



Wild ungulate effects on soil fungal diversity in Mediterranean mixed forests

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ABSTRACT

The effect of wild ungulate density on ecosystems varies according to different factors: climate and physiography conditions, forest type, management history, and herbivore identity. In this study, we evaluated the effect of historically high densities of red deer (*Cervus elaphus* L.) on the soil fungal communities in Mediterranean ecosystems using 30 paired plots, open on the one hand and enclosure plots on the other one. Plots were established at the end of 2020 in a perimeter-fenced hunting estate of 6600 ha in Toledo, Spain. Three months after plots were established, we analysed fungal communities in 60 soil samples using Illumina 250-bp paired-end sequencing. We estimated changes in total fungal richness and in the richness of trophic groups through Linear Mixed Effects models using fenced/unfenced type and deer habitat use as fixed variables and the location of the plots and the main tree host species as random variables. Fungal composition was analysed using non-metric multidimensional scaling and permutational multivariate ANOVA; edaphic characteristics were incorporated to explain differences. Soil fungal communities were not differentially affected by excluding ungulates for three months. Areas with high deer densities had a richer saprotrophic community and where lowland environments were dominated by the main tree hosts *Quercus faginea* and *Quercus ilex*. *Arbutus unedo* was found in mountain areas where there was less herbivore pressure, a greater richness of ectomycorrhizal and lichenized fungi and soils positively associated to nitrogen, phosphorus, potassium and organic matter levels.

1. Introduction

Large wild herbivores in forested areas are one of the main direct controls on vegetation composition and structure due to its nature (Robin Gill, 1990). They are considered keystone species and ecosystem engineers (Rooney, 2001) because by trampling, grazing, browsing and deposition of faeces they can modify the structure and dynamics of the entire ecosystem, from small scale modifications, such as the diversity of trees in a stand, to landscape scale, creating open areas (Russel et al., 2001; Rooney and Waller, 2003). Modifications in species composition, plant structure and function due to large wild herbivores have occurred historically, which has led several species to develop adaptations, as changes in their palatability or shoot architecture, that have allowed their long-term persistence (Perevolotsky and Seligman, 1998; Díaz et al., 2007). Understanding the effect that large herbivores can have on

ecosystems is of vital importance given that more than 25 % of terrestrial Earth's surface is affected by animal trampling, grazing, browsing or deposition of excreta (Asner et al., 2004). Scientists are worried that wild ungulate population densities have reached a critical tipping point in some areas. This could result in a negative impact on the ecosystem in some parts of the Mediterranean region as soil compaction and over-nitification, plant recruitment through diminishing seedlings density or changes on plant species through seed dispersal or through affectation of palatable plants density (Côté et al., 2004; Mysterud, 2010; Valente et al., 2020; Velamazán et al., 2020).

High densities of wild ungulates can negatively affect the regeneration processes of woody species, particularly of the most palatable species, due to its predation (Kuijper et al., 2010; Perea et al., 2014; Perea and Gil, 2014), which can lead to the decline of local plant diversity (Perea et al., 2014). However, wild ungulates can facilitate the seed

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dispersal of numerous species (Gill and Beardall, 2001; Gómez, 2003; Baltzinger et al., 2019). Moreover, it could increase local biodiversity (e.g., the intermediate disturbance hypothesis, (Wilkinson, 1999), and maintain habitat heterogeneity, which is necessary to conserve biodiversity at large scales. Many other effects of large wild herbivores via direct and indirect pathways have already been described in the literature. It can be found effects on grassland production (Frank and McNaughton, 1993), nutrient cycling (Mohr et al., 2005; Molvar et al., 1993), soil properties (Abdel-Magid et al., 1987; Tanentzap and Coomes, 2012; Lecomte et al., 2019), fungal communities (Dudinszky et al., 2019), invertebrate assemblages (Bernes et al., 2018; Bugalho et al., 2011) and other animals (Dermer et al., 2009). However, the context (i.e., climate, forest type, management history, herbivore identity and density) affects the effects of ungulate herbivory on biodiversity (Bernes et al., 2018). In this sense, Mediterranean ecosystems, which are characterized by winter rainfall and drought stress in summer, are global hotspots for biodiversity (Myers et al., 2000; Underwood et al., 2009). Mediterranean ecosystems often include the southernmost distribution boundary of species in Europe (e.g., *Pinus sylvestris* L.) (Castro et al., 2004), have species that are specific to Mediterranean ecosystems (e.g., *Quercus ilex* L., *Quercus faginea* Lam., *Pinus pinaster* L., *Pinus pinea* L., and *Arbutus unedo* L.), and are disturbance-dependent habitats (Navarro and Pereira, 2015). However, not many studies have analysed the impact of different deer densities on these ecosystems (Velamazán et al., 2020).

In this study, we were interested in understanding the impact of elevated densities of red deer (*Cervus elaphus* L.) on the belowground communities and processes of Mediterranean ecosystems (Shelton et al., 2014). High densities of large herbivores are associated with altered soil moisture and bulk density (Asner et al., 2004). Animal trampling can reduce soil aeration, particularly in the top-soils, where the concentration of oxygen in the pores is considerably reduced (Donkor et al., 2002). These changes in soil moisture and bulk density can affect nutrient cycling, such as the soil carbon (C) and nitrogen (N) cycles (Olofsson et al., 2004), regardless of litter input quality or quantity (Powers et al., 2005). The input of nutrients through the deposition of urine and faeces, along with selective trampling and browsing, causes the aboveground ecosystem structure and species composition to change (Ramirez et al., 2019), which can have cascading belowground effects on soil quality, invertebrate diversity and abundance and the rate of organic matter decomposition (Allombert et al., 2005; Bressette et al., 2012). Fungi play an important role in decomposition producing biomass that can be used by other organisms as creating a more favourable habitat (Angel and Wicklow, 1974). Herbivore faeces can contain 4 % N (Kendrick, 2000), as well as vitamins, growth factors, minerals and water (Sarrocco, 2016). Faeces can be a major source of nutrients for saprotrophic fungi, which play an important role in the reallocation of nutrients in ecosystems (Hättenschwiler et al., 2005).

The aim of this study was to investigate the effect of wild ungulates on fungal communities (i.e., their richness and composition) by analyzing the use of Mediterranean ecosystems by red deer populations using 30 paired plots (open plots and enclosure plots to exclude wild ungulates). We hypothesized that no big differences should be found between fenced and non-fenced areas as a result of an immediate effect of fencing. We also hypothesized that there would be an association between fungal communities and the main tree host species, which has been reported previously for Mediterranean ecosystems (Moricca and Ragazzi, 2008). For achieving this we have studied the soil fungal community regarding the fencing state of the plots or the dominant vegetation. Moreover, we incorporated general habitat characteristics, as abiotic environmental variables into a unique variable called Location of the plot, to determine its association with fungal communities. The importance of this study relies on the complexity of the of ecosystem components evaluated in order to determine the fungal status and deer pressure interaction in relation to its local context. These findings should be important for understanding how the presence of wild ungulates affects fungal communities in Mediterranean ecosystems and could be applied

to the management of vegetation and large herbivores on hunting estates.

2. Material and methods

2.1. Study area and data collection

The study was located in the “Quintos de Mora”, a 6600 ha⁻¹ hunting estate in Toledo (Spain), which is enclosed by a perimeter-fence. This has allowed historically high densities of deer to inhabit this area (approximately 35 deer/km² for the past three decades). The study area comprises two elevated areas, Solana (S) in the north and Umbría (U) in the south, which delimitate the central lowland of Raña (R). There were also two intermediate areas at the interface between Solana and Raña (SR) and between Umbría and Raña (UR). This selection has been carried out in order to comprise differentiated forest composition and altitudinal characteristics. The higher and steeper areas were dominated by Mediterranean scrubland: *Arbutus unedo* L. and *Quercus ilex* L. were the main tree host species. There is a mixed forest with *Pinus pinea* L. reforestation and *Q. ilex* in some lowland areas and at the SR interface (sometimes accompanied by crops). The most favourable lowland areas for the deer presence were characterized by *Q. ilex* and *Quercus faginea* Lam. accompanied by crops, and there is a mixed forest of *Pinus pinaster* Ait., *Q. ilex* and/or *Q. faginea* in some lowland areas and at the UR interface.

A design of twin plots (i.e., 30 pairs of enclosure plots and open plots) distributed systematically throughout the hunting estate was established (Fig. 1). A proportional number of plots were established in all the different habitat types. Each plot was 20 20 m². Big game proof fencing was erected in November 2020 to exclude wild ungulates from enclosure plots.

The distribution and abundance of deer in an area is not homogeneous (Table 1) and they and their impacts are highly dependent on biotic and abiotic factors (Borowski et al., 2021; Bruggeman et al., 2007; Squires et al., 2013) (e.g., distance to water, busy roads or large urban areas, the presence of cultivated areas with shelter nearby or management activities like the installation of feeders or perimeter fences) (Martínez-Jauregui and Herruzo, 2014). In this study, we directly

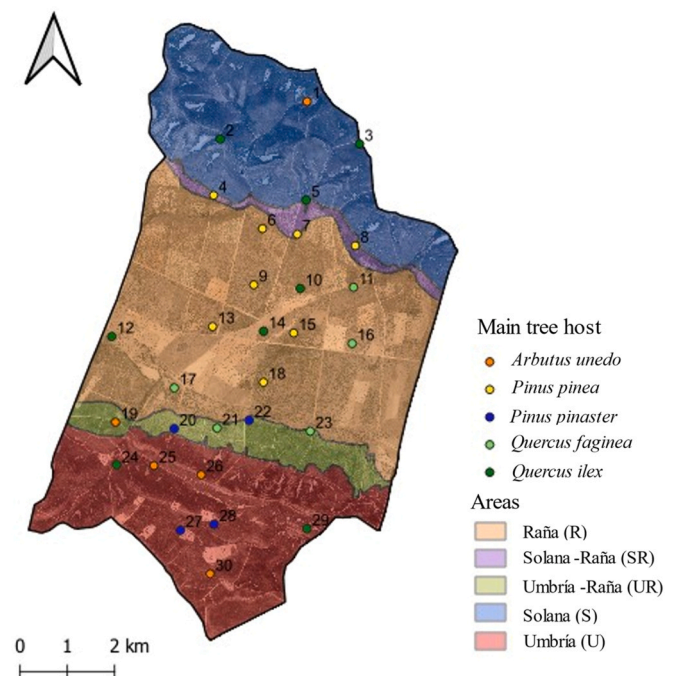


Fig. 1. Distribution map of the 30 pairs of enclosure and open plots.

Table 1
Distribution of deer use in the pair plots.

Deer use	Pair plots
High deer use	10,14,17,20
Medium deer use	3,4,6,7,8,12,13,16,18,23
Low deer use	1,2,5,9,11,15,19,21,22,24,25,26,27,28,29,30

measured red deer use of the plots (which is by far the main grazing/browsing species in the study area) by counting faecal pellet groups (Acevedo et al., 2008). Pellet group size was fixed at five pellets and were counted in the same 4 20 m² rectangle almost every month of 2021 in the open plots. Deer pellets were removed every time they were counted to enable the spatial and temporal use of the territory by deer to be estimated. A pellet group per month estimator was defined and, due to the spatial and temporal heterogeneity of the deer habitat use, a further hierarchical cluster analysis (Murtagh et al., 2012) was conducted using R (version 4.1.2, 'cluster' package, with Euclidean distance as the distance metric). This analysis identified 4 plots with significantly 'high' deer pressure (distance between 'high'-moderate-low' centroids = 30.58; Silhouette value = 0.215; mean = 14.16; SD = 11.80). A second cluster analysis was performed on the remaining 26 plots, revealing two distinct groups: 'moderate' (Silhouette value = 0.033; mean = 5.31; SD = 4.41) and 'low' (Silhouette value = 0.482; mean = 1.76; SD = 2.10) deer pressures, with a distance between their centroids of 9.11. These three categories were subsequently used as a categorical variable (i.e., Deer habitat use) in each plot.

In the first days of March 2021, we sampled inside and outside the 30 enclosure plots. We made two pulls of eight soil subsamples in every enclosure plots. Every subsample was a soil core of 10 cm deep with leaf litter removed first, with a minimum distance of 2 m to eliminate the edge effect of the plot. Therefore, we collected 60 soil samples in total, and the subsamples of each plot were pooled. Therefore, we obtained 30 soil samples within enclosure plots and another 30 samples represented the outside of these plots. The soil samples were stored at -20 °C and sent for physico-chemical performed by ITAGRA (www.itagra.com), and genetic analysis. Regarding the soil properties the N was analysed through Kjeldahl method (Bremner, 1960), P through Olsen method (Olsen and Khasawneh, 1980) and K through an amonic acetate solution and ICP-MS measurements (Warncke and Brown, 1998).

2.2. Molecular work

DNA isolation, library preparation and Illumina 250-bp paired-end sequencing were carried out by AllGenetics & Biology SL (www.allgenetics.eu). DNA was isolated from samples using a DNeasy PowerSoil Pro kit following the manufacturer's instructions. An extraction blank (Bex) was included in each round of DNA extraction and processed to check for cross-contamination. DNA was resuspended in a final volume of 50 µL. The DNA concentration was quantified by performing a Qubit High Sensitivity dsDNA Assay (Thermo Fisher Scientific). For fungal library preparation, a fragment of the ITS2 region was amplified using fITS7_nodg (5' GTGARTCATCGAATCTTTG 3') forward (Ihrmark et al., 2012) and ITS4 (5' TCCTCCGCTTATTGATATGC 3') reverse (White et al., 1990) primers.

In the first amplification step, PCRs were carried out in a final volume of 12.5 µL, containing 1.25 µL of template DNA, 0.5 µM of each primer, 3.25 µL of Supreme NZYTaQ 2x Green Master Mix (NZYTech) and ultrapure water to make up the volume to 12.5 µL. The reaction mixture was incubated as follows: an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 47 °C for 45 s, 72 °C for 45 s and a final extension step at 72 °C for 7 min.

Oligonucleotide indices required for multiplexing different libraries in the same sequencing pool were attached in a second amplification step, which involved the same conditions as those used in the first amplification step except for the use of only five cycles and an annealing

temperature of 60 °C. A negative control that contained no DNA (Bpcr) was included in every PCR round to check for contamination during the library preparation.

Library size was verified by running libraries on 2 % agarose gels stained with GreenSafe (NZYTech) and imaging them under UV light. Then, libraries were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek) and following the manufacturer's instructions. Finished libraries were pooled in equimolar amounts according to the results of a Qubit dsDNA HS Assay (Thermo Fisher Scientific) quantification. The pool was sequenced in a fraction of a NovaSeq PE250 flow cell (Illumina). Raw reads of amplicons were submitted to the European Nucleotide Archive of the European Molecular Biology Laboratory as part of BioProject PRJEB52639, under accession numbers ERX9472898–ERX9472957.

2.3. Bioinformatic analyses

The quality of the demultiplexed Illumina paired-end raw data was assessed by fastQC (Andrews, 2010). We used the R-package DADA2 (Callahan et al., 2016) to construct an amplicon sequence variant (ASV) table and all previous steps following the ITS-specific analysis strategy recommended in the DADA2 tutorial v1.8 (https://benjjneb.github.io/dada2/ITS_workflow.html). Data quality was very high and, therefore, no quality filtering was performed. Owing to natural length variation in the ITS2 region, the reads were not truncated. Primer identification and removal were performed with Cutadapt v3.7 (Martin, 2011) implemented in DADA2. Merging of dereplicated reads, construction of an ASV table and removal of chimeras were performed as part of the DADA2 analysis workflow. The naive Bayesian classifier algorithm that DADA2 implements in the function *assignTaxonomy* was used to assign taxonomy to sequences based on the UNITE 10.05.2021 ITS database (Abarenkov et al., 2020). Given that the ASV table is a higher resolution version of an operational taxonomic unit (OTU) table, corresponding to a 100 % identity threshold, hereafter we will use the more commonly used term OTU instead of ASV.

To enable comparisons between samples, the OTU (or ASV) table was randomly subsampled to the lowest number of non-chimeric merged reads to ensure an equal number of sequences per sample using *rarefy_even_depth* function in the R-package Phyloseq (McMurdie and Holmes, 2013). OTUs with >90 % similarity to a fungal Species Hypothesis (Köljalg et al., 2013) with known ecological function were assigned to functional groups according to Pölme et al. (2020). Samples were rarefied to 60,221 reads per sample.

2.4. Statistical analyses

The effects of ungulate exclusion (called *Type of plot*: open or enclosure plots), *Deer habitat use* (low, moderate or high in the open plots), *Main tree host species* in the plots (the species that accumulates the highest crown cover, *A. unedo*, *Q. ilex*, *P. pinea*, *Q. faginea* or *P. pinaster*), and *Location of the plot* (which describes habitats combining both abiotic and biotic information in the study area: U, UR, R, SR, or S locations, described above) on total fungal richness and on the richness of different trophic groups were analysed separately using Linear Mixed Effects models (LME, $p \leq 0.05$, package nlme, (Pinheiro et al., 2015)). *Type of plot* and *Deer habitat use* were defined as fixed variables and *Main tree host*, *Location of the plot* and plot pairs were added to the model as random variables. *Deer habitat use* only took into account those non-fenced areas. To determine specific significant differences, Tukey's test was applied to analyse the fixed variables. These multiple comparisons were performed applying a family-wise confidence level of 95 %. Confidence intervals (CI) to compare the effect of random variables were estimated $\pm 1.96 \times$ the standard error.

To estimate the change in the composition of soil fungi, the relationship between soil fungal composition and edaphic parameters was visualized using non-metric multidimensional scaling (NMDS), based on

a Hellinger-transformed OTU matrix and environmental scaled data. The effects of exclusion (*Type of plot*), *Deer habitat use*, *Main tree host species* and *Location of the plot* were analysed using a permutational multivariate ANOVA (PerMANOVA) by adonis function, followed by a PERMANOVA (Anderson and Santana-Garçon, 2015) using a *betadisper* function, based both on 999 permutations function in the *vegan R-software package* (Oksanen et al., 2015). Bray-Curtis dissimilarities were used for NMDS and PERMANOVA. Then the edaphic characteristics were brought in through correlation. The correlation of NMDS axes scores with explanatory variables was assessed using the *envfit* function in R.

3. Results

3.1. Basic exploration and quality check of fungal sequencing

The per base quality of reads was assessed by FastQC analysis. No samples were excluded based on low sequence quality. The number of reads in the ASV table produced by the DADA2 workflow ranged from 60,221 reads (sample 27 A) to 117,554 (20 C). They were assigned to 14,400 OTUs (because no clustering was performed, each OTU corresponds to one ASV). After rarefying the OTU table, 407 OTUs were removed because they were no longer present in any sample after random subsampling.

3.2. Fungal richness

Analyses performed using Linear Mixed Effects models showed that soil fungal total richness estimations did not show significant differences based on *Type of the plot* (Table 2). In addition, none of the fitted guild models showed a significant difference based on the fixed effects of *Type of plot*: the mycoparasite guild showed the most difference (90 % of significance; $p = 0.09$). However, richness of total fungi was significantly affected by the Deer pressure ($F=3.697$; $p = 0.03$). Specifically, those guilds affected were the AM fungi, mycoparasite ($p = 0.04$, both), dung ($p = 0.05$) and soil saprotrophs ($p < 0.01$).

Our analyses showed that *Main tree host species* ($p < 0.01$) (Table 2) was significantly associated with the richness of dung saprotrophs ($p < 0.01$), ectomycorrhizal fungi (ECM) ($p < 0.01$), lichenized fungi ($p < 0.01$), litter saprotrophs ($p < 0.01$), plant pathogen ($p < 0.01$), root endophytes ($p = 0.026$), unspecified saprotrophs ($p < 0.01$) and soil and wood saprotrophs ($p < 0.01$). Among the host species analysed, *P. pinaster* and *P. pinea* in general had a more negative influence on the mean values of the Linear Mixed Effects model (Fig. 2). Dung saprotrophs, litter saprotrophs, unspecified saprotrophs and wood saprotrophs showed a higher richness estimate when the host species was *Q. faginea*. By contrast, when the host species was *A. unedo*, richness values were higher owing to higher richness estimates for lichenized fungi, root endophytes and soil saprotrophs.

Table 2

F and p-values of Linear Mixed Effect models showing the effect of *Type of plot*, *Deer habitat use*, *Main tree host species* and *Location of the plot* variables on the richness estimation of total fungi and fungal trophic guilds in soil samples. Significant p-values (0.05) are shown in bold.

Group	Type		Deer use		Host		Location	
	F test	p value	F test	p value	F test	p value	F test	p value
Overall	0.058	0.810	3.697	0.031	12.413	0.000	4.733	0.002
Arbuscular mycorrhizal	0.214	0.646	3.249	0.046	2.028	0.103	4.185	0.005
Animal parasite	2.606	0.112	1.507	0.230	2.027	0.103	1.323	0.273
Dung saprotroph	0.156	0.694	3.143	0.051	5.161	0.001	1.325	0.272
Ectomycorrhizal	0.936	0.337	2.894	0.063	5.390	0.001	9.015	0.000
Mycoparasite	2.965	0.090	3.251	0.046	1.684	0.167	4.193	0.005
Lichenized	0.053	0.818	1.933	0.154	8.954	0.000	5.930	0.000
Litter saprotroph	0.245	0.623	0.569	0.569	4.364	0.004	0.497	0.738
Plant pathogen	0.000	0.990	0.198	0.821	8.639	0.000	1.403	0.245
Root endophyte	0.433	0.512	0.804	0.452	3.007	0.026	3.589	0.011
Unspecified saprotroph	0.049	0.826	1.977	0.148	6.028	0.000	2.413	0.059
Soil saprotroph	0.025	0.874	6.633	0.003	7.749	0.000	9.686	0.000
Wood saprotroph	0.168	0.683	0.239	0.788	6.461	0.000	1.308	0.278

The *Location of the plot* (Table 2) was significantly associated with the ectomycorrhizal fungi (ECM) ($p < 0.01$), arbuscular mycorrhizal fungi (AM) ($p < 0.01$), mycoparasite ($p < 0.01$), lichenized ($p < 0.01$), root endophyte ($p < 0.01$) and soil saprotroph guilds ($p < 0.01$). The R and SR locations presented a negative richness dynamic than the other sites. In the case of ECM fungi, there was no confidence interval overlap between the S location, which showed a higher richness, and the R, SR and UR locations, which had lower estimated richness values. Root endophytes showed higher richness levels at S and UR sites, likewise soil saprotrophs, which also showed higher richness levels at the U location (Fig. 3).

3.3. Fungal communities' composition

Based on the Adonis test, soil fungal communities in enclosure plots (*Type of plots*) were not significantly different ($p = 1$, Table 3) to those in plots without deer exclusion (Fig. 4a).

Fungal communities were influenced by *Deer habitat use* (Table 3), with fungal communities present in low and moderate deer pressure plots differing the most, whereas fungal communities detected in high deer pressure plots were intermediate between the two (Fig. 4c). Plots with low deer pressure were positively related to potassium (K), N, organic material ($p = 0.001$) and phosphorus (P) ($p = 0.002$) contents, whereas fungal communities detected in the high and moderate deer pressure plots were largely influenced by the soil pH level ($p = 0.001$).

Fungal communities associated with the *Main tree host species* and *Deer habitat use* differed significantly ($p = 0.001$, Table 3). To assess the effect of each of these variables on the total fungal community, four NMDS analyses were carried out: all presented a type 1 stress (stress = 0.108, weak ties). *Deer habitat use* showed the lowest *Betadisper* p-value (0.023), indicating a possible homogenization of the community. The ordination of the relationship between fungal communities and the main tree host species revealed five well-differentiated fungal communities (Fig. 4d). Fungal communities associated with *A. unedo* and *Q. ilex* were similar to those associated with the low deer pressure stands (Fig. 4c), and where the communities were much more positively related to inorganic K, N, organic material and P (Fig. 4d). Fungal communities associated with *Q. faginea* and *P. pinea* were similar to those associated with the high and moderate deer pressure plots, with pH showing an influence on all these fungal communities. The ordination of fungal communities associated with *P. pinaster* placed them in an intermediate position relative to those associated with the rest of the host species but separated from communities associated with the other host species. Moreover, its low variability showed a possible specialized community. However, the fungal community associated with *Q. ilex* had the largest and most dispersed ordination pattern, which included all the environmental influences, and was similar to the ordination patterns of fungal communities associated with high, moderate and low deer pressure

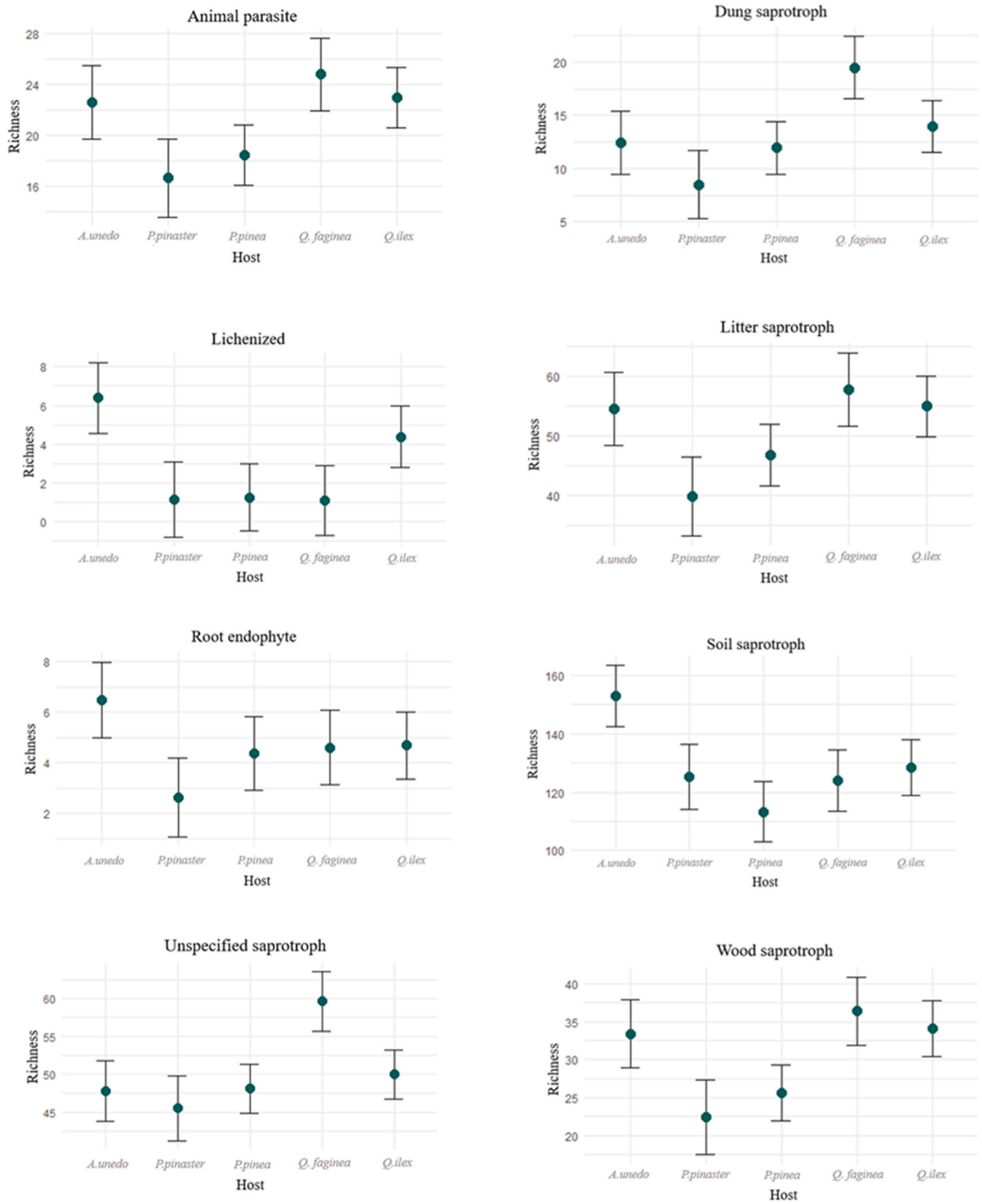


Fig. 2. Fungal richness depending on the effect of the random variable Main tree host on the richness levels of different guilds. Bar represent $\pm 1.96 \times$ the standard error (SE).

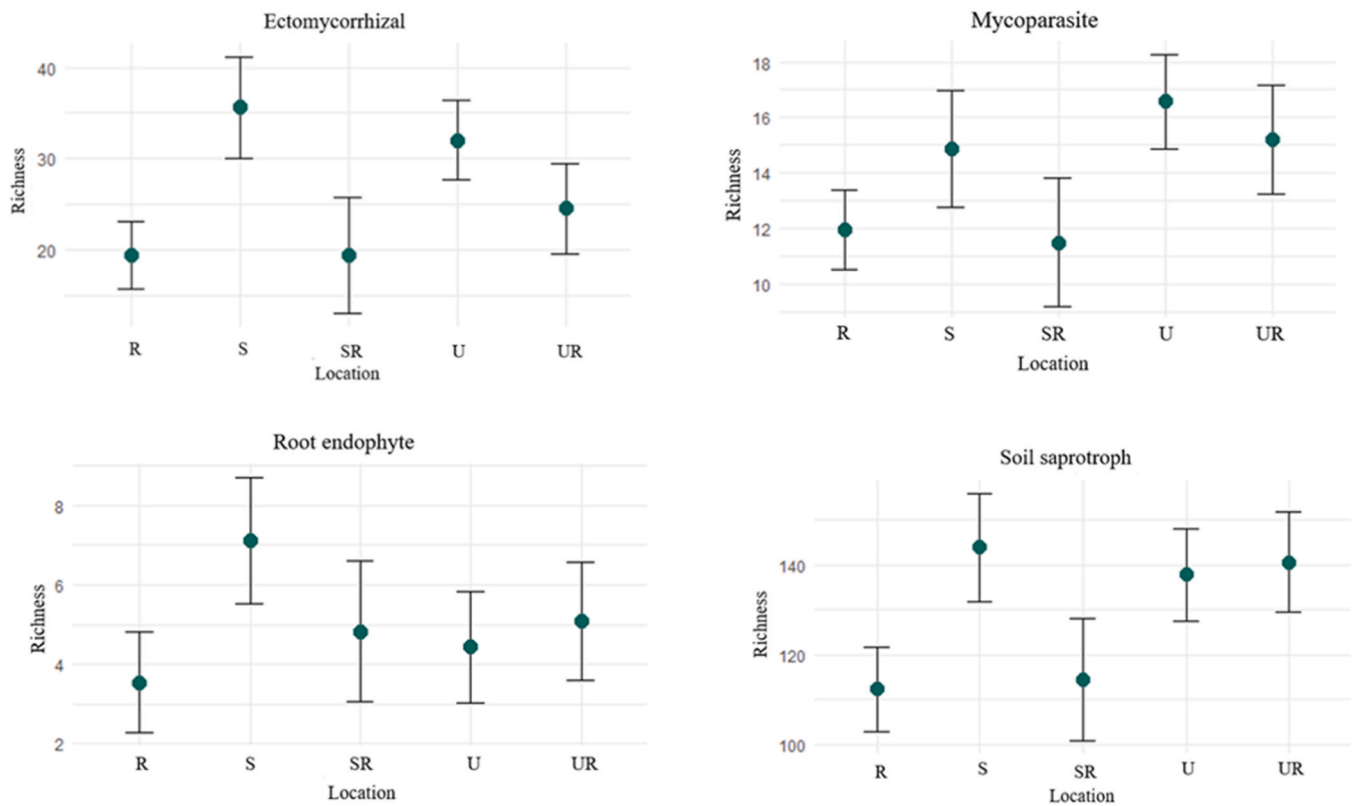


Fig. 3. Fungal richness depending on the effect of the random variable Location on the richness levels of different guilds. Bars represent $\pm 1.96 \times$ the standard error (SE). Plot locations: R, Raña; S, Solana; SR, between Solana and Raña; U, Umbría; UR, between Umbría and Raña.

Table 3

R², F and p-values of PERMANOVA and BETADISPER analyses of Type of plot, Deer habitat use, Main tree host species and location of the plot. Significant p-values (0.05) are shown in bold.

	R2	F	p-value	Betadisper p-value
Type	0.146	0.009	0.533	0.883
Location	0.200	3.447	0.001	0.092
Deer use	0.071	2.194	0.001	0.023
Host	0.215	3.765	0.001	0.006

plots.

Fungal communities associated with the Location of the plot differed significantly ($p < 0.01$). Fungal communities associated with some locations showed some overlap, particularly communities associated with S, UR and U plots (Fig. 4b). The soil variables influencing the community composition for the Location of the plot effect were N, K and the organic matter ($p < 0.01$). The Location of the plot significantly influenced the trophic nature of the studied areas ($p < 0.01$), showing a larger ubiquitous diversity of guilds in U stands. The composition of guilds in R stands differed to those of U stands whereas UR and SR showed some overlap with S and R stands respectively. More than one soil variable influenced the trophic guild composition, particularly N, K and organic matter ($p < 0.01$).

4. Discussion

4.1. Effect of deer exclusion on fungal communities

Our findings that variations in deer densities within a hunting estate influenced the composition of soil fungal communities further support their role as ecosystem engineers (Carpio et al., 2014). There are many perimeter-fenced hunting estates in central and southern Spain that

have very high densities of wild ungulates and where the animals are not distributed homogeneously throughout the territory (Carpio et al., 2015). Our analyses showed that the soil fungal community (total fungal richness and total trophic taxa richness) showed no signs of change following the erection of fencing to exclude red deer for 3 months (Tatsumi et al., 2021). Our study supports the view of Tatsumi et al. (2021) that direct human-intervention, such as the application of soil treatments, is necessary to enhance the diversity of the soil fungal community. Furthermore, previous studies have shown that when grazing and browsing (which are the main ungulate impacts) are prevented by fencing, in general, ungulate exclusion did not affect the soil fungal community (Che et al., 2019; Cheng et al., 2016). In our study, soil samples were analysed three months after deer exclusion, which may not have been long enough for the microbial community to recover from the effects of ungulate grazing and browsing (Burke et al., 2019) given that high deer densities on the hunting estate do cause changes in the fungal community of the soil. Future measurements will be needed in order to deeply evaluate the fencing effect. However, deer habitat showed a low betadisersion p-value indicating a low variability and a possible homogenization effect of the fungal community, that could be linked to a reduction of it (Burke et al., 2019; Larkin et al., 2012).

4.2. Fungal richness affected by host and deer pressure

We did not find differences in richness within guilds when the Deer habitat use was considered. However, significant differences in the overall fungal richness and of specific guilds were found when the host species was considered. Higher densities of ungulates in Quercus-dominated areas may lead to the development of a more saprophytic environment. In our study area, plots dominated by Quercus species, which were always relatively close to areas of pasture, were in the most favourable lowland areas, which is the habitat type most intensively used by deer, especially during winter (Beck et al., 2013).

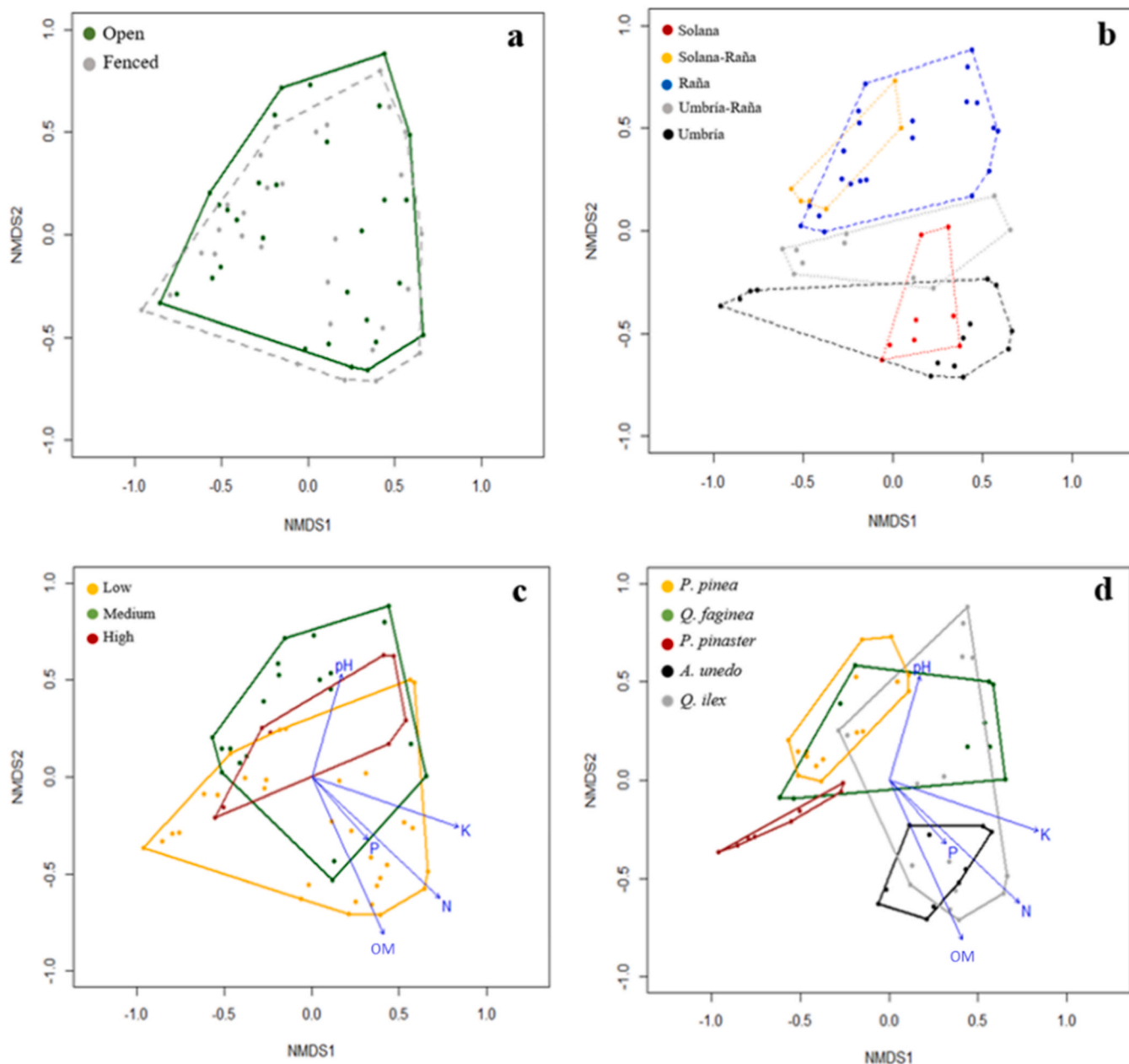


Fig. 4. Non-metric multidimensional scaling (NMDS) ordination showing the relationship between the fungal community and the type of plot (a), the location of the plot (b), deer habitat use (c) and the main tree host species (d).

Leaf litter quality may have influenced the fungal community as climate and litter quality are the main factors controlling litter decomposition over large spatial ranges (Zhang et al., 2008). Conifer litter is of lower quality than deciduous species (Cornwell et al., 2008) and, therefore, decomposer communities in coniferous forests must have a greater ability to decompose litter (Zhang et al., 2008). In this study, litter saprophytic fungal richness was greater in stands dominated by the hardwood species *Q. ilex*, *Q. faginea* and *A. unedo*. This relates to the finding of Lidman et al. (2017) who found that the decomposition of coniferous litter (in our case *P. pinaster* and *P. pinea*) was much slower than in deciduous litter (in our case *Q. faginea*). The latest is attributed to the greater quantity of structural compounds (lignin and cellulose) and refractory compounds (tannins and polyphenols) in pine species (Ardón et al., 2009). Lignin content negatively affects decomposition because specialized enzymes are needed to process this recalcitrant C (Alvim et al., 2014; König et al., 2014). A high tannin content may prevent litter

decomposition by inhibiting microbial growth and soil enzyme activity (Chomel et al., 2016). In our case study, the pressure of ungulates on habitats dominated by conifers was high, although somewhat lower than that on habitats dominated by *Quercus*. This could explain why the dung saprophytic fungal community was poorer in the pine forest area and the soil saprotrophic fungal community was richer in elevated areas (i.e., S, U and UR) owing to the lack of soil alteration derived from trampling (Köster et al., 2013; Kumpula et al., 2014; Mysterud, 2006).

AM fungi are highly dependent on their herbaceous symbiotic association, differing greatly depending on the availability of C or P in the soil (Pearson and Jakobsen, 1993). However, AM fungi can maintain symbiosis with most terrestrial plants (Wang et al., 2020). Therefore, the presence of ungulates and grazing on one of the plant species may affect the other through this bond. Grazing and browsing can modify the richness of these species and their composition because herbivores not only affect soil compaction and nutrient cycling (as explained above) but

also compete for the host resource allocation (Eom et al., 2001). This is in line with the results of our analysis as AM fungi are significantly affected by deer use.

There was a greater richness of ECM fungi in *A. unedo*-dominated stands. These stands also had lower densities of ungulates and were the location of greater ECM fungal richness (i.e., S and U sites). However, our study disagrees with that of Santalahti et al. (2018) who reported that the ECM fungal community was not altered by grazing pressure. Herbivores can indirectly compete with mycorrhizal fungi for C from host plants, which may lead to a decrease in mycorrhizal colonization of severely damaged plants (Gehring and Bennett, 2009). Stress conditions such as herbivory increase the plant's need for resources, thus reducing the amount of photosynthates that the plant can provide to ECM fungi it associates with (Nehls, 2008), leading to negative effects on the ECM fungal community (Markkola et al., 2004).

Lichenized fungal richness was greatest in stands dominated by *A. unedo*. This may be because there were low densities of ungulates in this vegetation type given that the main impact of grazing on the forest floor is a reduction of lichen biomass (Stark et al., 2000; Susiluoto et al., 2008) which is mainly due to trampling (Köster et al., 2013; Kumpula et al., 2011; Myrsterud, 2006).

Root endophyte richness values were also slightly higher in *A. unedo* stands, which could explain the higher richness levels at location S where *A. unedo* dominates. Root endophyte richness values were quite similar in open plots. Some studies have observed a positive relationship between the presence of ungulates and the natural frequency of endophytes (Bazely et al., 1997; Koh and Hik, 2007). The association between endophytic fungi and plants has been postulated to directly increase the host plant's defences against herbivores (Clay, 1988) through mycotoxin production (Faeth and Bultman, 2002). However, the maintenance of these organisms implies that there is a cost for the plant (Omacini et al., 2012). McInenly et al. (2010) observed that cutting *Festuca campestris* to simulate grazing caused the reallocation of resources to aboveground growth, which reduced root production. However, Rudgers et al. (2016) observed that the prevalence of endophytes in grasses corresponded with their regulation by ungulates and that exclusion of these herbivores for 7–10 years resulted in the decline of endophyte communities. It should be stated the difficulties about study in a disentangled manner the parameters of Deer habitat use, host and location due to it interdependence and intrinsically correlated, but progress needs to be made in studying this interaction to improve the management planification regarding wildlife impacts over territory conservation.

4.3. Environmental factors influencing fungal composition

Firstly, lowland sites (R) appeared to have a more diverse fungal community than the highland sites (S). This could be due to the negative effect of altitude on fungal communities (Shen et al., 2020), particularly root-associated fungi (Park et al., 2021) which could explain the effect of location on community composition.

In our case study, moderate and high *Deer habitat use* plots highly correlates on pH. Grazing can affect pH in several ways. For example, Denyer et al. (2010) also found that excluding large herbivores had a significant effect on soil pH. However, stands with high densities of large herbivores were more influenced by pH. Changes in soil pH influence soil C and nutrient cycling, particularly N, because it affects microorganisms involved in the transformation of compounds containing either of these two elements (Kunhikrishnan et al., 2016). The soil pH level also plays an important role in determining the availability of other soil nutrients such as P and K (Awad et al., 2019; Guo et al., 2020). Therefore, variations in pH owing to deer grazing activities might have indirectly influenced the availability of these nutrients in this study.

Soil fungal communities of stands with a low density of ungulates correlated more with K, N, P and organic matter than stands with moderate or high densities of ungulates. These soil variables were the

ones that most influenced the dispersed ordination pattern of soil fungal communities. Animals participate indirectly in nutrient cycling through forage consumption. There is a temporary immobilization of nutrients during digestion and then nutrients are returned to the soil in excrement and urine (Cantarutti et al., 2002). There is a positive correlation between the N content of an ungulate's diet and the N content of the dung (Sitters et al., 2017). N and P concentrations and N/P ratios in vegetation are determined by the vegetation species composition and factors that control the availability, uptake and loss of nutrients (Güsewell, 2004). Mediterranean terrestrial plant production is generally more P-limited than N-limited (Du et al., 2020), which suggests that P may be a more important nutrient trader than N in these ecosystems (Adamo et al., 2021). It has been suggested that plants allocate more resources to P uptake as their P limitation increases (Simard et al., 2012); however, Frank (Frank, 2008) reported that grazing had no influence on plant P uptake. Therefore, it cannot be assumed that the presence of ungulates will affect different elements in the same way (Sitters and Andriuzzi, 2019). Another nutrient measured was K. Although it is an abundant element on Earth, the forms in which it is mostly found are not available for plant use and its availability depends mainly on cation exchange reactions (Haro and Benito, 2019). Zarekia et al. (2012) found an increase in K in grazed areas, possibly owing to an accumulation of K as a result of trampling and droppings (urine and faeces). The availability of K to plants has been shown to be crucial for reducing herbivory (Warren et al., 1999). A possible explanation for the correlation of K in stands with a low deer density could be the lack of activity of ungulates in these stands, which might have enhanced the uptake of K by plants. However, Assmann et al. (2017), reported that analyses of manure revealed that most of the ingested P (60%) was returned to the soil. K cycling followed the same trend. Sigcha et al. (2018) observed that the exclusion of grazing increased many soil properties, such as electrical conductivity, exchangeable K, availability of P, total N, and total and oxidizable organic matter. The influence of organic matter was greater in stands with low or moderate deer densities than in high deer density stands. This could be because more litter falls on the soil in stands with low or moderate deer density levels; foliar litter is the main input of organic C in forest soils (Zhang et al., 2008). Litter is an important pathway for the transfer of nutrients from plants to soil during C and N cycling (Miao et al., 2019). Forest species associated with a low density of ungulates were mainly conifers or the less palatable species. Conifer stands with recalcitrant litter could accumulate more litter over time than broadleaf stands (Ramirez et al., 2021) and, even though the production rates of these species (conifers) are lower than those of broadleaf stand species (Ramirez et al., 2018), they are not as affected by the presence of ungulates as broadleaf species.

5. Conclusion

High deer habitat use, mostly in *Quercus*-dominated areas, have favoured a saprotrophic environment, according to our first hypothesis. This could be owed to the greater quantity of excreta as well as the quality of the leaf litter and the lack of specialization that these fungi need to decompose this kind of litter compared with the more recalcitrant conifer litter. AM fungal communities were not altered by any of the studied variables, except deer use. The richness of ECM fungal communities was higher in areas with low deer pressure and in elevated areas. The richness of endophytes and lichenized fungi was greater in *A. unedo*, being the only tree specifically associated with concrete guilds. This could be associated with a not as heavy frequentation by ungulates due to both their higher elevation and lower palatability despite being a broadleaf species. The correlation of pH was greater in stands with moderate or high deer pressure, possibly because of higher organic matter and nutrient inputs, which could have produced variations in the soil pH value. However, in stands with low to moderate deer pressure, this influence was not detected, although fungal communities were correlated with N, P, K and organic matter. No differences were found

between fenced and unfenced areas, contradicting our second hypothesis. This could be due to the short amount of time that had elapsed between fencing and sampling (3 months) and the possible cessation of vegetative activity during winter. Regarding soil fungal communities, neither beneficial nor negative effect of fencing has been found, being impossible for us suggest the implement of this ecosystem management strategy. Future studies should include a more in-depth assessment of the effects of fencing and ungulates on Mediterranean forests and its implementation as part of forest management.

CRedit authorship contribution statement

Rafael Villafuerte-Jordán: Methodology, Investigation. **María Martínez-Jauregui:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Alba Magarzo:** Writing – original draft, Investigation. **Pablo Martín-Pinto:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Ignacio Sanz-Benito:** Writing – review & editing. **Sanna Olsson:** Writing – review & editing, Methodology, Formal analysis. **Juan Andrés Oria-de-Rueda:** Writing – review & editing, Supervision. **Olaya Mediavilla:** Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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