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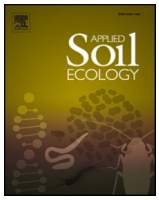

Relation of the soil microbiota of cork oak groves and surrounding grasslands to tree decline☆

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ABSTRACT

Cork oak (*Quercus suber* L.) form low-density silvopastoral systems of great ecosystemic and socioeconomic importance in the Mediterranean Basin, as they provide bark of great industrial value. Since the 1950s, these trees have been increasingly suffering from a deadly disorder known as decline, due to different biotic and abiotic factors. Associated with these forests, grasslands of great pastoral value and as carbon sequestrators develop. The aim of this work was to study the bacterial and fungal diversity present in the soils of healthy and diseased cork oaks (due to decline) and their associated grasslands, an ecosystem whose microbiome has not been studied so far by metagenomics. Soil samples were collected from cork oak forests in southern Spain and their microbial diversity was analyzed by metabarcoding with Illumina MiSeq. With respect to bacterial families, no differences were reported between cork oak forest soils and their associated grasslands, possibly due to the presence of endemic bacteria and similar environmental conditions. However, there were differences in fungal diversity between healthy cork oak forests and their associated grasslands. In the healthy cork oak soils, the families Gemmatimonadaceae and Nocardioidaceae were massively present, while in the diseased soils the fungal genus *Geminibasidium* was found. Regarding the functional niche, healthy cork oaks presented mainly ectomycorrhizae in their soils, while their associated grasslands presented fungal endophytes, less present in areas with diseased trees. Therefore, fungi, but not bacteria, present in the soils of cork oaks and associated grasslands could play a key role in the presence/absence of decline in cork oaks.

1. Introduction

Cork oak (*Quercus suber* L.) is an evergreen tree, taxonomically belonging to the family Fagaceae. *Q. suber* has a long life cycle of 200–205 years, reaching heights of 25 m and stem diameters of up to 2 m (Silva et al., 2023). Cork oak woodlands areas are widely distributed in the Mediterranean Basin, covering some 2.1 million hectares (Mechergui et al., 2024). Mainly, these woodlands are found forming low-density silvopastoral systems, which are called “montados” in Portugal and “dehesas” in Spain (Bicho et al., 2024). These silvopastoral systems present important biodiversity hotspots and provide valuable ecosystem services (carbon storage, cork, timber and firewood), in addition to coexisting with agriculture and grazing (Bicho et al., 2024; Mechergui et al., 2024). In this sense, the main raw material of high added value obtained from the cork oak is its bark, which is used in the manufacture of bottle stoppers and agglomerates, or to obtain metabolites used as fragrances and antioxidants in the pharmaceutical, nutraceutical and cosmetic industries (Rego et al., 2023).

In recent decades, the area of cork oak has decreased steadily. This is due to many different causes, such as longer and more intense periods of drought in summer, wildfires, predation of its seeds and their difficult establishment as seedlings, its slow growth, and the different pests and diseases that attack it (Mechergui et al., 2023). In this context, there is the so-called oak decline, a complex disorder caused by multiple stressors (Gosling et al., 2024). In the specific case of cork oak trees, the decline phenomenon was initially described in the 1950s, in the Mediterranean Basin, accelerating since the 1980s (Kim et al., 2017). Climate change has been described as the main cause of this increased decline, due to more frequent, longer and intense of high temperatures and droughts, a wider distribution of pests and diseases, and excessive human exploitation of these trees (Kim et al., 2017). Among the biotic stresses, the following pests have been pointed out in different works as

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predisposing factors to the development of decline in *Q. suber*, *Platypus cylindrus* (oak pinhole borer) (Inácio et al., 2022; Nones et al., 2022) and *Coraebus florentinus* (buprestid beetle) (Pinna et al., 2019), *C. undatus* (cork punch) (Tiberi et al., 2016), *Cerambyx cerdo* (great capricorn beetle) (Tiberi et al., 2016), *C. welensii* (Tiberi et al., 2016) and *Prinobius myardi* (Tiberi et al., 2016), or the fungal pathogens *Ceratocystiopsis quercina* (Inácio et al., 2022), *Apiognomonia quercina* (Linaldeddu et al., 2009), *Biscogniauxia mediterranea* (Linaldeddu et al., 2009), *Botryosphaeria corticola* (Linaldeddu et al., 2009) and *Pleurophoma cava* (Linaldeddu et al., 2009), the bacteria *Brenneria* spp. (Moradi-Amirabad et al., 2019), *Pseudomonas syringae* (Gosling et al., 2024) and *Rahnella victoriana* (Moradi-Amirabad et al., 2019), and the oomycete *Phytophthora cinnamomi* (González et al., 2020), along with other species of the same genus (Seddaiu et al., 2020).It is important to point out that cork oak decline is considered a complex, multifactorial disease, where these organisms tend to act as predisposing, triggering or contributing factors, generally in combination with abiotic factors such as prolonged droughts, climate change and inadequate forest management practices. The strategies developed so far for the prevention and control of decline in cork oak woodlands include the protection of the forests through international certification by the Forest Stewardship Council (FSC) or the Programme for the Endorsement of Forest Certification (PEFC) (Mexia et al., 2024), training of forestry professionals in more sustainable resource management, reforestation with cork oaks genetically resistant to identified abiotic and biotic stresses, or understanding of plant-microorganism interaction for the possible development of protective bioinoculants (Lopes-Fernandes et al., 2024).

As a consequence of silvopastoral activity, grasslands emerge within cork oak forests, providing fundamental ecosystem services, such as forage production and soil carbon sequestration (Seddaiu et al., 2018). Cork oak forests increase the ecological complexity of their associated grasslands by influencing soil properties, and the diversity and quantity of herbaceous species, along with animal and soil microbial species (Rossetti et al., 2015).

The microbiota associated with plants and soils is very complex and dynamic, ranging from symbiont to phytopathogenic microorganisms. The decline of woody plants has been related to modifications or imbalance in this microbiota, as these organisms perform essential functions in the maintenance of holobiont fitness. Understanding how this microbiota functions and what it contributes to the ecosystem is fundamental to developing biosystem engineering to improve the health and management of forest declines (Bettenfeld et al., 2020). Both soil microorganisms and endophytes play a fundamental role in the management of decline in different forest species (*Castanea sativa*, *Quercus ilex*, *Q. suber* and *Q. pyrenaica*), as they have potential biocontrol activity against disease-associated pathogens (Diez-Hermano et al., 2024). In addition, tree tissues and the rhizosphere present two closely related niches of microorganisms with a constant microbial exchange related to plant resilience (Diez-Hermano et al., 2024). Specifically referring to soil microbiota and its relationship with forest health status, it has been described in pine trees how bacterial and fungal diversity plays a fundamental role in the presence or absence of decline (Morales-Rodríguez et al., 2024), or, on the contrary, how they have no relationship at all (Gazol et al., 2024); however, the non-microbial mechanisms of action involved were not addressed in these works.

In cork oak, Maghnia et al. (2019) reviewed the work developed so far on the role of rhizospheric microbiota in decline, confirming its fundamental importance in the presence or absence of forest disease (Maghnia et al., 2019). Subsequent work has identified that the presence of bacteria of the Proteobacteria family and ectomycorrhizal fungi are fundamental for the absence of decline in cork oak (Gómez-Aparicio et al., 2022; Diez-Hermano et al., 2023).

On the other hand, in grasslands it has been shown that soil microbial diversity depends on the diversity of plants present and environmental conditions (Mészárošová et al., 2024). Specifically in grasslands associated with cork oak groves, no work has been developed (to our knowledge) on their microbiome. It is important to emphasize that by "grasslands associated with cork oak groves" we refer to plant cover near cork oak groves, but not within them.

The aim of this work is to analyze by metabarcoding the bacterial and fungal diversity of cork oak forest soils and surrounding grasslands and its relationship with the presence or absence of tree declines; this being, as far as we know, the first work carried out in this respect. In relation to this, the hypothesis of the work is that there are common aspects between the rhizospheric microbiome of cork oak forests and surrounding grasslands, as well as a relation between both microbiomes and the presence or absence of tree decline.

2. Materials and methods

2.1. Characteristics of the study area and sampling

Soil samples were collected from two study areas: Cortes de la Frontera and Jerez de la Frontera, located in the provinces of Málaga and Cádiz, Spain, between August 31 and September 5, 2021 (Fig. 1). These areas are characterized by a typical Mediterranean climate, with the following characteristics: (1) In Cortes de la Frontera, the mean annual temperature is 15.6 °C and the average annual precipitation of 939.8 mm. The predominant soil types include eutric cambisols, chromic luvisols, and lithosols, along with dystric cambisols and rankers. (2) In Jerez de la Frontera, the mean annual temperature is 17.6 °C and the average annual precipitation of 550.1 mm. The predominant soil types to the east include eutric cambisols, chromic luvisols, and lithosols, along with dystric cambisols and rankers. In the west the predominant soil types include chromic vertisols, vertic cambisols, calcic cambisols, calcareous regosols and pelic vertisols. In Los Alcornocales Natural Park, the main soil type is forest brown earth, developed on siliceous sandstones of the Aljibe. These soils, formed from sandstone bedrock, are acidic, poor and sandy. The sand compacted and cemented by diagenesis favors the growth of cork oaks, which are the dominant plant species in the area. These were the soil types chosen.

Our sampling strategy prioritized sites with similar soil properties across treatment groups (healthy cork oak forests, diseased cork oak forests, and associated grasslands). This approach allowed us to better isolate the effects of vegetation type and tree health on the soil microbiota, without the added complexity of varying soil types. The cork oak associated grasslands are defined as open spaces without tree cover with a minimum area of 100 m^2 located within the cork oak ecosystem, but with a distance of at least 30 m from the trunk of the nearest cork oak.

The selection of plots and sampling locations was conducted using a systematic approach based on several key criteria. First, we identified areas with contrasting tree health status (healthy vs. diseased cork oak trees) and vegetation structure (forested vs. non-forested grasslands). Within these categories, we further refined our selection by prioritizing sites with similar ecological characteristics including biodiversity patterns, altitudinal range, and climatic conditions to minimize confounding variables. Accessibility was also an important practical consideration in our study design, as we needed to efficiently transport equipment and samples. Therefore, we selected sites with reasonable vehicle access while ensuring this did not compromise the ecological validity of our sampling design. This multi-criteria selection process allowed us to establish a robust experimental framework for comparing microbial communities across different vegetation types and tree health statuses while controlling other environmental factors that might influence soil microbiota.

For the study, 24 soil composite samples were extracted, corresponding to two health conditions of *Q. suber* stands: healthy and declining trees. Each sample was taken under a single vegetation cover type. Additionally, soil samples were collected from grasslands adjacent to these stands to analyze potential similarities and differences. The distances within a type of coverage between the samples were about 10–100 m (average of 40 m). The distances between the healthy plots

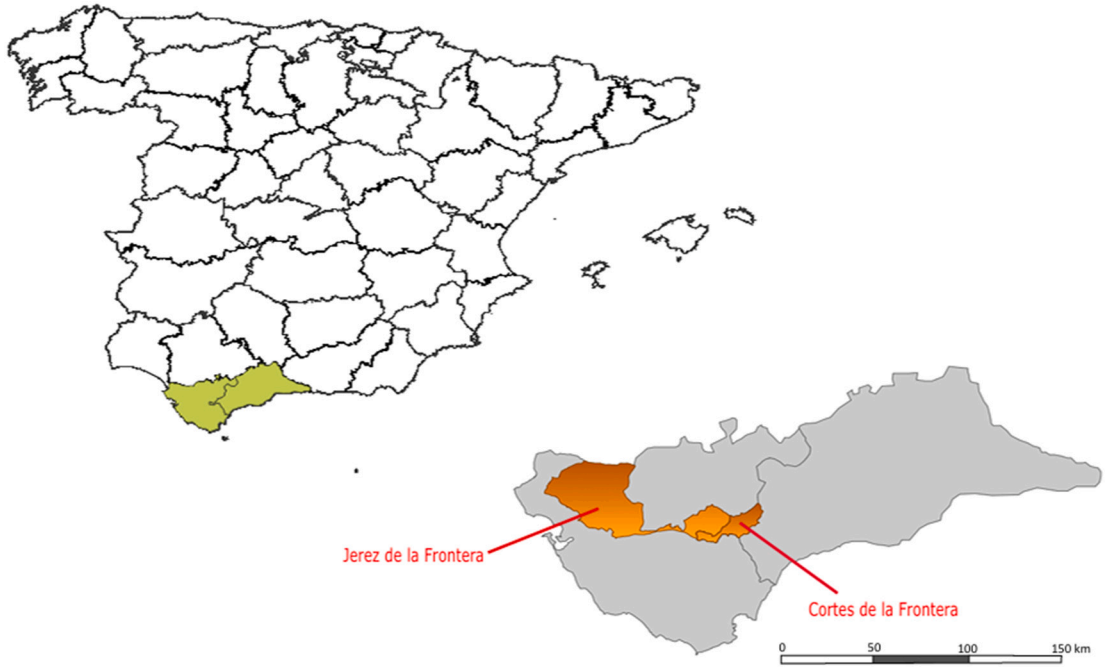

Fig. 1. Location of sampling sites. (Left) Map of Spanish provinces; the provinces of Cádiz and Málaga sampled are highlighted in red. (Right) Detail of the provinces of Cádiz and Málaga. The sampled municipalities are highlighted in yellow. The red dots correspond to the sampling sites.

and the diseased plots were about 30–150 m.

Soil sampling was conducted to a depth of approximately 15 cm. Surface debris was removed prior to sampling to avoid contamination, and coarse roots and stones were discarded. After each extraction, sampling tools were disinfected with a 2 % sodium hypochlorite solution. All soil cores from the same plot were combined into a single composite sample. In total, 24 composite samples were obtained (2 areas × 2 health conditions × 3 replicates per condition). Samples were labeled, placed in sterile containers, and stored at −20 °C until laboratory processing.

In both analyzed areas, we have observed vascular plants typical of the cork oak vegetation series: (1) Cork oak formations that, when in good conservation condition, acquire a closed thickness and are usually accompanied by a significant number of shrubs. When the cork oaks are in poor condition, the number of trees decreases and trees are often accompanied by shrub species; and (2) grasslands adapted to the prevailing climate and animal grazing, which appear in clearings of cork oak forests and near boundaries and paths. The series of vegetation detected in our study areas were mainly: (1) *Q. suber, Arbutus unedo, Crataegus monogyna* subsp. *brevispina, Chamaerops humilis,Juniperus oxycedrus* subsp. *oxycedrus, Myrtus communis, Olea europaea* var. *sylvestris, Phillyrea angustifolia, P. latifolia, Pistacia lentiscu, Quercus coccifera, Rhamnus oleoides, Smilax aspera* and *Viburnum tinus*; (2) *Agrostis curtisii, A. castellana, A. pourretii, Anthoxanthum ovatum, Avena barbata, Bromus diandrus, B. hordeaceus, Dactylis glomerata* subsp. *hispanica, Festuca ampla* subsp. *ampla, Gaudinia fragilis, Melica magnolii, Ornithopus compressus, Poa bulbosa, Trifolium angustifolium, T. campestre, T. glomeratum, T. scabrum* and *T. subterraneum.*

2.1.1. Categorization of healthy and diseased cork oaks

The evaluation of the health status of the cork oaks has been carried out by visually analyzing the symptoms of apparent decline, mainly the level of defoliation and discoloration of each tree, following the adapted version of the standard European forest health monitoring protocol. To analyze the level of discoloration, 4 levels have been used by visually observing the live branches of the tree and observing the color changes of the leaves, comparing them with a standard tree. Discoloration levels (0–4): (0) no discoloration (0–10 %); (1) slight discoloration (11–25 %); (2) moderate discoloration (26–60 %); (3) severe discoloration (61–99 %); and (4) dead/completely discolored (100 %).

To analyze the level of defoliation, the percentage of leaf loss in the crowns of the analyzed trees has been assessed in comparison to the reference tree. This assessment, if the crown is homogeneous, was carried out for the entire crown at once. If the crown was not homogeneous, the tree's crown was divided into different parts, analyzing them separately, and subsequently, the average of all the obtained values was calculated.

2.2. Sample processing and sequencing

Sample processing and sequencing of DNA fragments were performed using the methodology previously described by Morales-Rodríguez et al., 2024. DNA extraction was performed using the DNeasy PowerLyzer PowerSoil DNeasy kit (Qiagen, Germany). The V4 domain of bacterial 16S rRNA genes was amplified using primers F515 (5′-NNNNNNNNNNNNGTGTGCCAGCCAGCMGCCGC GGTAA-3′) and R806 (5′-GGACTACHVGGGGGTWTCTAAT-3′), with the forward primer modified to contain a unique 8 nt barcode and a 2 nt linker sequence at the 5′ end. Fungal internal transcribed spacer (ITS) loci 1 were amplified with primers BITS (5′-NNNNNNNNNNNNNNNNNNCTACCTGCGGARGGATCA-3′) and B58S3 (5′-GAGATCC RTTGYTRAAAGTT-3′), with a unique 8 nt barcode and linker sequence (bolded portion) incorporated into each forward primer. Samples were sent to Biome Markers (Valladolid, Spain) for processing and sequencing using Illumina MiSeq 2 × 300.

2.3. Bioinformatic analysis

Bioinformatic analysis of the sequences obtained was performed using the methodology previously described by Diez-Hermano et al. (2024). Illumina adapters and chimeras were removed and the quality of the reads was trimmed. Sequencing data were processed following the DADA2 pipeline (Callahan et al., 2016). Parameter values were as follows: filtering and trimming (maxN = 0, maxEE = 2, truncQ = 2, minLen = 50, rm. phix = TRUE, compress = TRUE), learning error rates (nbases = 1e+08, nreads = NULL, errorEstimationFunction =

loessErrfun, MAX_CONSIST = 10, OMEGA_C = 0), merging of paired reads (errorEstimationFunction = loessErrfun, selfConsist = FALSE, pool = FALSE) and chimera elimination (method = "consensus").

To obtain amplicon sequence variants (ASVs), no clustering based on similarity percentages was applied. Taxonomic assignment and abundance estimation were performed by comparing the ASVs with the SILVA v138.1 database (Quast et al., 2012) for bacteria, and the UNITE database version 10.0 (https://unite.ut.ee) for fungi. Rarefaction curves were used to assess the relationship between sequencing depth and number of ASVs.

2.4. Statistical analysis

For the statistical analysis of the data obtained, the methodology previously described by Diez-Hermano et al. (2023) was followed. Prior to analysis, raw ASV readings were aggregated to genus level to have higher confidence and avoid misidentification of closely related species. Differences between mycobiome communities were assessed in terms of alpha and beta diversity. Alpha diversity was assessed using Hill's diversity indices (Roswell et al., 2021). Differences between sanitary conditions were contrasted using the Wilcoxon rank test.

Beta diversity was assessed in terms of differential abundance, keeping in mind that high-throughput sequencing counts should be considered compositional data. Analyses followed the ZicoSeq procedure (Yang and Chen, 2022), while compositional effects were addressed by adopting a reference-based approach (selecting taxa close to invariants as reference abundances). Association tests were conducted using Smith permutation tests based on the linear models of the LDM (Hu and Satten, 2020) and DACOMP (Brill et al., 2022) methods. Reference taxa were adjusted for health status (factor with two levels: healthy, declining) as a covariate. Taxa were filtered out if their prevalence was <20 % and their mean relative abundance was <0.2 %. The percentage of top outliers replaced by winsorization was 10 %. Abundances were square root transformed. Correction of *p*-values by multiple testing was based on 500 permutation tests.

All bacterial and fungal genera were included in the functional analyses based on the assignment of a functional guild using BactoTraits V2 (Cébron et al., 2021) and FungalTraits 1.2 (Põlme et al., 2020) databases. Only ASVs classified with a confidence level of "Probable" or "Highly probable" were used. The numbers of raw readings for each guild were summed by health condition and expressed as log2(guild abundance/total abundance).

All analyses were performed in the R 4.1.3 environment (R Core Team, 2022). Sequencing data analysis and ASV identification were performed using the *Biostrings* (Pagès et al., 2022), *dada2* (Callahan et al., 2016) and *ShortRead* (Morgan et al., 2009) packages. Hill diversity analysis was carried out using the *MeanRarity* package (Roswell and Dushoff, 2022). The *GUniFrac* package (Chen et al., 2022) was used for compositional analysis. Taxonomic information was handled and plotted with the *metacoder* package (Foster et al., 2017).

3. Results

3.1. Characterization of tree decline

The most important dendrometric values for understanding the state of trees are diameter at breast height (DBH), total height and the height of the first live branch (HFLB). Declining trees showed a slightly smaller diameter and height than healthy trees, although without statistically significant differences. As for the height of the first live branch, it was slightly lower in healthy trees, where most trees have the first live branch at a height of between 2 and 2.5 m (Fig. 2).

Regarding the level of defoliation, healthy trees showed a low level, while diseased trees showed high rates (values between 20 and 100 %). The levels of discoloration were significantly different between healthy and diseased trees. Most healthy trees showed a low level of discoloration (level 0), while declining trees mainly exhibited high levels of discoloration (level 4) (Fig. 2).

3.2. Description of bacterial communities

The analysis of the bacteria present in all the soils sampled resulted in 12 phyla and 107 orders. Specifically, 12 phyla and 101 orders were found under the cork oaks. As far as bacterial families are concerned, most of them correspond to the families Proteobacteria (26.29 %), Firmicutes (17.14 %) and Actinobacteriota (17.14 %) (Fig. 3).

The detailed analysis of the bacterial families present under healthy and diseased cork oak and grasslands shows that there is a great coincidence of families associated with both diseased (n = 45, 77.6 %) and healthy (n = 43, 67.2 %) states of both types of vegetation. As for the

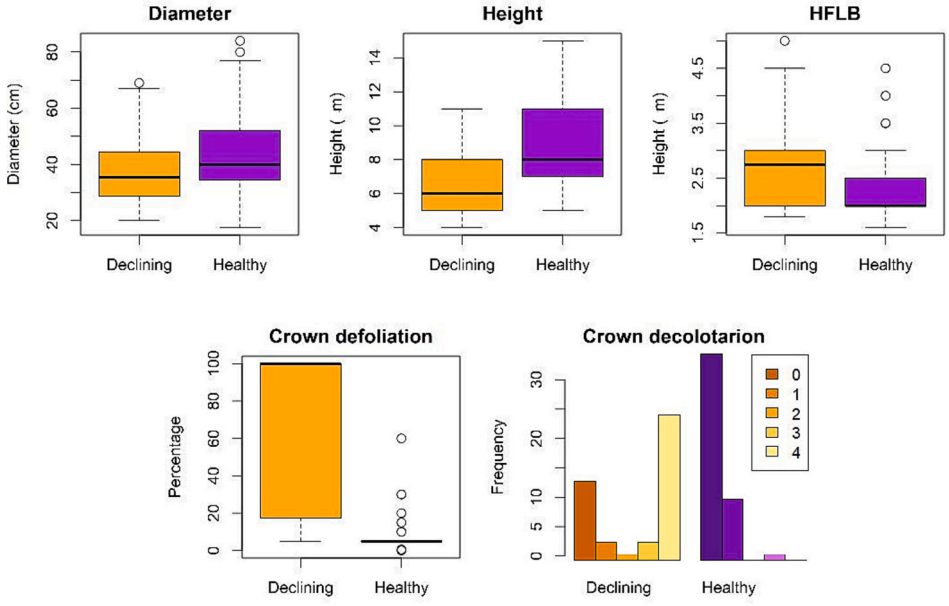

Fig. 2. Dendrometric measurements and level of defoliation and discoloration of trees. Boxplot corresponding to declining and healthy areas. Median height declining vs healthy: 6 vs 8, pval = 0.011. Median crown defoliation declining vs healthy: 100 vs 5, pval = $9{,}75^{-11}$.

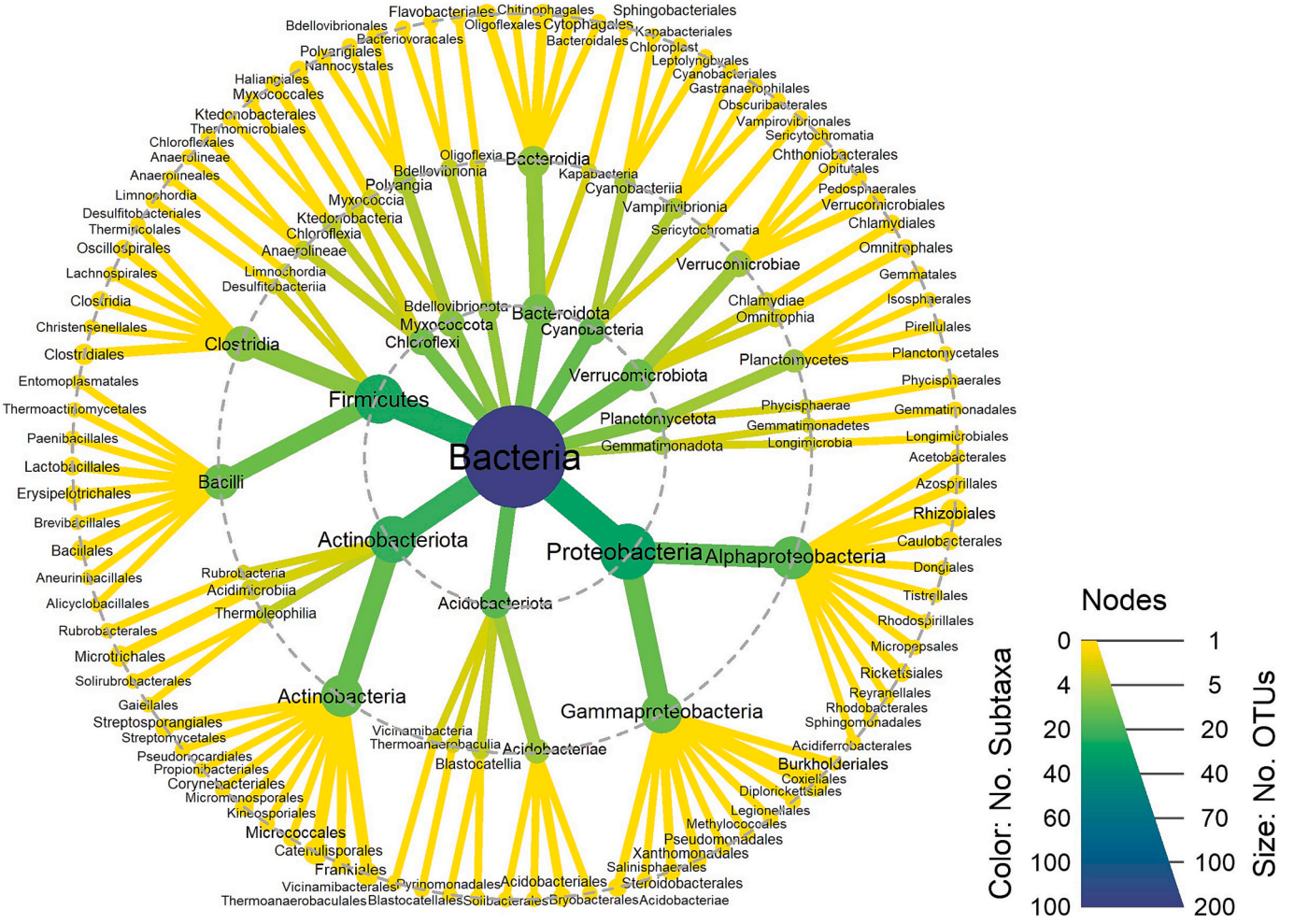

Fig. 3. Taxonomic tree of bacteria in cork oak forest. The size of the node represents the number of taxonomic units and the color scale indicates the number of subtaxa. Concentric circles indicate taxonomic levels: phylum, class and order.

specific bacterial families under some type of cover and state of health, those observed under healthy grasslands (n = 12, 18.8 %) and under cork oaks in good condition (n = 9, 14.1 %) stand out, in comparison with both plant covers associated with tree decline (Fig. 4).

3.3. Description of fungal communities

In all the samples analyzed under cork oak and grassland the fungal communities resulted in 68 orders encompassed in 6 phyla, of which 5 phyla and 60 orders were found under cork oak. Most of the genera collected in the samples corresponded to the phyla Ascomycota, with a total of 360 genera (64.98 %), and Basidiomycota, with a total of 183 genera (33.03 %) (Fig. 5).

A more detailed analysis of the fungal genera in certain conditions showed that in the grasslands associated with disease states, the coincidence between both plant covers is greater (n = 35, 66 %). However, in

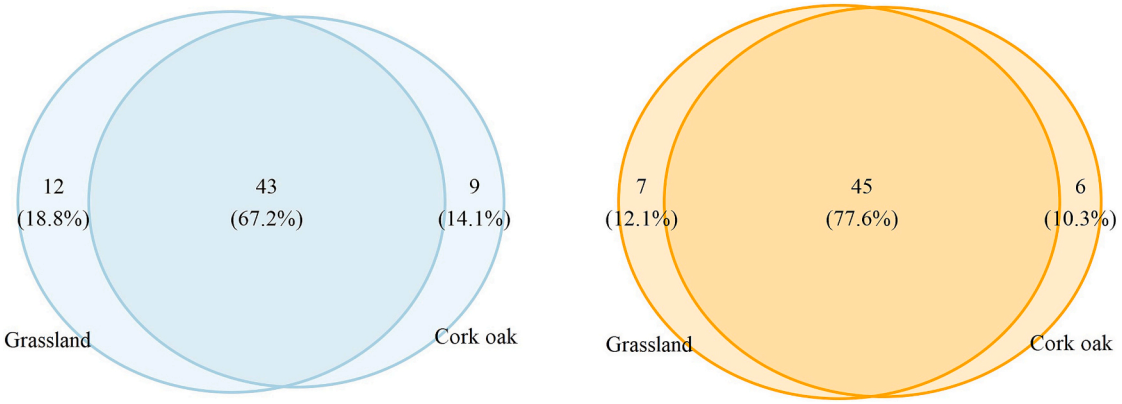

Fig. 4. Venn diagrams for the bacterial families of each condition under study. The Venn diagram indicates the coincidences and differences between bacterial families in at least 70 % of the samples collected in healthy (in blue) and diseased (in orange) plots.

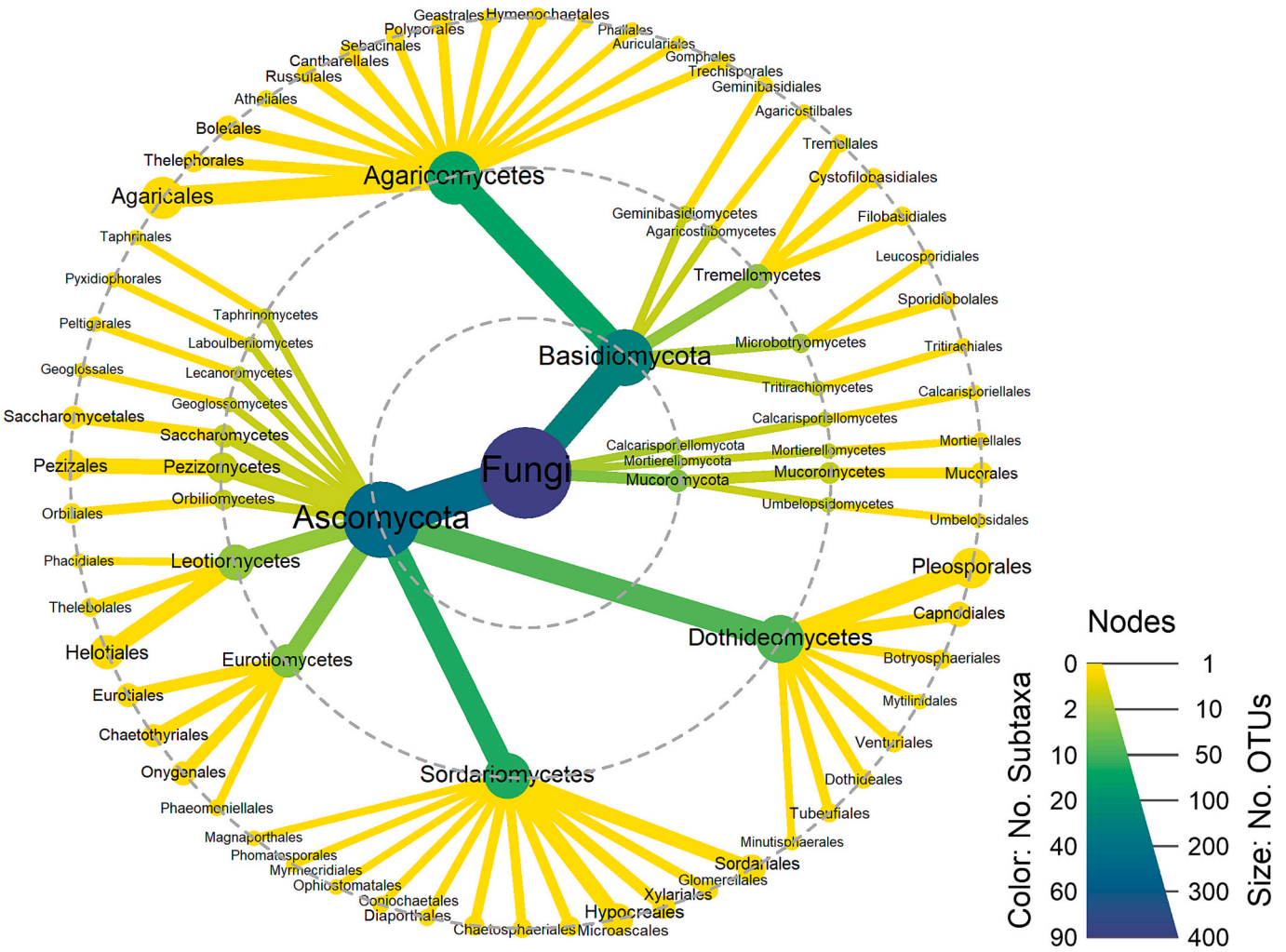

Fig. 5. Taxonomic tree of fungi in cork oak forest. The size of the node represents the number of taxonomic units and the color scale indicates the number of subtaxa. Concentric circles indicate taxonomic levels: phylum, class and order.

the grasslands associated with healthy cork oak forests, the coincidence is lower ($n = 21$, 39.6 %). In both healthy and diseased cork oak forests, the presence of specific genera is very low ($n = 7$, 13.2 % and n = 12, 22.6 %), unlike in the grasslands associated with healthy cork oak forests, where there are a large number of specific fungal genera ($n = 25$, 47.2 %). The presence of specific genera in the grasslands associated with diseased cork oak forests is lower ($n = 6$, 11.3 %) (Fig. 6).

3.4. Rarefaction analysis and measurement of biodiversity

The rarefaction curves of bacteria and fungi show in all cases that a reasonable number of genera have been sampled in both healthy and diseased areas, therefore conducting a greater number of samplings would not yield new data (Supplementary Fig. 1). Specifically in the case of cork oaks, those bacterial rarefaction curves corresponding to healthy areas stand out compared to those corresponding to sick areas, as they

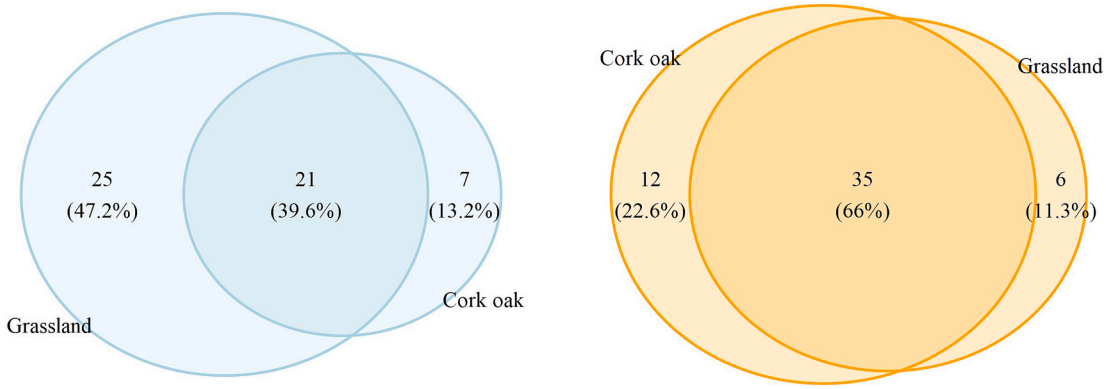

Fig. 6. Venn diagrams for the fungal genera of each condition under study. The Venn diagram indicates the coincidences and differences between the fungal genera obtained for the samples collected in healthy plots (in blue) and diseased plots (in orange).

acquire an asymptotic shape with a higher number of genera in many cases. The same does not happen with grasslands, where the evolution of the curves shows a similar behavior in both healthy and diseased areas (Supplementary Fig. 1). In the fungal rarefaction curves, those corresponding to healthy areas show a disparate trend, with some curves exhibiting a low taxonomic richness level and others around 150 genera. In the case of the curves corresponding to fungi located under declining trees, their evolution has been similar in most cases, taking an asymptotic shape around 100 genera. In the case of grasslands, those curves corresponding to healthy areas stand out, where the richness levels of a large part of the curves have reached values above 100 (Supplementary Fig. 1).

3.5. Microbial biodiversity analysis

The study of the microbial biodiversity of the soils was carried out separately, analyzing fungi and bacteria according to the health status of the predominant vegetation cover in each case. Regarding the alpha diversity of bacterial families, no significant differences were observed under cork oak or grassland (Fig. 7a). As for fungal genera, significant differences were reported between the different sanitary states, especially for the dominant genera (Hill = −1) and those with intermediate abundance (Hill = 0) (Fig. 7b). Furthermore, in the case of cork oak trees, the curve for diseased areas is above, while in grasslands the curve above corresponds to healthy areas (Fig. 7b). A comparison between the bacterial and fungal curves under cork oak and grassland allowed highlighting how the fungal communities present a higher proportion of dominant genera (Hill = −1), while there is a lower number of bacterial families acting as dominant.

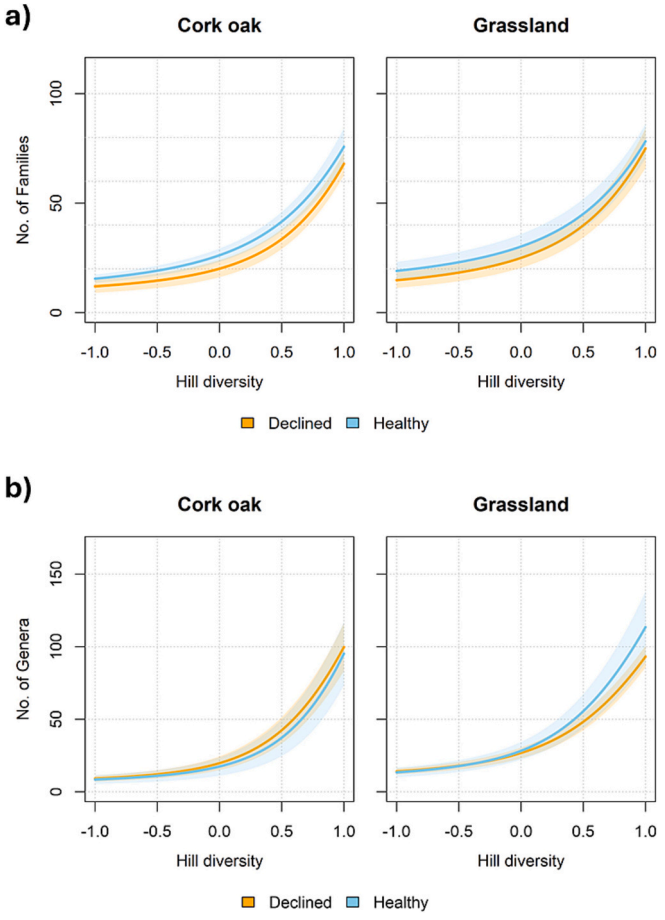

Fig. 7. Bacterial (a) and fungal (b) diversity according to sanitary status and vegetation cover. The horizontal axis represents the exponent l of Hill diversity, which can be interpreted as equivalence-corrected versions for richness (l = 1), Shannon (l = 0) and Simpson (l = −1) diversity estimators. Shadowed intervals correspond to standard error.

3.6. Differential abundance analysis

A correlation analysis was carried out between the abundance of the different microbial genera in healthy and diseased cork oak stands. With respect to bacterial families, no differential families were reported in the grasslands. However, two bacterial families (Gemmatimonadaceae and Nocardioidaceae) were differently present in the soils of healthy cork oaks (Fig. 8a). On the other hand, in relation to fungal genera, no differential genera were reported in the grasslands, while the fungal genus *Geminibasidium* appeared differentially in the soils of the diseased cork oaks (Fig. 8b).

3.7. Functional niche analysis

The most abundant bacterial functional niches, both under cork oak and in healthy and diseased grasslands, were those corresponding to aerobic heterotrophic bacteria, chemotrophs and aerobic organotrophs. However, facultative lithotrophic and facultative autotrophic bacteria were practically nonexistent under healthy grasslands and nonexistent under grasslands associated with diseased grasslands and cork oaks in good and poor condition (Fig. 9a).

As for the fungal communities sampled, soil saprotrophs, saprotrophs and ectomycorrhizae appear as main groups under cork oak forest. While under grassland there are mainly saprotrophs, soil saprotrophs and plant pathogens. A more specific analysis with respect to the plant cover and its sanitary status, highlighted under cork oak in good condition ectomycorrhizal fungi and to a lesser extent soil saprotrophs and saprotrophs, while algal parasites are absent, and parasitic lichens are scarcely represented. As for the grasslands, saprotrophs and soil saprotrophs are present under these plant coverts associated with healthy and diseased trees, while algal parasites and root endophytes are very scarce under declining areas, but somewhat more abundant in areas of healthy grassland (Fig. 9b).

4. Discussion

Microorganisms, including soil bacteria and fungi, play an important role in the proper development of plant species, by contributing to fundamental soil processes, such as the carbon cycle and the nitrogen and phosphorus cycles (Lladó et al., 2017), intervening in the processes of nutrient absorption by plants (Oliverio et al., 2020), or by acting as pathogens on them (Mohammad-Razdari et al., 2022). In this context, metabarcoding studies represent a great technical advance to know the microbial diversity associated with soils and to be able to analyze these microbial communities in relation to the health status of trees (Diez-Hermano et al., 2023, 2024; Morales-Rodríguez et al., 2024).

Our study has described a wide bacterial and fungal diversity in cork oak and associated grassland soils. Although mycorrhizal fungal diversity is a widely studied aspect in cork oak-associated soils, bacterial diversity has been poorly characterized (Maghnia et al., 2019). On the other hand, our study represents the first to address the study of soil microbial diversity of cork oak-associated grasslands.

With respect to bacteria, the families found in the soils of the cork oak trees and their associated grasslands were very similar, regardless of the health status of the trees. This could be due to ecological processes reported in previous works, where it is described how forests and associated grasslands present a very similar bacterial diversity due to the presence of endemic bacteria and the strong conditioning of bacterial diversity presented by the common environmental conditions in both types of vegetation cover (Wang et al., 2021), such as pH (Kaiser et al., 2016), precipitation or temperature (Reis et al., 2019). However, there are also different environmental factors in both canopies that have been

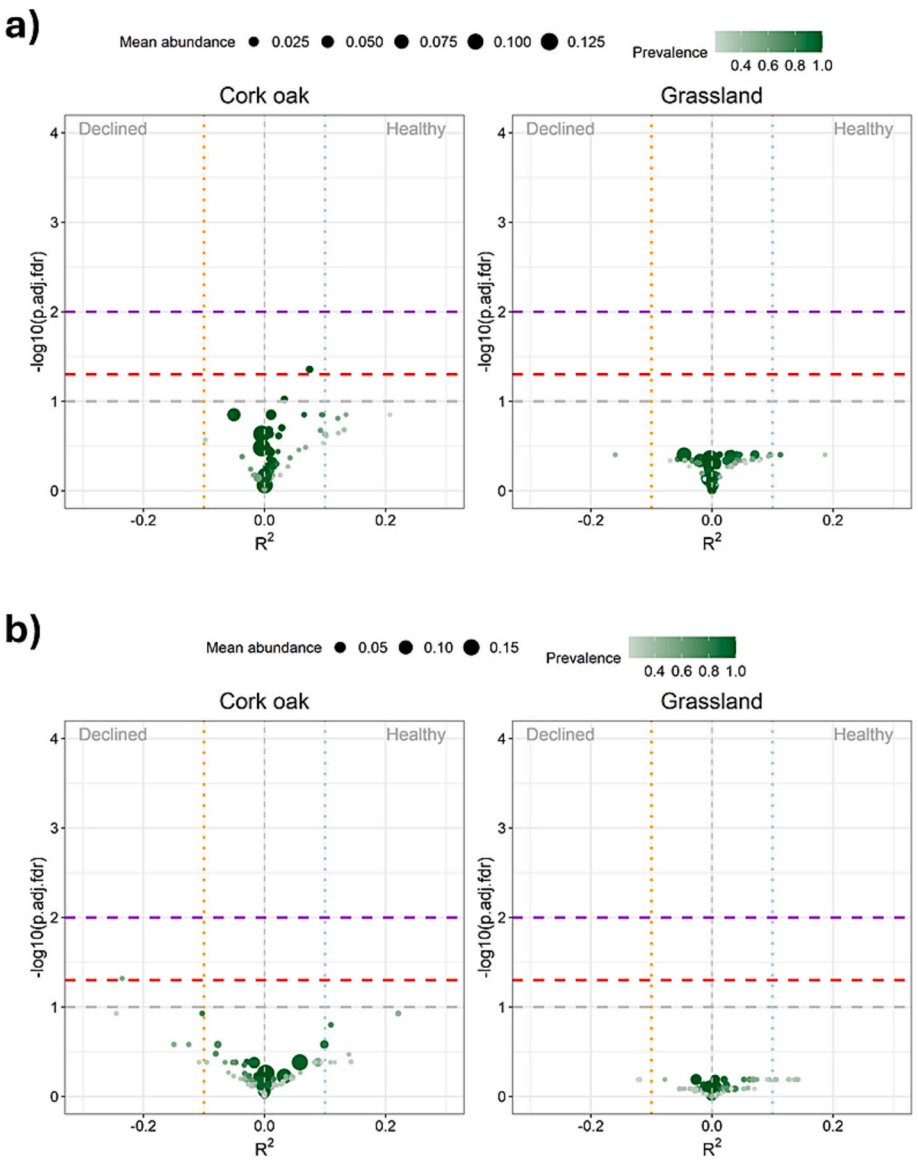

Fig. 8. Differential bacterial (a) and fungal (b) abundance. Volcano plot of differentially abundant families (in bacteria) and genera (in fungi) detected by ZicoSeq analysis. Each family (in bacteria) or genus (in fungi) is depicted by a dot. Dot size indicates the mean abundance across all samples and color indicates the proportion of samples in which the family (in bacteria) or genus (in fungi) is present. Vertical axis represents value of p adjusted by false discovery rate (FDR) in logarithmic scale (1 = 0.1, 1.30 = 0.05, 2 = 0.01 and so on). Horizontal axis represents the strength of association between abundance and health condition, with the sign indicating the association direction (negative for declining, positive for healthy). Families (in bacteria) and genera (in fungi) that surpass the horizontal dashed line are differentially abundant (p-val < 0.1).

described as important determinants of bacterial diversity in forests and associated grasslands, such as solar radiation (Gömöryová et al., 2009). The absence of differences in bacterial diversity in healthy and diseased cork oak soils agrees with what was reported in a previous work conducted in different Portuguese cork oak forests (Reis et al., 2019).

In the case of fungi, differences were reported between the genera present in the soils of healthy cork oaks and associated grasslands, but not when there were diseased trees. The presence of different fungal communities in forest and associated grassland soils has been previously described as being strongly influenced by plant diversity, soil moisture, soil nutrient content, or pH (Rossel et al., 2022). With respect to forest decline, the results reported in our work suggest that the presence of decline reduces fungal diversity in the soils of both canopies analyzed when diseased trees were present, which reduced the existing differences between them.

On the other hand, the presence of diseased trees also implied a different fungal diversity in the soils present in the cork oaks and in the associated grasslands, making the comparison in the same type of vegetation cover. Although our work is the first to study fungal diversity in cork oak-associated grassland soils, the higher fungal diversity in healthy cork oak versus diseased soils has been described in previous works (Diez-Hermano et al., 2023, 2024).

With respect to differential abundance, no bacterial families or fungal genera were reported to be differentially present in healthy versus diseased pastures, nor vice versa. These results could indicate that the health status of the trees does not condition the specific microbial diversity in the associated grasslands, but, as this is the first work carried out in cork oak forests, further studies are required to confirm the results obtained in this work.

In the soils of healthy cork oaks, we found two bacterial families differentially present, Gemmatimonadaceae and Nocardioidaceae. The bacterial family Gemmatimonadaceae has been described as differentially present in soils of healthy versus diseased plants of the medicinal plant *Angelica sinensis* (Liu et al., 2021), as well as in Chinese cabbage (Li

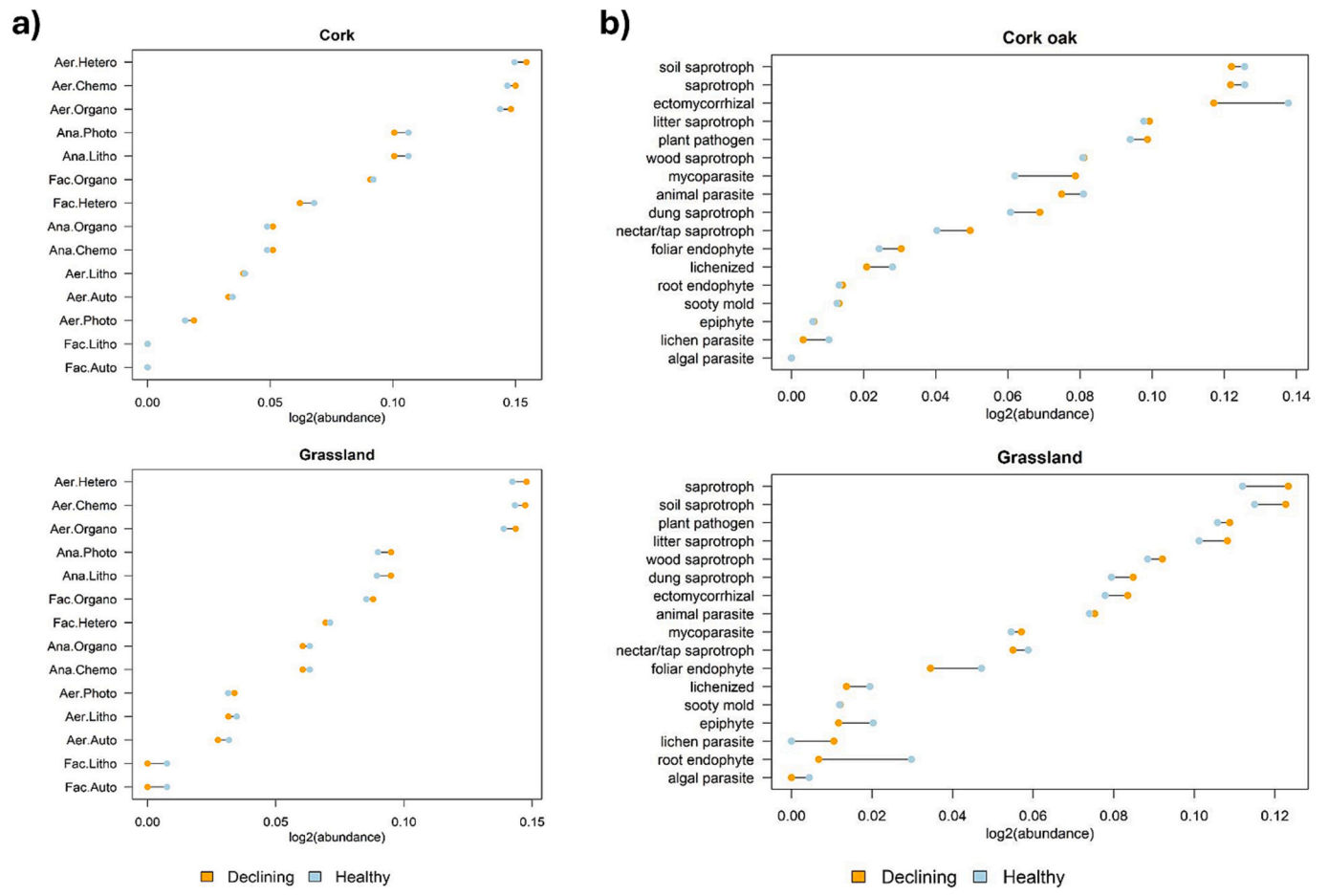

Fig. 9. Bacterial (a) and fungal (b) functional niche analysis. The X-axis corresponds to the abundance of bacterial functional traits on a logarithmic scale, and the Y-axis corresponds to a functional trait (oxygen utilization and feeding style). The distance of functional abundance between healthy and diseased samples is indicated by the line joining samples.

et al., 2024). However, it has also been described as the bacterial family differentially present in the soils of diseased versus healthy wheat plants (Jia et al., 2022). So far, no biological control agent has been described in this bacterial family, so its role in the possible control or prevention of cork oak decline cannot yet be hypothesized. On the other hand, the Nocardioidaceae family, in addition to being bioremediating agents (Ma et al., 2023), its member bacteria have been described as possible biocontrol agents against soil pathogens in ginseng (Cho et al., 2024), or against nematodes in *Cucumis* crops (Song et al., 2023). Therefore, the bacterial family Nocardioidaceae differentially present in healthy cork oak soils could include bacteria effective in the biocontrol of the causal agents of decline in these trees. These results differ from those reported in other works, where the family differentially present in healthy cork oaks was the phylum Proteobacteria (Gómez-Aparicio et al., 2022).

With respect to fungi, the genus *Geminibasidium* was found to be differentially present in diseased cork oak soils. This fungal genus was originally described as high temperature tolerant and xenotolerant, but not as a possible plant pathogen (Nguyen et al., 2013). So far, in its relationship with plants, this fungal genus has been described as especially selected to relate to the plant species *Atractylodes lancea* (a medicinal plant) (Li et al., 2018) and peanut (Li et al., 2014), through its root exudates, but its actual role in plant interaction is ignored. Although in our work the genus *Geminibasidium* appeared differentially present in the soils of diseased cork oaks, the current knowledge about this fungal genus prevents us from relating this data with a possible pathogenic behavior in the arboreal decline.

The analysis of functional niches found in the different soils according to their sanitary status reported no differences with respect to bacterial families. This is consistent with the bacterial diversity data discussed above and suggests that bacteria may not play an important role in the microbial dynamics of cork oak and associated grassland soils when they suffer tree decline.

However, different functional niches were found in the case of soil fungi. The main group of fungi present in the soil of healthy versus diseased cork oak trees were the ectomycorrhizal fungi. These fungi have been widely described as effective biological control agents against tree diseases (Poveda et al., 2024), and even as possible suppressors of decline in cork oak previously (Diez-Hermano et al., 2023), therefore, they could be microorganisms involved in the absence of disease in the trees analyzed in this study. In addition, it was reported that grasslands associated with healthy cork oaks had differentially present fungi belonging to the root endophyte functional niche. Endophytic fungi have been widely described as biological control agents of plant pests and diseases (Muhammad et al., 2024), so their presence could be implicated in the absence of disease in cork oaks, but further research is needed.

In conclusion, cork oak forest soils and their associated grasslands present similar bacterial diversity when no diseased trees are present. The bacterial families Gemmatimonadaceae and Nocardioidaceae were massively present in healthy cork oak forest soils, and may include some decline biocontrol species. While the fungal genus *Geminibasidium* was found to be massively present in diseased cork oak soils, although it does not include, so far, described phytopathogenic species. Moreover, the massively differential functional presence of ectomycorrhizae in healthy cork oak soils and fungal endophytes in associated grasslands could be implicated in the prevention and/or control of decline. Therefore, fungi,

but not bacteria, present in cork oak soils and associated grasslands could play a key role in the presence/absence of tree decline.

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CRediT authorship contribution statement

José Carlos Marcos-Romero: Methodology, Investigation. **Jorge Poveda:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis. **Julio Javier Diez:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

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

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Data availability

Data will be made available on request.

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