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Metabarcoding reveals rhizosphere microbiome shifts between healthy and declining *Quercus robur* trees

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ABSTRACT

Oak dieback affecting *Quercus robur* L. (pedunculate oak) in Northern Europe, is driven by a complex interaction of abiotic and biotic factors, such as pests, diseases, and environmental stress, including drought. To better understand the role of the soil microbiome in oak dieback, we analysed the diversity and composition of the microbial communities in the rhizospheres of declining and visibly healthy trees. We used metabarcoding to describe the microbiome and baiting (i.e., the use of plant tissues to act as baits) to isolate species of *Phytophthora*, a protist genus known for its contribution to the decline of oak trees. Our findings revealed significant differences in bacterial alpha diversity and fungal beta diversity between the rhizospheres of healthy and declining trees. Viable isolates of several species of *Phytophthora*, such as *Phytophthora plurivora, P. cactorum*, and *P. gonapodyides* were obtained using the baiting technique. The results underscore the stand level diversity of rhizosphere soil microbiota and support our initial idea that microbial communities vary with tree health conditions.

1. Introduction

According to recent research, terrestrial plants including forest trees are considered holobionts, comprising the host plants and their associated microbiota (Vandenkoornhuyse et al., 2015; Sánchez-Cañizares et al., 2017; Berg et al., 2020). The plant microbiome (phytobiome) comprises all microbial genomes associated within the host plant phyllosphere, rhizosphere, and endosphere (Guttman et al., 2014; Terhonen et al., 2019). It is widely recognized that these microbial communities play a crucial role in plant growth and health (Vandenkoornhuyse et al., 2015; Terhonen et al., 2019) and enhance the adaptability of plants to diverse environmental conditions (Bulgarelli et al., 2013; Vandenkoornhuyse et al., 2015; Gehring et al., 2017). Consequently, this plant-associated microbiome is referred as the second genome of the plant (Berendsen et al., 2012; Vandenkoornhuyse et al., 2015; Gehring et al., 2017). The root-soil interface, known as the rhizosphere, includes both endophytic (host plant internal) and epiphytic (external) microbial communities, primarily consisting of bacteria and fungi associated with the root surface and the surrounding soil layer (Christian et al., 2015; Mardanova et al., 2019; Terhonen et al., 2019). Plants secrete exudates from their roots which can drive plant-soil feedback by either attracting or repelling specific members of the microbiome and therefore shaping the rhizosphere community (Hu et al., 2018). As the location for nutrient uptake, this zone significantly contributes to various functions of both individual trees and forest ecosystems, including growth, defense, litter decomposition and nutrient cycling (Kuzyakov et al., 2007; Hu et al., 2018; Ribbons et al., 2022). Advancements in sequencing technologies have greatly increased our understanding of these interactions (Barberán et al., 2012; Lebeis, 2015; Terhonen et al., 2019). A deeper understanding of these processes is essential for assessing forest resilience in the face of escalating environmental stressors associated with climate change and the growing threat of invasive pathogens (Trumbore

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et al., 2015; Vandenkoornhuyse et al., 2015; Gehring et al., 2017; Lehmann et al., 2020).

Oak decline —a phenomenon characterized by the gradual decline in oak tree health driven by complex interactions between abiotic and biotic factors—has become a significant ecological concern in forest ecosystems (Vettraino et al., 2002; Pérez-Sierra et al., 2013; Jung et al., 2018a; Seddaiu et al., 2020; Macháčová et al., 2022; Kowsari and Karimi, 2023; Horta-Jung et al., 2025). Pathogenic organisms, including some introduced species within the oomycetes such as *Phytophthora*, have been identified as primary driving factors of oak decline, which is characterized by a variety of symptoms, including loss of fine roots, necrotic root lesions, collar rot cankers, crown transparency and mortality (Brasier et al., 1993; Vettraino et al., 2002; Balci and Halmschlager, 2003; Jung et al., 1996; Pérez-Sierra et al., 2013; Jung et al., 2000, 2016; Jung et al., 2018b; Horta-Jung et al., 2025). Soil-borne pathogens, such as *Phytophthora* species, release signals that alter plant root exudation profiles and modify the composition and activity of the rhizosphere microbiome (Stringlis et al., 2018). Understanding these alterations in microbial communities is crucial, as they may contribute to suppressing or facilitating soil-borne pathogens through production of bioactive chemicals or niche competition (Rolfe et al., 2019). For example, certain species of *Pseudomonas, Bacillus*, and *Trichoderma*, that are native inhabitants of temperate soil ecosystems, have demonstrated the ability to reduce crop vulnerability to soil-borne pathogens in agricultural settings (Durairaj et al., 2018; Adnan et al., 2019). Despite the recognized importance of soil microbiota in plant health, the impact of biotic factors such as invasive soil-borne pathogens on microbial communities in mature *Q. robur* production forests remains poorly understood.

We investigated the diversity of soil microbiota within the rhizosphere of pedunculate oak (*Quercus robur* L.) trees in southern Sweden and explored their relationships with tree health. Using a metabarcoding approach, we compared the composition of the rhizosphere microbiome in soil samples collected around visibly healthy (trees exhibiting minor symptoms) and declining oak trees growing in a managed forest stand where decline symptoms had been observed for about a decade. Additionally, we used the baiting method, which enables the formation of sporangia and motile zoospores in water, to isolate viable *Phytophthora* species. Our study addressed two questions: 1) Do significant differences exist in soil microbiome diversity and abundance between visibly healthy and declining trees? 2) Are tree-pathogenic *Phytophthora* species more diverse or abundant in the rhizosphere of declining trees?

2. Material and methods

2.1. Field site and soil sampling

The study location was a production oak (ca. 70 years old) forest located in the region of Skåne, southern Sweden (55°30′ 16.3″ N and 13°25’ 26.9” E) where dieback and advancing decline had been observed during the past decade. The dominant plant species in the ground layer were *Urtica dioica*, *Dryopteris* spp., *Pteridium aquilinum* and *Poaceae* spp. Tree health was assessed using a rating scale based on average crown defoliation following ICP Forest Monitoring standards (Müller and Stierlin, 1990; Eichhorn et al., 2020). Defoliation was visually assessed and categorized at 5 % intervals (from 0 to 100 %) and included leaf loss, twigs and branch loss. The presence of epicormic branches on the main stem was considered as a sign of lowered vitality (see Fig. 1). Two distinct vitality classes were defined for the analyses based on the following criteria (See supplementary data, Table S2):

Healthy (H); with minimal visible crown damage, exhibiting up to 5–15 % damaged branches;

Declining (D); up to 65–90 % damaged branches, the presence of wilting leaves in the crown and epicormic branches on the main stem.

In July 2020, rhizosphere soil samples were collected surrounding trees classified as healthy (H) and declining (D) from three directions around the trunk (3 samples per tree) at a distance between 150 cm and 200 cm from the base of the stem to a depth of 15–35 cm (Drenth and Sendall, 2001; Jung et al., 1996, 2000). The litter layer was removed with a long-handled shovel, and three soil samples (each with volumes

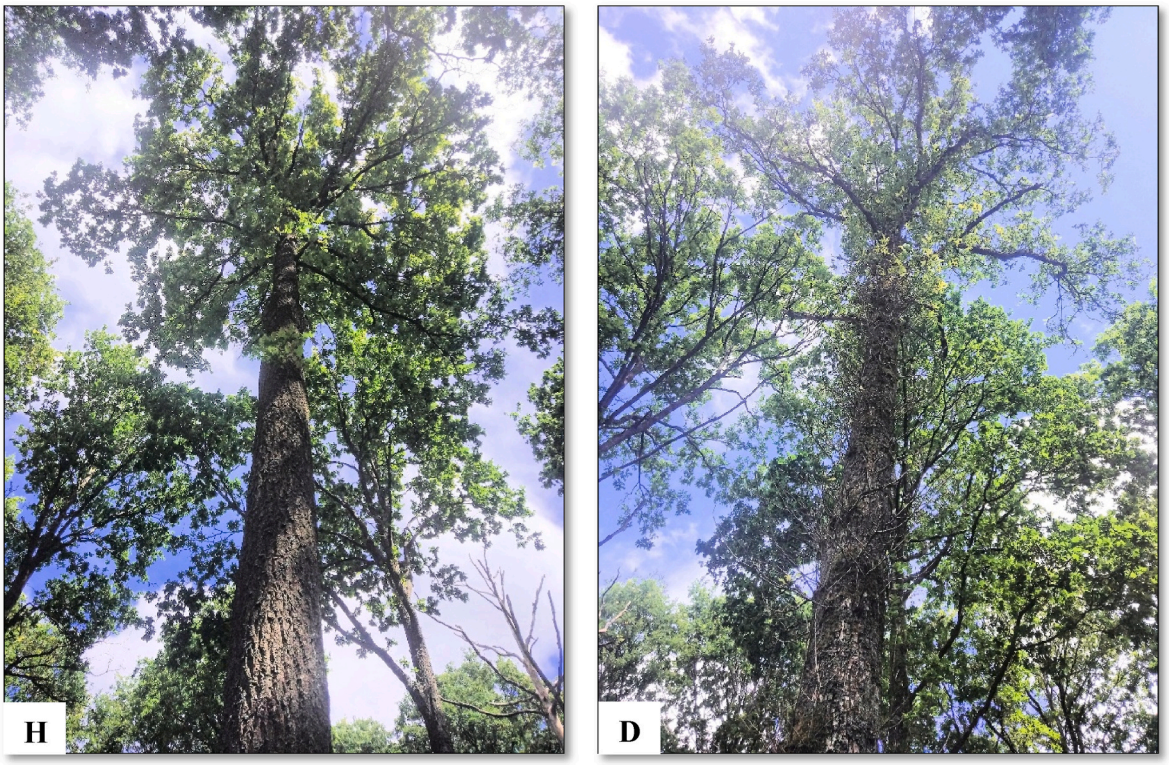

Fig. 1. Photographs of the sampled trees. The image on the right (D) shows a declining tree, while the image on the left (H) depicts a healthy tree.

ranging from approximately 0.95 to 1.56 L of soil) collected in a plastic bag using a hand shovel. The three samples were combined and mixed thoroughly to yield a single rhizosphere soil sample for each tree, ensuring the inclusion of a substantial amount of fine roots (total n = 20 samples, 10 from each vitality class).

2.2. Metabarcoding, sequencing and bioinformatics of soil microbiome samples

To analyse the composition of the rhizosphere microbiome, 25 g of rhizosphere soil from each sample (n = 20) was transferred into 25 mL centrifuge tubes and sent to Biome Makers Inc. in Spain for analysis using amplicon sequencing methods. PCR assays were prepared using sterilized material and equipment, while negative controls containing nuclease-free water were run alongside the samples. The entire fungal ITS1 and bacterial 16S rRNA gene V4 regions were amplified with WineSeq® primers (Patent No.:WO2017096385; Becares and Fernández, 2017). After a quality control check by gel electrophoresis, the 16S rRNA and ITS libraries were pooled in equimolar concentration and subsequently sequenced using pairs of sequences of 2 x 300bp (base pairs) MiSeq® Reagent Kit v3 kit (Illumina, San Diego, CA, USA) on the Illumina MiSeq® platform (Illumina Inc., San Diego, CA, USA), according to the Biome Makers proprietary protocol.

The bioinformatics analysis of both the bacterial and fungal raw reads was carried out using the DADA2 pipeline (Callahan et al., 2016) in R studio version 4.3.2 (R Core Team, 2023). Default settings through the pipeline were used for filtering and trimming of reads. Identical sequencing reads were combined using the *dereplication* function. Further, paired-end reads were merged, chimeras were removed, and the reference database of amplicon sequence variants (ASV) table was built using internal manually curated taxonomies from the latest version available of SILVA 138.1 (Glöckner et al., 2017) for 16S sequences, and the UNITE 10.0 database (Nilsson et al., 2019) for ITS sequences. Finally, DADA2 data outputs were combined into a phyloseq object using the *phyloseq*-package (McMurdie and Holmes, 2013) and the phyloseq object converted into a MicroEco-object using the *MicroEco*-package (Liu et al., 2021) for post-processing data analysis.

2.3. Diversity and statistical analyses

All statistical analyses were performed in R studio v. 4.3.2 (R Core Team, 2023) and tests were considered significant at $P < 0.05$. Values from species richness were utilized to perform a sample-size-based rarefaction (interpolation) curve (Colwell et al., 2012; Chao et al., 2014). Rarefaction curves were visualized in the *iNEXT* package (Hsieh, Ma and Chao, 2023). The package *rstatix* (Kassambara, 2023, 2023) was employed to calculate different statistical tests, including Wilcoxon test, *t*-test, ANOVA and correlation analyses. All boxplots were performed using the package *ggplot2* (Wickham et al., 2023).

Differences in community composition of rhizosphere fungi and bacteria among healthy and declining trees were assessed using permutational multivariate analysis of variance [PERMANOVA test based on 999 permutations (Anderson, 2001) using the function *adonis2* of the *microeco* package (Liu et al., 2021)]. Amplicon sequence variants (ASVs) richness and evenness, including Simpson's (D), Shannon (H), and Chao 1 diversity, were analysed using multivariate analysis of variance (MANOVA) to compare the relative abundance of fungi and bacteria in the rhizosphere of healthy and declining trees. Non-metric Multidimensional Scaling (NMDS) was performed on Bray–Curtis dissimilarity matrices to evaluate differences in beta diversity between healthy and declining trees using the function *ordinate* and plotted using the function *plot ordination* of the *microeco* package.

Pearson correlation coefficients were calculated to assess the co-occurrence relationships between bacterial and fungal taxa in the rhizosphere soil samples. Correlation matrices were constructed using the *cor* function from the *stats* package (R Core Team, 2023) and applied to the transformed taxonomic abundance dataset. Prior to analysis, taxonomic abundance data were filtered to retain only the most abundant taxa and reformatted into a wide format using the *pivot wider* function from the *tidyverse* package (Wickham et al., 2019). Unidentified taxa were excluded from the dataset using the filter function in combination with *str detect* from the *dplyr* package (Wickham et al., 2023). To visualize clustering patterns among taxa, correlation heatmaps were generated using the *pheatmap* function from the *pheatmap* package (Kolde, 2018). Hierarchical clustering was applied to both rows and columns to group taxa with similar correlation structures.

2.4. Baiting and isolation of Phytophthora spp.

In the laboratory, a sub-sample of 500 g was separated from each soil sample. Each sub-sample (n = 20) was placed in a plastic container and flooded with distilled water at a 4:1 water-to-soil ratio, following the protocol described by Drenth and Sendall (2001). After 20 h, any litter and debris floating on the surface were cautiously removed with a paper tissue, before covering the surface of the water with young rhododendron leaves sterilized in 1 % sodium hypochlorite for 30 s, washed three times in sterile water, and blotted dry on filter paper. The samples were then incubated at room temperature (21 °C). After three to four days when dark brown lesions or necrotic spots appeared, leaves were dipped in deionized water, dried on sterile tissue paper, cut aseptically into approximately 3 × 3 mm pieces from both the infected and healthy sections of the tissue and plated on PARP(H) + B V8 agar containing 10 mg/L rifampicin, 250 mg/L sodium ampicillin, 5 mg/L pimaricin, 100 mg/L pentachloronitrobenzene [PCNB], 50 mg/L hymexazol, and 10 mg/L benomyl (Jeffers, 1986). Petri dishes were examined daily for the presence of emerging hyphae. When growth was observed, small plugs of mycelium plus agar were transferred from the colony margin to potato dextrose agar (PDA) (Merck KGaA, Darmstadt, Germany), to obtain pure cultures. Isolates were categorized based on morphological traits, and subsequently, 23 representative samples of each morphotypes, were selected for molecular analyses.

2.5. DNA extraction and sanger sequencing of samples derived from baiting

To prepare for DNA extraction, plugs of the 115 preselected isolates were transferred to 50 mL Falcon tubes containing 35 mL liquid malt extract broth (MEB), (Merck KGaA, Darmstadt, Germany). After incubation at room temperature for two weeks in the dark, the cultures were filtered on sterile filter paper placed in a ceramic funnel and any residues of PDA removed manually. The mycelia were rinsed three times in 50 mL deionized sterile water, transferred to 2 mL Eppendorf tubes with four glass beads (3 mm) and freeze–dried for 2–3 days before homogenizing to fine powder using a Rescht MM400 ball mill (Retsch, Haan, Germany). DNA was extracted using an E.Z.N.A. SP Plant DNA Kit (Omega Bio-Tek, Inc., Norcross, USA), following the manufacturer's instructions. DNA concentration was measured using NanoDrop® ND1000 (Wilmington, USA). The region spanning the internal transcribed spacer ITS of ribosomal DNA was amplified by Polymerase Chain Reaction (PCR) using the primers ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) (White et al., 1990) and ITS6 (5′–GAAGGTGAAGTCGTAACAAGG–3′) (Cooke et al., 2000). PCR was carried out in 25 μL reaction volumes containing: 2 μL (5 ng/μL) DNA template, 1 μL ITS4 (0.4 μM) and 1 μL ITS6 (0.4 μM) primers, 12.5 μL DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific Inc.) and 8.5 μL sterile water (Tedersoo et al., 2014). All PCR reactions were carried out in an Eppendorf Mastercycler DNA Engine Thermal Cycler (Hayward) under the following conditions: initial denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C. A final elongation step was carried out for 10 min at 72 °C. PCR products were visualized by electrophoresis on 1.2 % agarose. PCR products were

purified using the DANAGENE Clean PCR Kit following the manufacturer's instructions. Purified PCR products were quantified using NanoDrop® ND1000 (Wilmington, USA) before sending for sequencing in both directions ITS4 (forward) and ITS6 (reverse) regions by automated Sanger sequencing at the National Genomics Infrastructure (NGI) at the Science for Life Laboratory (Scilifelab, Solna), Sweden. Sequences were checked manually, aligned and edited using Geneious Prime Software Version 2021.0.3, before comparison with known reference sequences in three different sources: GenBank (National Center for Biotechnology Information, NCBI) using the Basic Local Alignment Search Tool (BLAST); "*Phytophthora*-ID" (Grünwald et al., 2011) (http://phytophthora-id.org/) a resource for the identification of *Phytophthora* species and genotypes using BLAST; and finally using The Barcode of Life Data System (BOLD) (http://www.boldsystems.org/) (Ratnasingham and Hebert, 2007). Isolates were determined to species level based on 99 % sequence similarity or above and to genus based on 98 % sequence similarity. The sequences obtained for all isolates were registered in GenBank under the accession numbers PP759664 to PP759724.

3. Results

3.1. Relative abundance of bacteria and fungi within the rhizosphere community

To assess differences in the microbial communities associated with healthy or declining *Quercus robur* trees, we took a metabarcoding approach. From sequencing of the ITS1 and 16S genes, from rhizosphere soil samples from 10 healthy and 10 declining trees, a total of 2563 fungal Amplicon sequence variants (ASVs) and 6647 bacterial ASVs were recorded. Rarefaction analysis revealed that the abundance of ASVs reached a plateau, indicating that the sequencing depth for the samples was saturated (see supplementary data, Fig. S1). No significant differences were observed in the overall relative abundance of bacterial (p = 0.7477) and fungal (p = 0.6587) ASVs between healthy and declining trees (Fig. 2A–D). The most abundant bacterial ASVs were associated with the phyla Proteobacteria, Acidobacteriota, and Planctomycetota, which together accounted for over 65 % of the total relative abundance. Particularly, the phylum Proteobacteria showed higher relative abundance in declining trees (31 %) compared to healthy ones (25.2 %). Acidobacteriota was also more abundant in declining trees

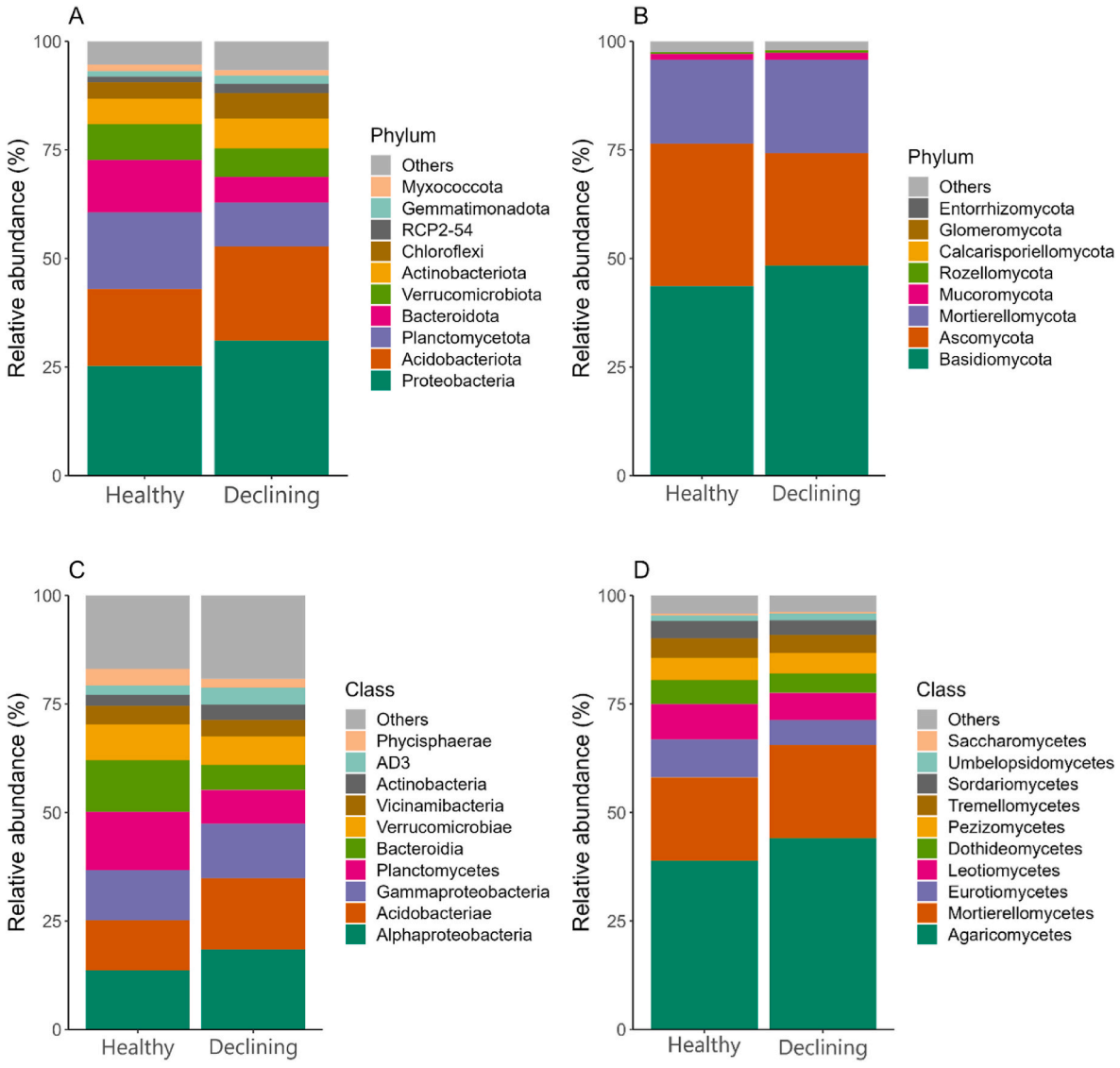

Fig. 2. Group mean of the relative abundance of the top 10 phyla and classes between healthy and declining *Quercus robur* rhizosphere soil samples, where (**A**) represents the bacterial and (**B**) the fungal phyla, and (**C**) represents the bacterial and (**D**) the fungal classes.

(21.7 %) than in healthy trees (17.8 %). Conversely, the phylum Planctomycetota had greater abundance in the healthy trees (17.6 %) than in the declining ones (10.1 %). The most dominant fungal ASVs belong to the phylum Basidiomycota, followed by the phyla Ascomycota and Mortierellomycota, respectively, and collectively contributed over 90 % of the relative abundance. Basidiomycota were slightly (about 4 %) more abundant in soil samples collected around declining trees whereas Ascomycota were about 7 % more abundant in soil around healthy trees (Fig. 2A–D). These trends were not significant.

At the class level, bacterial ($p = 0.9487$) ASVs were dominated by Alphaproteobacteria, Acidobacteriae, Planctomycetes and Gammaproteobacteria which collectively contributed over 50 % of the relative abundance. The relative abundance of the class Alphaproteobacteria was higher in rhizosphere soil samples from declining trees (18.5 %) compared to healthy trees (13.7 %). Similarly, Acidobacteriae showed a greater relative abundance in rhizosphere soil samples from declining trees (16.4 %) than in healthy trees (11.5 %). Fungal communities from both healthy and declining rhizosphere soils samples were dominated at the class level ($p = 0.6994$) as well as at the order level by Agaricomycetes, Mortierellomycetes, Eurotiomycetes, Leotiomycetes and Dothideomycetes, which contributed over 80 % of the relative abundance collectively. The relative abundance of Agaricomycetes was higher in rhizosphere soil samples from declining trees (44.1 %) compared to healthy ones (38.9 %). Mortierellomycetes also exhibited a greater relative abundance in declining trees (21.5 %) than in healthy ones (19.2 %) (Fig. 2A–D).

Upon examination at the genus taxonomic level for bacteria, the dataset generated by metabarcoding yielded 713 different genera, with widely scattered relative abundances for each genus. *Candidatus udaeobacter* emerged as the predominant genus from both healthy and declining trees, although it tended to be more prevalent in healthy trees (see supplementary data, S4). For fungi, metabarcoding identified 345 different genera with broadly dispersed relative abundances. *Mortierella* was the predominant genus across all samples, but it tended to be more common in declining trees. At the species taxonomic level, metabarcoding revealed 564 different fungi species with diverse relative abundances. Individual bacterial species were not identified at this level (see supplementary data, Taxa abundance).

3.2. Differences in microbial diversity and composition

In order to analyse the diversity (species richness and evenness)

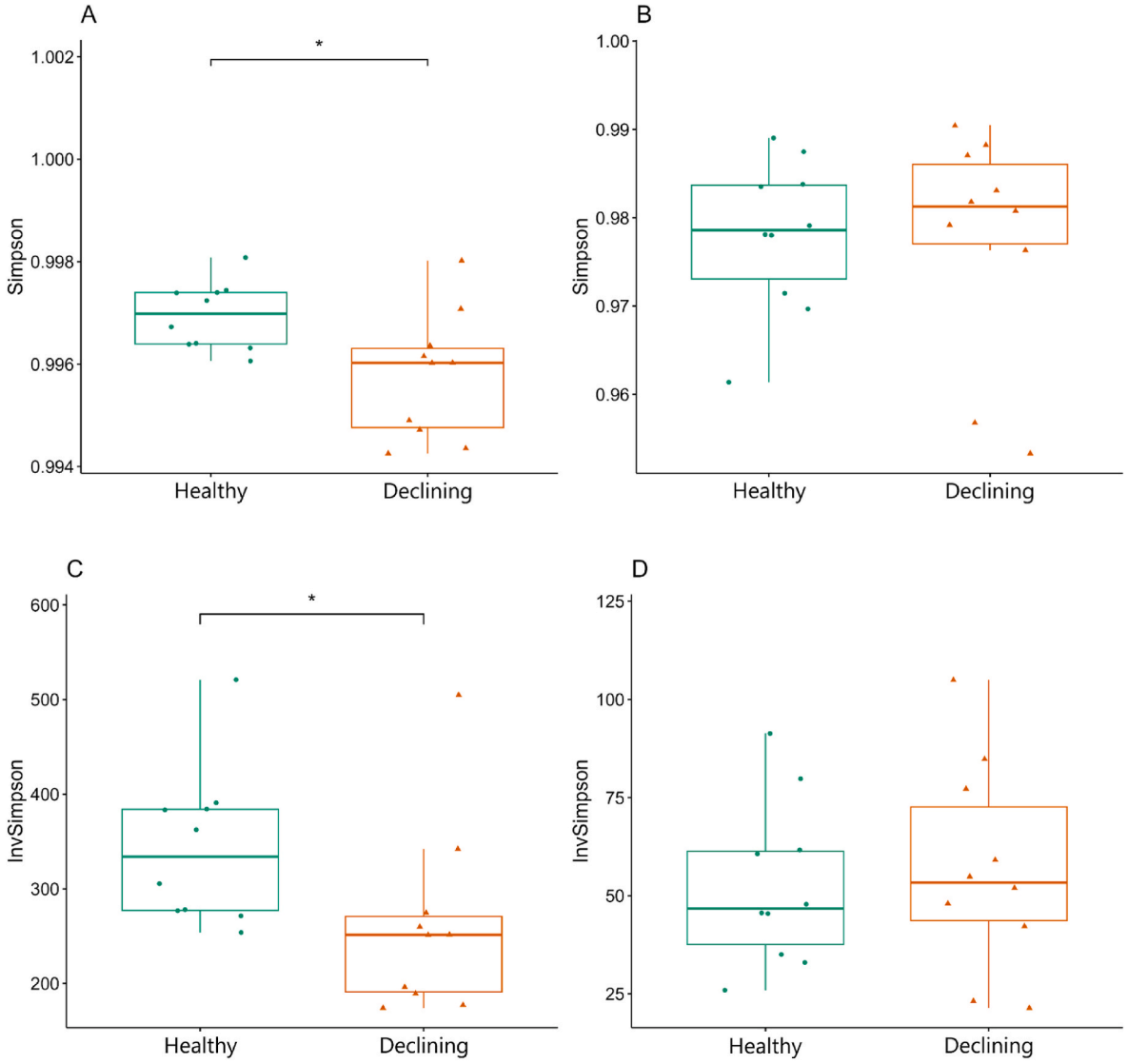

Fig. 3. Alpha diversity shown as Simpson (D) and InvSimpson indices between healthy and declining *Quercus robur* rhizosphere soils samples, where results of pairwise comparisons using the Wilcoxon rank sum test were (**A**; **C**) bacterial ($p = 0.011$) and (**B**; **D**) fungal ($p = 0.795$) species diversity composition (n = 10). Asterisk across vitalities indicate significant differences ($p < 0.05$).

within each rhizosphere sample, we assessed the alpha diversity, which measures both the number of species present in a sample and the evenness of their distribution (the relative abundances of those taxa). We applied three commonly used diversity indices—Chao1, Shannon, and Simpson's indices—each capturing different aspects of community diversity. The Chao1 index estimates species richness by considering both observed taxa and rare species, providing an estimate of the total species pool. The Shannon index incorporates both richness and evenness, placing greater emphasis on rare species, whereas the Simpson's indices are more influenced by the dominance of abundant taxa and less sensitive to rare species. Alpha diversity analysis, using Simpson's indices, revealed significant differences in the bacterial community structure between samples collected around healthy and declining trees (Fig. 3). However, no differences were observed using the Chao1 and Shannon indices, suggesting that the observed differences may be driven by changes in the relative abundance of dominant bacterial taxa rather than shifts in overall species richness or the presence of rare species. The lack of significant differences in Shannon and Chao1 indices further supports the idea that rare taxa and overall species richness remained relatively stable between conditions. In contrast, alpha diversity analysis of rhizosphere fungal communities showed no significant differences between healthy and declining trees across all diversity indices (Chao1, Shannon, Simpson, and Inverse Simpson) ($p < 0.05$) (see supplementary data, Fig. S2).

Differential abundance at the order level was explored to determine whether there are major differences in the bacterial and fungal diversity of the rhizosphere microbiome among the samples from the two vitality classes. Significant differences were found in the abundance of several bacterial orders between the two vitality classes in the study (Fig. 4). However, there were no significant differences in the abundance of fungal orders across these vitality classes (Fig. 5).

Differences in fungal and bacterial community composition at the beta diversity level were determined (Fig. 6). There were no significant differences in the composition of the rhizosphere bacterial communities associated with healthy or declining trees (PERMANOVA $R^2 = 0.072$, $p = 0.109$). In contrast, significant differences were observed in the rhizosphere fungal community composition between samples from

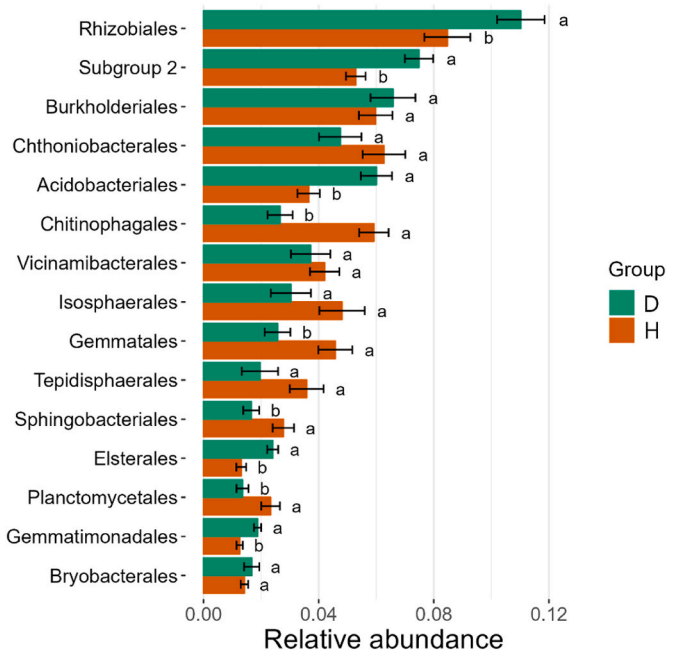

Fig. 4. Differential abundance analysis of bacteria at the order level, showing pairwise comparisons between rhizosphere soil samples from healthy (H) and declining (D) *Quercus robur* trees (n = 10). Distinct letters across vitalities indicate significant differences ($p < 0.05$).

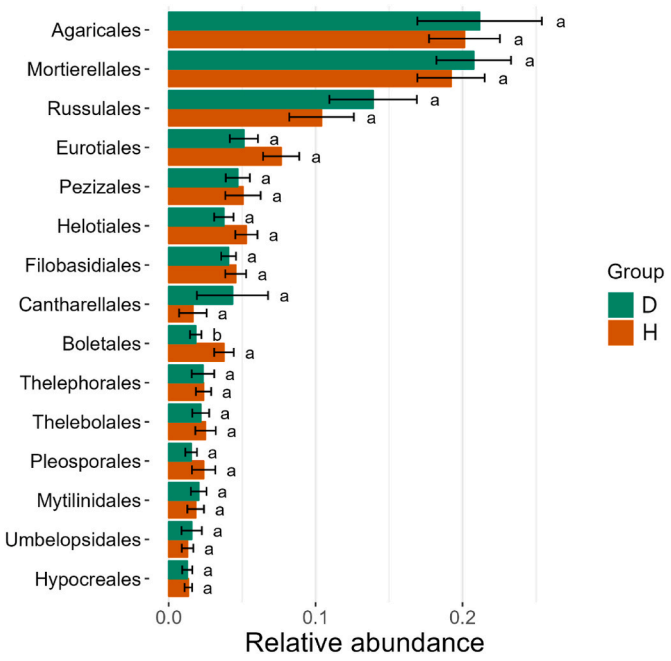

Fig. 5. Differential abundance analysis of fungi at the order level, showing pairwise comparisons between rhizosphere soil samples from healthy (H) and declining (D) *Quercus robur* trees (n = 10). Distinct letters across vitalities indicate significant differences ($p < 0.05$).

healthy and declining trees (PERMANOVA $R^2 = 0.067$, $p = 0.001$).

The PERMANOVA analysis showed that the health status of the trees significantly influenced the diversity of the fungal community. The Wilcoxon Rank Sum test ($p = 0.035$) revealed significant differences between the fungal communities of healthy and declining trees. In contrast, no differences were observed in the bacterial community analysis with these statistical analyses.

The overall assessment for homogeneity, evaluated using a test with 999 permutations (see supplementary data) produced non–significant results in multivariate dispersions among bacterial ($p = 0.834$) and fungal ($p = 0.41$) community groups. Pairwise comparisons of bacterial and fungal communities in rhizosphere soil samples from healthy and declining trees revealed no significant differences in multivariate dispersions ($p < 0.05$).

The Pearson correlation analysis revealed significant relationships among various microbial phyla, highlighting both positive and negative associations (Fig. 7). Specifically, Acidobacteriota exhibited strong negative correlations with Bacteroidota ($r = -0.77$, $p < 0.001$), and Planctomycetota ($r = -0.64$, $p < 0.01$), while showing a significant positive correlation with Proteobacteria ($r = 0.60$, $p < 0.01$).

Actinobacteriota showed a highly significant negative correlation with Planctomycetota ($r = -0.86$, $p < 0.001$) and a strong positive correlation with Proteobacteria ($r = 0.85$, $p < 0.001$). Bacteroidota was negatively correlated with Chloroflexi ($r = -0.49$, $p < 0.05$), Proteobacteria ($r = -0.48$, $p < 0.05$), and RCP2-54 ($r = -0.54$, $p < 0.05$), but positively correlated with Planctomycetota ($r = 0.54$, $p < 0.05$).

Chloroflexi exhibited significant positive correlations with Calcarisporiellomycota ($r = 0.62$, $p < 0.01$) and Rozellomycota ($r = 0.51$, $p < 0.05$). Planctomycetota showed a strong negative correlation with Proteobacteria ($r = -0.96$, $p < 0.001$) and a positive correlation with Verrucomicrobiota ($r = 0.54$, $p < 0.05$). Proteobacteria was negatively correlated with Verrucomicrobiota ($r = -0.57$, $p < 0.01$) and Ascomycota ($r = -0.46$, $p < 0.05$), but positively correlated with Basidiomycota ($r = 0.52$, $p < 0.05$).

Among fungal phyla, Ascomycota and Basidiomycota exhibited a strong negative correlation ($r = -0.79$, $p < 0.001$). Calcarisporiellomycota was positively correlated with Glomeromycota ($r = 0.56$, p

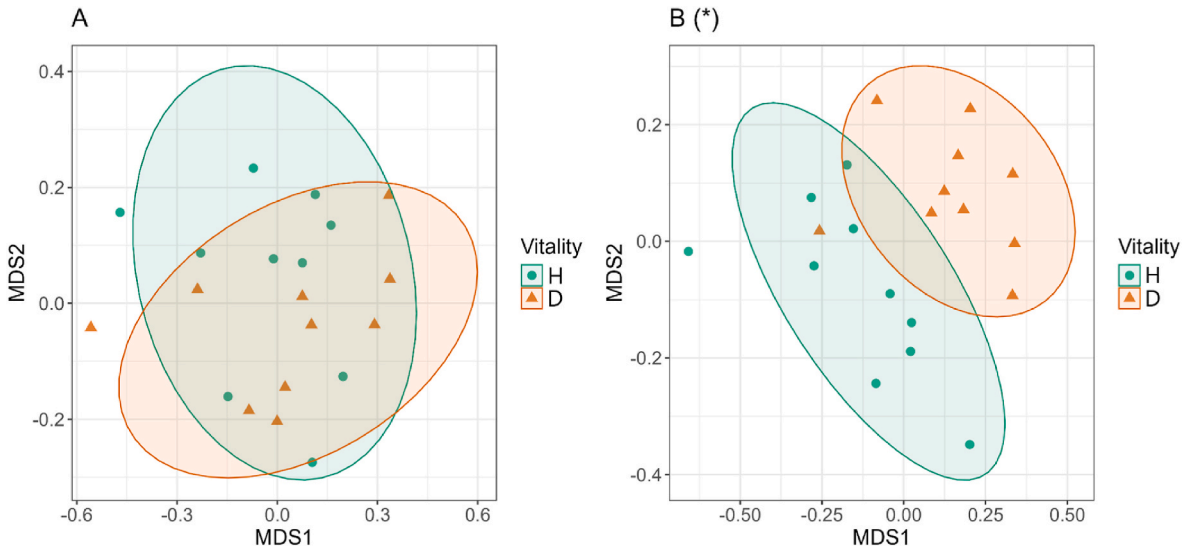

Fig. 6. Beta diversity shown as non-metric Multidimensional Scaling (NMDS) of Bray–Curtis dissimilarity matrices which display the differences in (**A**) bacterial (p = 0.088) and (**B**) fungal (p = 0.001) community composition in rhizosphere soil samples of healthy (H) and declining (D) *Quercus robur*. The axes show the first two coordinates (NMDS1 and NMDS2).

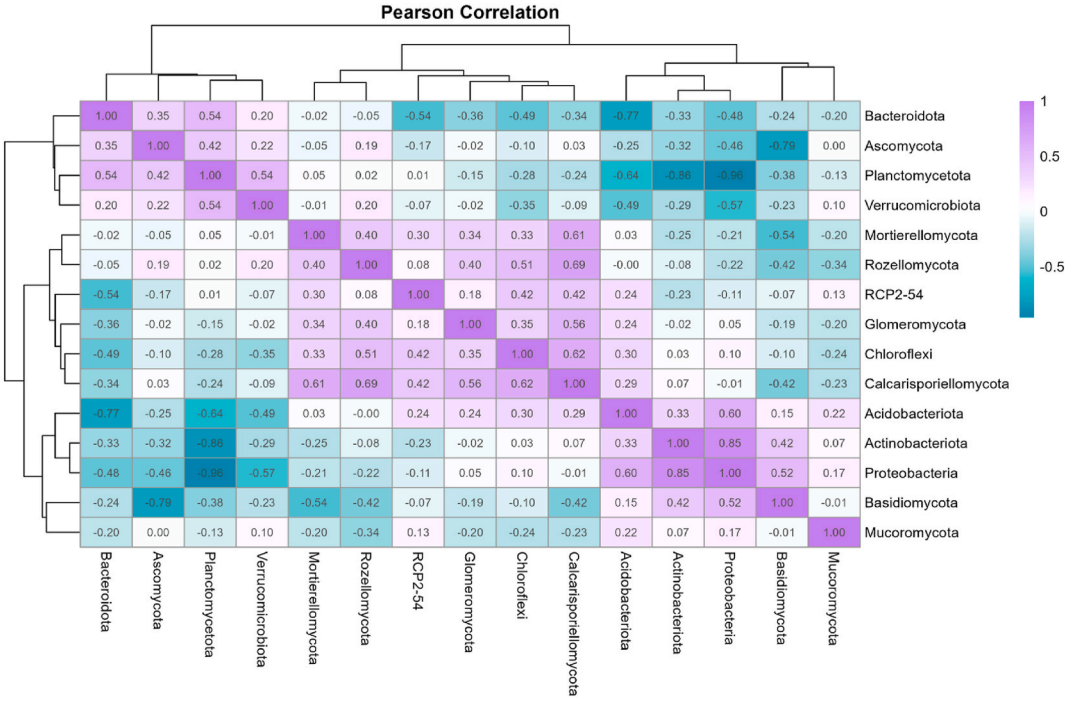

Fig. 7. The Pearson correlation matrix shows the relative abundance of fungal and bacterial phyla in the studied plot, highlighting significant correlations ($p < 0.05$). Positive correlations are indicated in violet, while negative correlations are shown in green. Asterisks denote the significance levels: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

< 0.05), Mortierellomycota (r = 0.61, $p < 0.01$), and Rozellomycota (r = 0.69, $p < 0.001$). Basidiomycota also showed a negative correlation with Mortierellomycota (r = −0.54, $p < 0.05$).

3.3. Baiting and molecular analysis of Phytophthora species isolates

Of the 84 isolates selected for Sanger sequencing, 52 were identified as *Phytophthora* species, including *P. gonapodyides* (H.E. Petersen) Buisman, *P. plurivora* T. Jung & T.I. Burgess, and *P. cactorum* (Lebert & Cohn) J. Schröt. The remaining 29 isolates were identified as *Pythium* spp., and *Phytopythium* spp., including *Globisporangium intermedium* (de Bary) Uzuahshi & Tojo, *Globisporangium irregulare* (Buisman) Uzuhashi, Tojo & Kakish., and *P. litorale* (Nechw.) Abad, de Cock, Bala Robideau, A.M. Lodhi & Lévesque, as well as *Phytopythium citrinum* (B. Paul) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque, and *Phytopythium litorale* (Nechw.) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque. Almost 70 % of the selected isolates were obtained from baits of rhizosphere samples from healthy trees. A table displaying the presence and absence of *Phytophthora* species isolated in the studied samples across different vitality classes is given in the supplementary data (Table S1).

4. Discussion

The primary objective of this study was to assess the rhizosphere microbial community dynamics and taxonomic composition differences between healthy and declining *Quercus robur* trees affected by decline. The significant differences in fungal beta diversity and bacterial alpha diversity between the two vitality groups suggest potential distinctions in soil microbial communities around oaks with different health statuses, though the effect sizes were limited. These results are consistent with previous studies that have reported changes in the soil microbiome in connection to biotic disturbances in forests, with soil microbial communities associated with declining trees diverging from those linked to healthy trees (Pinho et al., 2020; Gómez-Aparicio et al., 2022).

4.1. Alpha and beta–diversity of bacterial and fungal communities in the rhizosphere of healthy and declining Q. robur trees

The observed differences in bacterial alpha diversity within the rhizosphere of healthy and declining *Q. robur* trees underscore the dynamic nature of soil bacterial communities and their spatial variability at the stand level. The presence of dominant ASVs suggests that certain bacterial taxa may have a profound influence on overall community diversity, potentially reflecting their roles in nutrient cycling, organic matter decomposition, or plant-microbe interactions within the rhizosphere. We found that bacterial communities in the soil around healthy trees differed in community structure compared to those around declining trees, as indicated by significant differences in Simpson's indices. However, the lack of significant differences in Chao1 and Shannon indices implies that these changes were not driven by variations in overall species richness or the presence of rare taxa. Similar findings were reported in Chinese Fir plantations, where bacterial dominance shifted, but total species richness remained stable (Zhang et al., 2024). In contrast, fungal communities exhibited no significant differences in alpha diversity across all indices, indicating a relatively stable fungal composition regardless of tree health status. This is consistent with research on Acute oak decline, where fungal diversity remained unchanged despite differences in host health (Pinho et al., 2020). The observed decrease in bacterial diversity and microbial imbalance in this community may be indicative of a shift toward a more specialised microbial community, with saprotrophic bacterial taxa potentially outcompeting other groups due to the presence of decaying roots from *Phytophthora* infections (Ruiz-Gómez et al., 2019; Gómez-Aparicio et al., 2022). This shift could represent a natural ecological response in the rhizosphere of declining oak trees, where saprotrophic bacteria proliferate as a result of increased root necrosis. However, it may also be interpreted as a form of dysbiosis, among the bacterial communities of declining trees, a condition often associated with their increased vulnerability to stress in declining trees (Pinho et al., 2020; Han et al., 2022; Ketehouli et al., 2024). Thus, both the preferential growth of saprotrophic bacteria and the disruption of microbial balance linked to tree stress may contribute to the observed changes in bacterial community composition. Furthermore, our findings suggest that host health status may be shaped by, or shape the bacterial community composition in forest soil, as previously observed by others (Habiyaremye et al., 2021; Hubbard et al., 2023; Liu et al., 2023). This underscores the complex interplay between microbial dynamics and tree health, highlighting the need for further studies that classify bacterial and fungal taxa into functional groups to better understand their ecological roles and interactions.

The beta-diversity analysis revealed significant differences in the fungal, but not in bacterial, rhizosphere communities between healthy and declining *Q. robur* trees. While root exudates can influence microbial composition (Broeckling et al., 2008; Hu et al., 2018; Li et al., 2023), many rhizosphere bacteria and fungi, particularly saprotrophic taxa, rely on the decomposition of organic matter such as leaf litter and dead roots (Marañón-Jiménez et al., 2021). Thus, the microbial differences observed in declining trees could thus reflect not only changes in exudate composition but also shifts in organic matter availability and decomposition dynamics, as supported by findings from previous studies (Marañón-Jiménez et al., 2021; Wang et al., 2025). Unlike saprotrophic fungi, ectomycorrhizal fungi primarily acquire carbon from their host plant rather than from soil organic matter or root exudates (Lebreton et al., 2021), Therefore, the reduced fungal diversity observed in declining trees is likely a consequence of fine root loss rather than direct changes in exudate composition. This finding is consistent with studies showing that oaks affected by *Phytophthora* spp. exhibit significantly reduced fine root length and fewer root tips compared to healthy oak trees (Jung et al., 2000, 2013, 2018b; Vettraino et al., 2002; Balci and Halmschlager, 2003; Pérez-Sierra et al., 2013; Horta-Jung et al., 2025). Since ectomycorrhizal fungi colonize fine roots, their diversity and abundance are expected to decrease in infected trees, as observed in our study. Given the well-documented impact of *Phytophthora* spp. on fine root loss, it is likely that these pathogens contributed to the fungal community shifts observed in declining trees. However, this study did not use a metagenomic approach specifically designed to detect *Phytophthora* spp. and other oomycetes in the soil samples (e.g., Català et al., 2015; Burgess et al., 2021; Riddell et al., 2019). Thus, further studies should be addressed to assess their role in microbial community shifts and oak decline.

4.2. Differential and relative abundance analysis of rhizosphere fungal and bacterial communities

The differential abundance analysis further revealed significant differences in certain bacterial genera between the two vitality classes. Bacteria in the orders Rhizobiales, Subgroup 2, Acidobacteriales, Elsterales, and Gemmatimonadales exhibited higher differential abundance in declining trees, while Chitinophagales, Gemmatales, Sphingobacteriales, and Planctomycetales were more differentially prevalent in healthy trees. Rhizobiales are a diverse order of bacteria that include well-known nitrogen-fixing symbionts (e.g., *Rhizobium, Bradyrhizobium*) as well as some opportunistic plant and animal pathogens (Kohlmeier et al., 2025). However, to our knowledge, no species within this order have been demonstrated to be pathogenic to oak roots. Acidobacteriales, Subgroup 2, Elsterales, and Gemmatimonadales are key players in microbial community structure and nutrient cycling (Conradie and Jacobs, 2020; Kalam et al., 2020; Liu et al., 2022; Mujakić et al., 2022; Huang et al., 2023), potentially influencing nutrient dynamics in declining trees. In contrast, the taxa predominant in healthy trees—Chitinophagales, Gemmatales, Sphingobacteriales, and Planctomycetales—are recognized as indicator taxa in specific plant rhizospheres and are crucial for organic carbon cycling (Nunes da Rocha et al., 2009; Dedysh, 2020; Zhang et al., 2021; Kuang et al., 2023).

While there were variations in the differential abundance of fungal taxa, most differences between healthy and declining trees were not statistically significant (Fig. 5). The only exception was Boletales, a primarily ectomycorrhizal group, which was significantly less differentially abundant in the rhizosphere of declining trees. This aligns with previous studies showing that *Phytophthora* infections reduce fine root length and mycorrhizal colonization, ultimately leading to a decline in mycorrhizal fungi abundance (Jung et al., 2000; Corcobado et al., 2020). The predominance of Basidiomycota in the soil around declining trees suggests a possible association with decline symptoms. Although previous studies have identified Basidiomycota as a dominant fungal division in declining trees, our results did not reveal a statistically significant correlation. However, it is essential to consider the functional diversity within this group. While ectomycorrhizal Basidiomycota—such as those in the Boletales—were significantly less abundant in declining trees, likely due to fine root loss caused by *Phytophthora* infections, other Basidiomycota members, including saprotrophic and opportunistic pathogenic fungi like *Armillaria* and *Collybia*, have been implicated as secondary root pathogens contributing to tree decline (Camy et al.,

2003; Sazonov et al., 2023). Similarly, Ascomycota, another dominant fungal division, likely plays a multifaceted role in the rhizosphere of declining trees. Many Ascomycetes function as saprotrophs, endophytes, or pathogens, and some, such as species within *Neonectria*, have been associated with root diseases in stressed trees (Gomdola et al., 2022). The correlation analysis (Fig. 7) reveals a strong negative relationship between Basidiomycota and Ascomycota, suggesting potential competitive exclusion, distinct ecological niches, or environmental filtering within the soil microbiome. This pattern may be driven by differences in substrate utilization, environmental preferences, or antagonistic interactions between these fungal groups. However, rather than direct competition, these shifts could reflect broader ecological adaptations to environmental conditions, aligning with findings from previous studies (Kutos et al., 2021; Viscarra Rossel et al., 2022).

In the bacterial community, the higher relative abundance of Proteobacteria and Acidobacteriota in declining trees suggests a potential involvement in tree health and disease progression. This pattern is consistent with previous findings identifying these bacterial phyla as dominant taxa in diseased tree rhizospheres, where they may contribute to decomposition, nutrient cycling, and interactions with soil pathogens (Denman et al., 2016; Ding et al., 2021; Gómez-Aparicio et al., 2022). The moderate positive correlation between Proteobacteria and Acidobacteriota (Fig. 7) further indicates potential co-existence or functional synergy within the microbial community. This association may reflect complementary metabolic activities, such as organic matter decomposition, nitrogen cycling, or responses to root exudates from stressed trees (Hassand and Atiqullah, 2024; Wu et al., 2024). However, it remains unclear whether these correlations in bacterial composition directly contribute to disease progression or are secondary responses to altered soil conditions driven by root deterioration and pathogen activity.

Rhizosphere soil samples from declining trees exhibited a greater relative abundance of Basidiomycota and Mortierellomycota compared to healthy *Q. robur*. While the overall abundance of Basidiomycota was only slightly higher (4 %) in declining trees, correlation analysis (Fig. 7) revealed a positive association between Basidiomycota and Proteobacteria, suggesting ecological interactions that may be linked to nutrient cycling and organic matter turnover (Lladó et al., 2017; Kirker et al., 2024; Lasa et al., 2024). Basidiomycota includes functionally diverse taxa, ranging from ectomycorrhizal fungi (e.g., *Amanita, Russula, Laccaria, Hebeloma*)—which are essential for nutrient exchange in forest soils (van der Heijden et al., 2015; Diez-Hermano et al., 2024)—to saprotrophs and secondary pathogens that may colonize declining trees (Sazonov et al., 2023). In our study, *Amanita* and *Hebeloma* were more abundant in healthy trees, whereas *Russula* and *Laccaria* showed similar abundance in both healthy and declining trees (see supplementary data, Fig. S3). The positive correlation between Basidiomycota and Proteobacteria likely reflects their shared role in decomposition processes (Purahong et al., 2016; Lladó et al., 2017; Anderson and Wu, 2024). Similar interactions have been reported in declining forests, where increases in saprotrophic fungi and copiotrophic bacterial groups indicate a shift toward a microbial community adapted to decomposing organic matter (Maillard et al., 2024; Tahovská et al., 2024). Additionally, samples from declining trees also exhibited an increased abundance of saprotrophic and potentially pathogenic fungi, including genera like, *Aspergillus* spp., an important genus within Ascomycota, known for its role in organic matter decomposition and nutrient cycling (Fang and Latgé, 2018; Rokas et al., 2020), (see supplementary data, Fig. S3). Furthermore, the moderate positive correlation between Proteobacteria, Actinobacteriota, and Acidobacteriota suggests potential microbial co-existence or functional redundancy in the rhizosphere of declining trees. These bacterial taxa have been consistently associated with diseased tree rhizospheres, where they contribute to organic matter decomposition, nutrient cycling, and interactions with soil pathogens (Denman et al., 2016; Ding et al., 2021; Gómez-Aparicio et al., 2022; Morales-Rodríguez et al., 2024).

Overall, the observed microbial shifts highlight the complexity of biotic interactions in declining oak trees. The relative increase in Basidiomycota and Proteobacteria suggests microbial responses to environmental stress. Further research using functional assays and soil metabolomics could help clarify whether these changes actively contribute to disease progression or represent adaptive responses to declining tree health.

4.3. Isolation and identification of different Phytophthora species

Using a baiting process with young rhododendron leaves, which are susceptible to many *Phytophthora* species (Junker et al., 2018; Taylor and Grünwald, 2021; Sapkota et al., 2022) we successfully isolated *P. gonapodyides, P. plurivora,* and *P. cactorum*. These species have also been previously identified in declining broadleaved forests of southern Sweden (Jönsson et al., 2003a, 2003b, 2005; Jönsson, 2006; Redondo et al., 2018). Specifically, a survey across 32 oak stands in southern Sweden by Jönsson et al. (2003b, 2003a, 2005) provided the first records of soilborne *Phytophthora* species in these ecosystems, with *Phytophthora quercina*—a fine root pathogen specific to oak—being the most frequently recovered and widely distributed species. However, we acknowledge that our baiting approach was not optimal for detecting *P. quercina*, the primary *Phytophthora* species associated with oak decline in temperate and Mediterranean regions of Europe, including the Skåne region (Jung et al., 1996, 2000, 2013, 2016, 2018b; Vettraino et al., 2002; Balci and Halmschlager, 2003; Pérez-Sierra et al., 2013; Seddaiu et al., 2020; Horta-Jung et al., 2025; Jönsson et al., 2003a, 2003b, 2005; Jönsson, 2006). Given that *P. quercina* does not efficiently colonize rhododendron leaves, its presence in the studied oak stands may have been underestimated. The presence of several plant pathogenic oomycete species, such as *Pythium* and *Phytopythium* species, underscores the potential impact of multiple pathogens on the observed tree decline. Large-scale surveys have further supported the role of multiple pathogens in forest decline worldwide (Corcobado et al., 2020; Horta Jung et al., 2025; Jung, 2009; Jung et al., 2013, 2018b, 2020).

In our study, we found that isolating *Phytophthora* species from baits in the rhizosphere of healthy trees was more successful than in declining trees. This suggests that healthy trees may already be affected by Phytophthora root rot, indicating potential future problems. This observation is consistent with the findings of Tkaczyk et al. (2014) and Jankowiak et al. (2014), who showed that trees with less crown defoliation and higher health status are more likely to harbour *Phytophthora* pathogens in the rhizosphere soil, which might have a detrimental impact on the host plants. Similarly, Jung et al. (2000), showed that oak trees infected with *Phytophthora* spp. experienced significantly greater fine root damage and faced a 280 % and 210 % increased risk of developing severe crown symptoms, respectively, compared to trees without *Phytophthora*. These findings highlight the potential for asymptomatic infections in seemingly healthy trees, reinforcing the importance of early detection and monitoring of *Phytophthora* in forest ecosystems to mitigate long-term impacts on tree health.

5. Concluding remarks

Our findings highlight the rich taxonomic diversity in forest soil microbiomes and their spatial heterogeneity at forest stand level. Overall, our results emphasize the connection between the soil microbiome and tree health, underlining the importance of considering rhizosphere microbial communities in the context of tree health status and disease symptoms. Future research should focus on elucidating the functional roles of these microbial communities and their potential implications for the management of dieback disease in *Q. robur* forests.

CRediT authorship contribution statement

Noelia López-García: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Carmen Romeralo:**

Writing – review & editing, Methodology, Conceptualization. **Christian B. Andersen:** Writing – review & editing, Methodology, Formal analysis. **Jonas Rönnberg:** Writing – review & editing, Supervision. **Laura J. Grenville-Briggs:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Johanna Witzell:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2025.101070.

Data availability

I have shared my data at the attach file step.

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