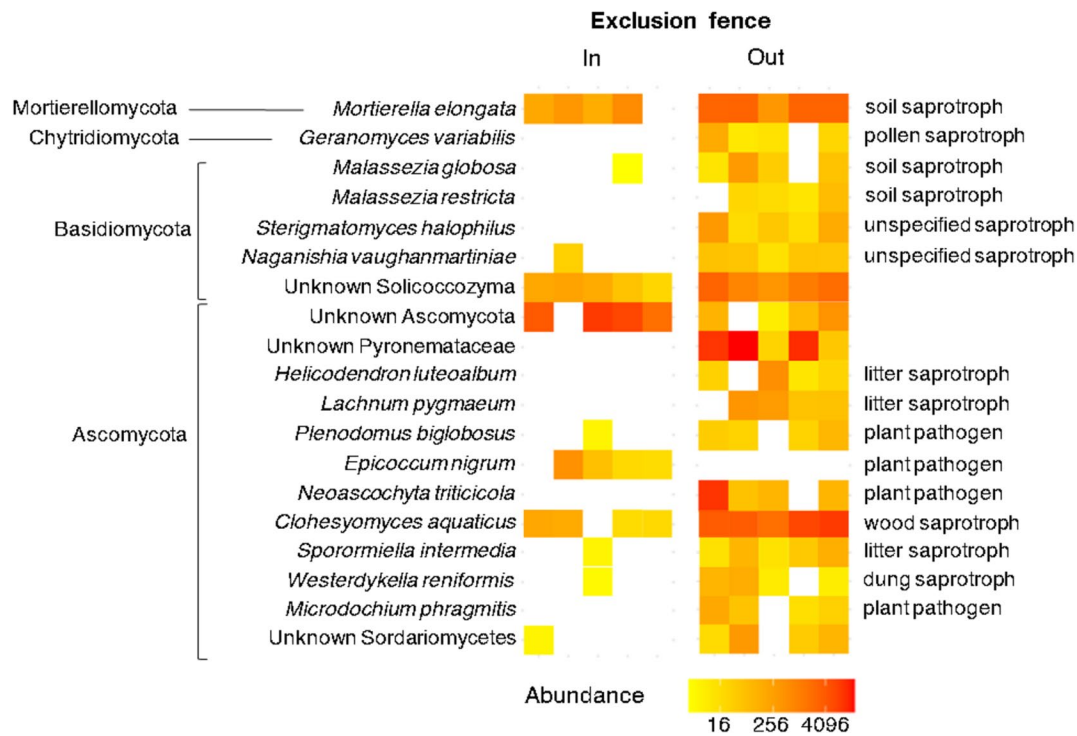

Fig. 3 Heatmap showing the absolute abundance of key ASVs associated with *Agrostis* roots in the field, identified through SIMPER analysis as most responsible for differences in root-associated fungal communities between soil from inside (In) and outside (Out) the herbivory exclosure. Taxonomic assignments and primary lifestyle categories (based on FungalTraits) are indicated. The full list of detected ASVs is provided in Supporting Information Table S2

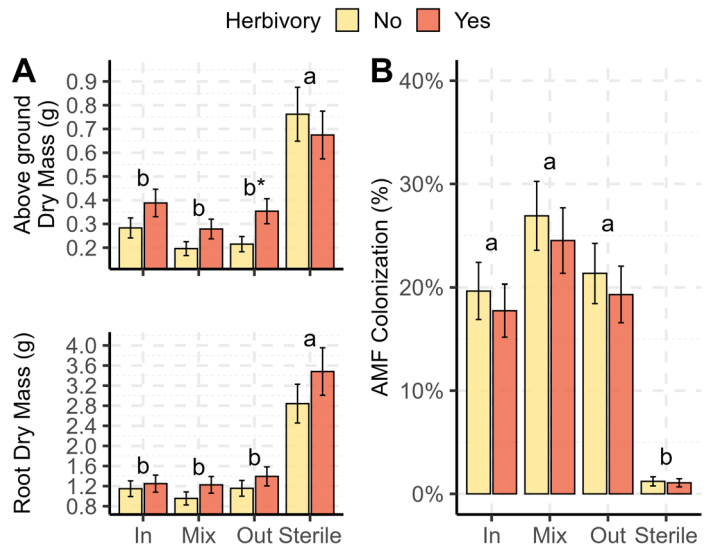

Fig. 4 **A**: Dry biomass from the aboveground parts (top) and the roots (bottom), **B**: proportion of roots colonized by arbuscular mycorrhizal fungi. Plots compare each soil type (x-axis) and herbivory (colors, yellow for control -no simulated herbivory- and orange for simulated herbivory). Different letters indicate significant differences for a post-hoc Tukey group comparing soil types. No differences were found for herbivory treatment within each soil type

phylogenetic diversity) were similar between the inside, mixed, and outside treatments; only the sterile treatment showed significantly lower values ($p < 0.01$, GLM; Fig. 5A). We found a substantial reduction in fungal ASVs in sterile soil, affecting alpha and beta diversity indices (Fig. 5). In contrast, no differences were found in simulated herbivory treatment in terms of alpha diversity (richness, $p = 0.891$; Shannon diversity, $p = 0.310$; phylogenetic diversity, $p = 0.888$, GLM) and beta diversity ($p = 0.917$, $R^2 = 0.04$, PERMANOVA). However, soil type had a significant effect on the composition of root-associated fungal communities ($p = 0.001$, $R^2 = 0.28$, PERMANOVA), with NMDS clearly separating roots of plants grown in sterile soil from others. Plants in mixed soil were placed between outside and inside soil treatments, as expected (Fig. 5B). Differences between In and Out soils with mixed soil in between maintained even when excluding sterile soils ($p = 0.001$, $R^2 = 0.63$, PERMANOVA). Higher dispersion of sterile samples, likely due to stochastic colonization, contributes to their separation in NMDS space.

The functionality of the fungal community also varied significantly between soil types ($p = 0.001$, $R^2 = 0.38$, PERMANOVA) but not between herbivory treatments ($p = 0.32$, $R^2 = 0.03$, PERMANOVA). Based on ASVs abundance across functional groups, samples from sterile soil differed notably from others, with a higher proportion of animal parasites, mycoparasites, and nonspecific saprotrophic fungi (Fig. 6). Differences between other soil types were less pronounced, showing high variability across samples (Fig. 6).

At the genus or species level, some soil saprotrophic genera, such as *Mortierella*, appeared exclusively in plants from outside-exclosure soil, while others, such as *Chaetothyriales*, were found only in roots of plants grown in inside-exclosure soil. Notably, within sequences identified as AMF, *Archaeosporales* sp. appeared mainly in inside-exclosure soil, whereas *Claroideoglomus* and *Scutellospora* were found mainly in outside-exclosure soil. Among Basidiomycota, *Atractiella rhizophila* was prevalent in outside-exclosure and mixed soils but absent in inside-exclosure soil, while *Rhizoctonia* sp., a plant pathogen, was found only in plants grown in inside-exclosure soil. Additionally, other Basidiomycota species appeared more frequently inside-exclosure soil and were absent in the sterile soil. Ascomycota, the most diverse fungal group, included numerous species and genera showing differential abundance between treatments, many of which have unknown functionality (Fig. 7). In sterile soil, we observed the presence of three species of *Trichoderma*, classified as mycoparasites (Fig. 7).

Discussion

Variation in vegetation and environmental conditions due to herbivory presence has been reported in previous studies (Harrison and Bardgett 2008), but

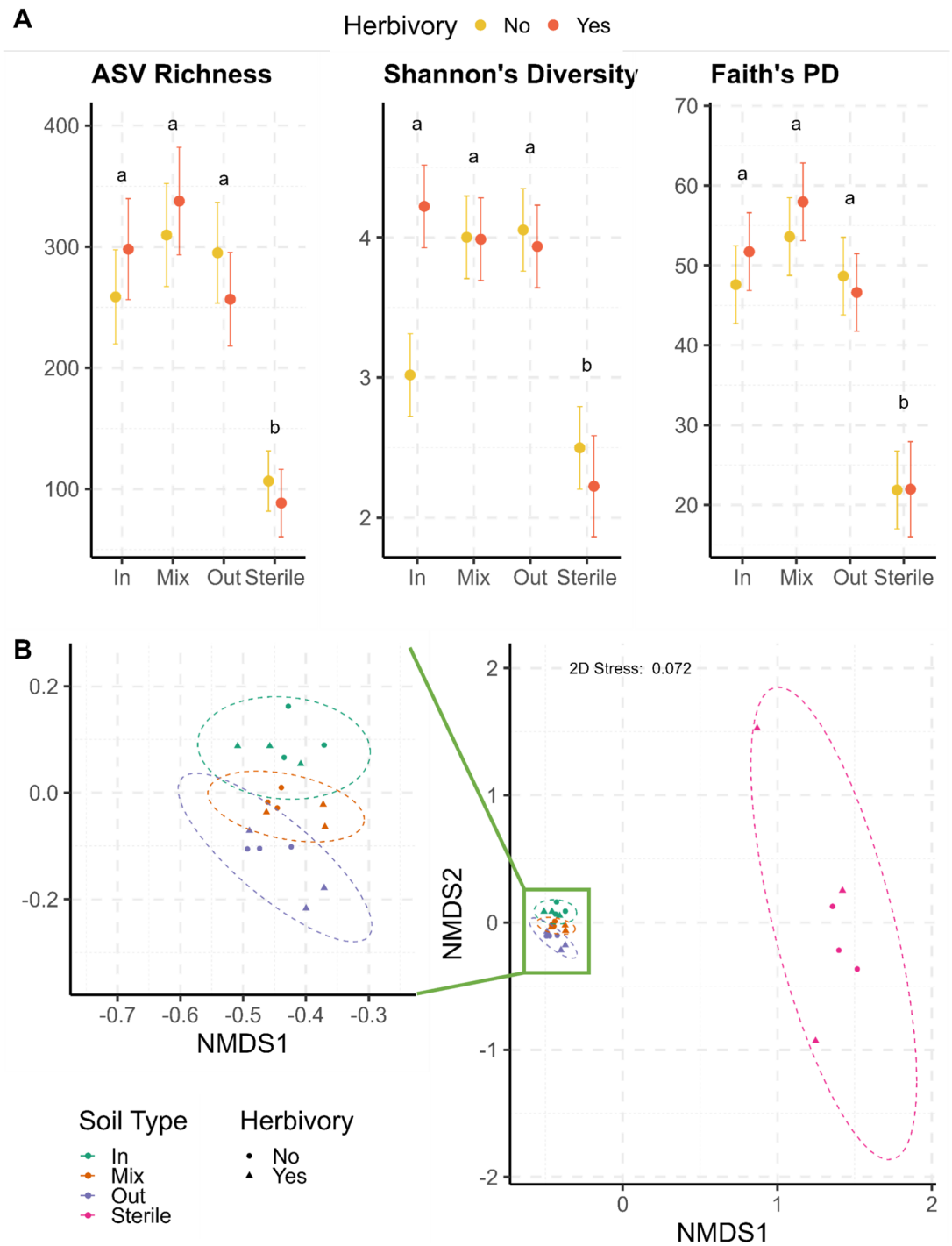

Fig. 5 Experimental assessment of the fungi microbiome associated to *A. stolonifera* roots comparing the experimental treatments: Soil inside the herbivory exclosure (In), outside the exclosure (Out), a mixture of both (Mix), and sterilised soil (Sterile); and with (Yes) or without (No) simulated herbivory treatment. Alpha diversity indexes considering richness, Shannon diversity and faith's phylogenetic diversity (**A**) and beta diversity based on the NMDS organization based on Bray–Curtis distances calculated from absolute ASV counts (**B**)

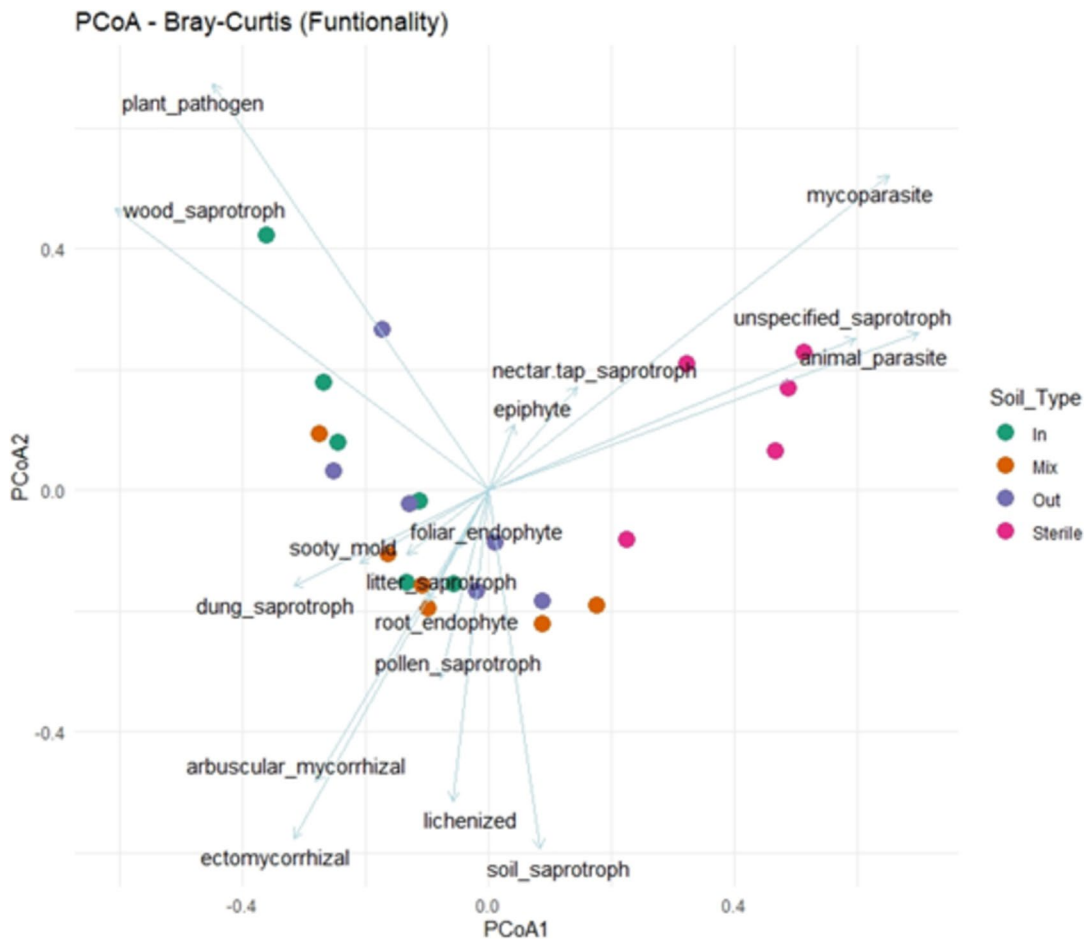

Fig. 6 Principal Coordinate Analysis (PCoA) plot based on the Bray–Curtis dissimilarity matrix, showing the relationship between soil types and fungal functional groups. Points represent soil samples, coloured according to soil type: soil inside herbivory exclosure (In), outside the exclosure (Out), a mixture of both (Mix), and sterilised soil (Sterile). The blue arrows indicate the projection of fungal functional groups, scaled by significance. The direction and length of the arrows represent the correlation between fungal functions and the ordination axes, where longer arrows indicate a stronger relationship. The key fungal functional groups were determined using the FungalTraits database

fewer studies have focused on variation of root fungal communities. For example, Ahonen et al. (2021) found that soils with a history of intense reindeer grazing in subarctic Finland hosted more diverse and stable soil fungal communities than those under light grazing. Similarly, we demonstrate a clear effect of ungulate (deer) exclusion on the root-associated fungal community of *Agrostis*. Globally, the presence of herbivores has modified the root-fungal community by increasing taxonomic richness, phylogenetic diversity, and variations in beta diversity. Although the variation in the specific composition was clear, with several fungal species only appearing in one of the environments (Fig. 3), the functional matrix was not different between fungal communities of *Agrostis* roots inside or outside the in-situ herbivory exclosures. This pattern may suggest a degree of functional redundancy in the root-associated fungal community of *Agrostis*, whereby shifts in species composition do not necessarily lead to changes in overall functional capacity (Persiani et al. 2008). Thus, the impact of ungulates on the community of *Agrostis* root-associated fungi may not affect their functions, such as maintaining symbiotic relationships, nutrient cycling, or regulation of competition mediated by parasites or diseases.

Despite the small dimensions of the exclusion fences (559 m^2 for the largest), differences in vegetation inside and outside the fence were visually observed after 14 years, and according to our results, also in soil composition. Ungulate exclusion significantly increased shrub cover (from 15% to 47.7%), shrub height, and woody species richness, enabling regeneration of *Fraxinus angustifolia*, *Acer monspessulanum*, and *Betula pendula*. Ungulate presence elevated bare soil (+ 11.64%) and reduced herbaceous height (− 48%) (Puyol-Castiñeira 2024). Soil nitrogen compounds were notably higher outside the fence, likely due to the large amount of urine and faecal deposition, which is common in areas with high ungulate density (Kaštovská et al. 2024), we did not find an increase in the amount of soil organic carbon. The exclusion of deer may have led to the accumulation of litter on the surface, but the lack of trampling could make it difficult for this to incorporate into the soil (Wei et al. 2021).

Magarzo et al. (2024), in a study conducted in the same area as our experiment, did not find any effects of ungulate exclusion on soil fungal communities after three months. However, our results highlight the importance of vegetation changes associated with ungulate exclusion, which can indirectly influence fungal communities over longer periods. If fungal community changes are primarily driven by these indirect effects via vegetation shifts rather than direct effects like trampling or nutrient deposition, extended exclusion periods, such as those used in our study, are necessary to capture these dynamics. This hypothesis is supported by our results from the greenhouse experiment, where differences were still present, although the proportion of soil in the sandy substrate was very low and artificial fertilization was applied, thus reducing a possible effect of soil physicochemical characteristics. This aligns with Vermeire et al. (2021), who found that herbivory exclusion shaped fungal communities through an increase in woody biomass in moist and dry savannas in Kruger National Park (South Africa). Thus, temporal scale is crucial: short-term studies may detect immediate direct impacts but might not fully represent cumulative or delayed indirect responses mediated through vegetation changes. Long-term experiments like ours are essential to comprehensively understand these complex belowground community dynamics (Wang et al. 2019).

Furthermore, the variations detected in the *Agrostis* root-associated fungal community inside and outside the exclusions may be mediated by the plants' response when consumed. Grasses vary their root exudates in response to herbivory, potentially altering the associated microbiome (Bardgett et al. 1998). The presence of herbivores could activate some plant mechanisms to stimulate the rhizospheric microbiome, thus indirectly stimulating the nutrient cycle (Hamilton and Frank 2001), as observed for nitrogen. This would lead us to hypothesize that *A. stolonifera* plants growing in the greenhouse with outside-exclosure soil could grow better and respond more favourably to herbivory compared to plants growing inside-exclosure soils. However, *A. stolonifera* grown in the greenhouse did not differ in biomass production or AMF formation when comparing soil from inside and outside the herbivory exclosures, or when mixed. Moreover, no effect of simulated herbivory treatment was found on total biomass, which shows the ability of *A. stolonifera* to maintain biomass production regardless of herbivory.

Surprisingly, plants grown in sterile soils were significantly larger than those grown in other soils, and this was observed independently of herbivory treatment. This observation could be explained by several processes; here, we suggest a few hypotheses: (1) the release of bioavailable micro- and macro-elements from organic biomass during the sterilization process could have benefited *A. stolonifera*; (2) the microbiome present in soils could be mostly pathogenic or saprophytic, and hence its absence benefitted *A. stolonifera*; or (3) sterile conditions allowed the colonization of opportunistic fungi present in the greenhouse, which favoured *A. stolonifera* growth.

The first hypothesis is unlikely, as the influence of soil sterilization for periods of 30 min on soil nutrient availability is generally limited and considered acceptable (Hu et al. 2020). Moreover, fertilizer was added to all other treatments to mitigate any potential effects of nutrient release caused by sterilization. The use of a fixed amount of sterilized soil as a control is common practice in similar studies involving grasses, where nutrient-related artifacts are rarely reported. For example, in a greenhouse trial with *Sorghum bicolor*, Frew et al. (2024) used 150 g of autoclaved field soil as a control and found no evidence that sterilization alone explained differences in plant performance.


Soil Treatment
In
Mix
Out
Sterile
Mucoromycota
Mortierellomycota
Glomeromycota
Basidiomycota
Ascomycota
Unknown Endogonales
Unknown Endogonomycetes
Umbelopsis sp.
Mortierella sp.
Unknown Archaeosporales
Archaeospora ecuadoriana
Unknown Scutellospora
Claroideoglomus sp.
Serendipita vermifera
Serendipita sp.
Russula sp.
Unknown Trechisporales
Athelopsis lembospora
Rhizoctonia sp.
Solicoccozyma sp.
Atractiella rhizophila
Unknown Ascomycota
Calycina sp.
Extremopsis radicicola
Unknown Capnobotryella
Unknown Lambiella
Scolecobasidium constrictum
Unknown Pseudosigmoidea
Unknown Teratosphaeriaceae
Unknown Gloniaceae
Poaceascoma sp.
Unknown Didymellaceae
Unknown Didymella
Pyrenophora triseptata
Pyrenophora biseptata
Unknown Ophiosphaerella
Unknown Pleosporales
Unknown Dothideomycetes
Clohesyomyces aquaticus
Unknown Ascomycota
Exophiala xenobiotica
Exophiala moniliae
Exophiala cancerae
Exophiala psychrophila
Unknown Chaetothyriales
Knufia sp.
Unknown Chaetothyriales
Cladophialophora sp.
Capronia sp.
Unknown Orbiliaceae
Rutstroemia sp.
Unknown Coniochaetaceae
Coniochaeta sp.
Unknown Diaporthaceae
Fusidium sp.
Myrmecridium schulzeri
Unknown Sordariales
Unknown Chaetomiaceae
Dictyosporella guizhouensis
Unknown Sordariomycetes
Phialemonium sp.
Phialemonium inflatum
Keithomyces cameus
Metacordyceps chlamydosporia
Trichoderma atroviride
Trichoderma asperellum
Trichoderma harzianum
Slopeiomyces cylindrosporus
Penicillago nodositata
Unknown Eurotiales
Unknown Pezicula
Unknown Leotiomycetes
Unknown Helotiales
Unknown Meliniomyces
Phialocephala sp.
Unknown Ploettnerulaceae
Pezoloma ericae
Unknown Lachnum
Scytalidium lignicola
soil saprotroph
soil saprotroph
arbuscular mycorrhizal
arbuscular mycorrhizal
arbuscular mycorrhizal
root endophyte
root endophyte
ectomycorrhizal
wood saprotroph
plant pathogen
soil saprotroph
unspecified saprotroph
wood saprotroph
litter saprotroph
lichenized
animal parasite
soil saprotroph
wood saprotroph
plant pathogen
plant pathogen
plant pathogen
plant pathogen
wood saprotroph
animal parasite
animal parasite
animal parasite
animal parasite
soil saprotroph
soil saprotroph
soil saprotroph
soil saprotroph
litter saprotroph
unspecified saprotroph
unspecified saprotroph
soil saprotroph
wood saprotroph
animal parasite
animal parasite
animal parasite
mycoparasite
mycoparasite
mycoparasite
root endophyte
root endophyte
root endophyte
soil saprotroph
root endophyte
litter saprotroph
wood saprotroph
Abundance
16
256
4096

◂**Fig. 7** Heatmap showing the abundance of key fungal ASVs associated with *Agrostis stolonifera* roots across different soil treatments: inside the herbivory exclosure (In), outside the exclosure (Out), a mixture of both (Mix), and sterilised soil (Sterile). The ASVs included were selected based on SIMPER analysis as the main contributors to differences in fungal community composition among soil treatments. Taxonomic assignments and primary fungal lifestyles (based on FungalTraits) are indicated. The complete ASV dataset, including all fungal taxa detected and their relative abundances, is provided in Supporting Information Table S3

The second hypothesis is not supported by the data, as important symbiotic species, such as arbuscular mycorrhizae and ectomycorrhizae, were more abundant in plants that grow in soils with the natural microbiome (Fig. 6). While pathogenic species and a high diversity of saprophytes, which could compete for nutrients, were also present, the balance between beneficial and pathogenic fungi may be crucial for plant growth and ecosystem function (Mishra et al. 2024). However, certain fungal species can behave as pathogens or symbionts depending on the plant species and environmental conditions (Johnson 2010). Furthermore, our data suggest that the ultimate effect of each fungal species associated with the root may be determined by the overall components of the fungal community. Thus, a significantly different fungal community in *A. stolonifera* roots, grown under different soil treatments, does not necessarily implicate differences in plant growth or simulated herbivory tolerance. That could be the case of *Epicoccum nigrum* a cosmopolitan Ascomycota species (a plant pathogen), which in our case only appears on *A. stolonifera* roots from inside the herbivory exclosure (Fig. 3). However, it also behaves as an antagonist for many other plant fungal pathogens. In fact, *E. nigrum* has been described as a biostimulant for the sugarcane crop because it is capable of increasing the root system biomass and controlling pathogens (Fávaro et al. 2012). Similarly, its application on cotton seedlings is well known, as it controls *Pythium* pathogen and enhances seedlings vigour and growth characteristics (Hashem and Ali 2004).

Our results support the third hypothesis, as the roots in the sterile treatment presented three species of *Trichoderma*, commonly used in phyto-optimization products (Fraceto et al. 2018; Stracquadanio et al. 2020). Moreover, inoculation with *Trichoderma harzianum* has been shown to stimulate *Capsicum annuum* growth and reduce feeding damage by the southern green stink bug (*Nezara viridula* L.; van Hee et al. 2023). The establishment of these fungi was probably suppressed by competition in treatments with soil containing the natural microbiome, but not on sterile soils, where we effectively removed most of the fungal species present, resulting in low alpha and beta diversity values.

This finding suggests that beneficial fungi such as *Trichoderma* could be strategically leveraged in ecological restoration, particularly in degraded soils with reduced microbial diversity. For instance, Cheng et al. (2025) demonstrated that the application of *Trichoderma longibrachiatum* significantly increased the above-ground biomass and leaf width of perennial ryegrass improving soil quality on mining waste dump sites. However, in systems where a well-established native fungal community is already present, the effectiveness of such inoculations may be limited due to biotic resistance and competitive exclusion. Recent research indicates that microbial inoculants can alter the composition of native microbial communities, but their success is often constrained by the established community's resistance mechanisms (Kaminsky et al. 2019; Jack et al. 2021). Therefore, the role of *Trichoderma* as a microbial bioinoculant should be carefully evaluated depending on soil context and community structure. Our results indicate that ungulate exclusion had no significant effect on the total abundance of sequences classified as *Glomeromycota* in *Agrostis* roots, under both in situ and controlled greenhouse conditions. Consistently, we found no statistically significant differences in the percentage of root colonization among *Agrostis* plants grown in soils originating from sites with different herbivory histories. The relationship between herbivore pressure and the presence, diversity, and colonization of AMF in vegetation is complex depending on herbivore species, plant species, environmental conditions, or herbivore pressure (Eldridge and Delgado-Baquerizo 2018; Faghihinia et al. 2020; Toledo et al. 2022). This makes it difficult to predict the outcome of both ungulate removal in natural systems and simulated herbivory in greenhouses.

Although several studies have found a significant reduction in AMF colonization in roots of grasses subjected to herbivory (see Frew 2021, e.g. for *Bothriochloa macra* and *Dichanthium sericeum*), this effect is less likely when herbivory treatment

is applied in a simulated manner (Barto and Rillig 2010), as in our case. This decrease in the mycorrhizae' response to a biomass loss stimulus, without the presence of herbivores, could occur in other root-associated fungal species. This may explain the lack of effect of simulated herbivory treatment on the root-associated fungal community of *A. stolonifera*.

Some key findings of this study refer to the root-associated fungal community of *A. stolonifera* cultivated across different soil treatments. Interestingly, the fungal community of plants growing in the soil of herbivore areas varied substantially in terms of beta diversity compared to those growing in herbivore exclosures. Still, the species that determined these differences were not the same as in the *in-situ* study. It is not the first study to suggest that the initial fungal community determines the final outcome. For example, Malacrinò et al. (2021) in a manipulative trial with two *Solanum* species grown in three soil microbial diversity environments (high or low diversity), and, with the presence or absence of a phloem-feeding insect showed that the initial soil microbiome diversity explained the largest variation in the plant- and herbivore-associated microbial communities. This is also known as the "priority effect" in which the outcome of species interactions depends on the order of their arrival, with the first colonizing species always being the dominant (Kennedy et al. 2009). Thus, the establishment of new organisms in a community may strongly depend on the order or timing of their arrival (Debray et al. 2022). Further studies should address the mechanisms of priority effects and search for evidence on their importance in soil microbial communities inhabiting herbivore-dominated environments. For effective grassland conservation in Mediterranean ecosystems, our findings highlight the importance of considering the long-term impacts of herbivory not only on plant communities but also on belowground biodiversity. Particular attention should be directed toward root-associated microbiomes, which are fundamental to ecosystem functioning, nutrient cycling, and plant resilience. Empirical evidence indicates that herbivory can induce significant shifts in soil microbial communities, with downstream consequences for plant productivity and overall ecosystem functioning (Eldridge and Delgado-Baquerizo 2018; Faghihinia et al. 2020). We therefore recommend that land managers strive to maintain balanced herbivore populations—not only to conserve aboveground vegetation but also to safeguard the integrity of soil fungal communities, which are essential for sustaining ecosystem stability (Wang et al. 2019; Toledo et al. 2022). Finally, our study highlights the urgent need for further research on how herbivory mediates plant–microbe interactions. Such knowledge is vital for informing adaptive management strategies in grassland restoration and herbivore regulation (Bardgett et al. 1998; Hamilton and Frank 2001).

Conclusions

Plants should no longer be considered as isolated entities; instead, a holistic perspective is necessary to fully understand their interactions with both biotic and abiotic components of ecosystems. Herbivores, through their presence or absence, can directly and/or indirectly influence plant-associated microbial communities. However, as demonstrated in our study, taxonomic shifts within these microbial communities do not always result in functional changes, nor do they necessarily affect plant growth or herbivory tolerance. This finding highlights a critical limitation of relying solely on microbial diversity metrics to predict ecosystem functioning. Although diversity serves as a valuable indicator, it may not accurately reflect microbial functional capacity or ecosystem-level processes. Functional redundancy, complex biotic interactions, and context-dependent dynamics can decouple community composition from ecological outcomes.

The broader implications of such shifts in root-associated fungal communities—particularly regarding plant community dynamics, competition, and facilitation—remain unclear and warrant further investigation. Addressing these complexities will require both experimental and long-term observational studies to elucidate the mechanisms underlying the interactions among root fungi, vegetation structure, and herbivore pressure.

Our findings emphasize the importance of evaluating both direct and indirect effects of herbivory on soil microbial communities, especially those associated with dominant grass species such as *Agrostis* spp. In Mediterranean grasslands, where herbivore exclusion is commonly used for conservation, it is essential to assess the long-term consequences on soil health and microbial diversity. While herbivory

exclusion can increase fungal and woody plant diversity, these changes do not necessarily correspond to shifts in microbial function. Therefore, effective conservation planning must consider both the taxonomic composition and functional potential of soil microbiomes, particularly in degraded systems influenced by altered herbivore regimes.

These recommendations align with broader conservation principles that emphasize ecosystem resilience and the importance of maintaining or restoring the natural balance between vegetation, herbivory, and microbial communities in Mediterranean ecosystems.

Linguistic review of the manuscript

Once the entire manuscript was drafted, AI-assisted enhancements were used in human-generated texts to improve readability and style, and to ensure that the texts were free of grammar, spelling, punctuation, and tone errors.

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Authors' contribution Conceptualization: Elena Baraza, Ramón Perea; Data curation: Daniel Gambra; Carlota Solano; Formal analysis: Elena Baraza, Joshua Borràs; Funding acquisition: Ramón Perea; Investigation: Elena Baraza, Joshua Borràs, Daniel Gambra, Carlota Solano, Miquel Capó, Ramón Perea; Methodology: Elena Baraza, Daniel Gambra; Project administration: Elena Baraza, Ramón Perea; Supervision: Elena Baraza, Daniel Gambra, Miquel Capó, Carlota Solano, Ramón Perea; Visualization: Elena Baraza, Joshua Borràs; Writing-original draft: Elena Baraza, Miquel Capó; Writing-review & editing: all authors.

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Data availability All raw sequencing data, associated metadata, and the complete ASV table used in this study are publicly available in Zenodo at the following https://doi.org/10.5281/zenodo.15311564.

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