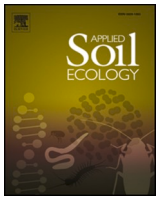
Applied Soil Ecology 185 (2023) 104790

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



# Soil microbiota impact on *Boletus edulis* mycelium in chestnut orchards of different ages

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ARTICLE INFO

*Keywords:*
Bacterial community
*Boletus edulis*
*Castanea* × *coudercii*
Fungal community
DNA metabarcoding
qPCR

ABSTRACT

Biotic factors (host plant age and soil microbiota) influencing *Boletus edulis* mycelium frequency and concentration have been investigated. The study was carried out in September, October and November 2018 and 2020, using six orchards of chestnut hybrid *Castanea* × *coudercii* of 40-, 10- and 3-years-old. Taxonomical identity of bacterial and fungal species was ascertained by DNA metabarcoding. Mycelium frequency and concentration of *B. edulis* were studied through quantitative PCR (qPCR) of soil samples. It was hypothesized that mature plants (40-years-old) have a greater soil microbiota associated at the rhizosphere, in comparison with young plants (10- and 3-years-old). This situation may reverberate on *B. edulis* mycelium that should be more abundant in 40-years-old orchards, also thanks to the higher number of beneficial interactions whit soil microbiota. It was found that host plant age significantly altered bacterial and fungal assemblages in both years. Bacterial fraction was dominated by the phylum *Proteobacteria*, *Acidobacteria* and *Actinobacteria* and showed a higher species richness and diversity in orchards of 10- and 3-years-old. Fungal phylum such as Basidiomycota was more abundant in 40- and 10-years-old orchards, whereas Ascomycota and Mortierellomycota were more abundant in the 10- and 3-years-old orchards. Mycelium frequency of *B. edulis* in soil samples was higher in 2020 (97 %) than in 2018 (19 %), although without differences between plant age and sampling month in each year. *Boletus edulis* mycelium concentration was higher in 2020 than in 2018, and in September 2020, whereas plant age did not have any effect. Of 173 bacterial taxa considered, the 12 %, 54 % and 82 % of the significant correlations with *B. edulis* mycelium concentration were found in 40-, 10- and 3-years-old orchards, respectively. On the other hand, of 180 fungal taxa, the 12 %, 24 % and 42 % of the significant correlations occurred in 40-, 10- and 3-years-old orchards, respectively. This investigation represents the first report about the role of soil microbiota on the ecology of *B. edulis* in *Castanea* agro-ecosystem.

## 1. Introduction

In Spain, the chestnut, *Castanea sativa* Mill. (Fagaceae), occupy 276,358 ha, being the dominant species in 154,500 ha (MAPAMA, 2013). Galicia (NW Spain) is the second main producing area in the country, and both forest stands and cultivated orchards cover a total surface area of 69,327 ha (MAPAMA, 2013). Chestnut trees establish ectomycorrhizal associations with a wide macro-fungal community, usually dominated by the families Amanitaceae, Boletaceae, and Cortinariaceae, being the genera *Amanita*, *Cortinarius*, *Inocybe*, *Lactarius*, *Russula*, and *Tricholoma*, the prevalent taxa above-ground (Baptista et al., 2010; Martins et al., 2011; Álvarez-La Fuente, 2015; Baptista et al., 2015). Among Boletaceae, the members of the *Boletus edulis* complex (*B. edulis* Bull.: Fr. sensu stricto, *B. aereus* Bull.: Fr., *B. pinophilus* Pilat et Dermek, and *B. reticulatus* Schaeff.) are indeed the most appreciated mushrooms (Hall et al., 1998; Sitta and Davoli, 2012). Despite its ecological and economic importance, autoecology and interspecific competition of the *B. edulis* complex have been rarely investigated, and field study have been carried out only in Italy (Meotto et al., 1999; Salerni and Perini, 2004; Peintner et al., 2007; Ambrosio and Zotti, 2015) and Spain (Alonso Ponce et al., 2011; Martínez-Peña et al., 2012; De la Varga et al., 2013; Mediavilla et al., 2017; Parladé et al., 2017; Mediavilla et al., 2019). The existence of significant correlations between *B. edulis* mycelium abundance and sporocarp production has been

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https://doi.org/10.1016/j.apsoil.2022.104790
Received 19 May 2022; Received in revised form 26 November 2022; Accepted 19 December 2022
Available online 7 January 2023

studied by Peintner et al. (2007), and De la Varga et al. (2012, 2013), as well as the impact of forest management on mycelium and sporocarp yields (Mediavilla et al., 2017; Parladé et al., 2017). The health and age of the host plants seems to also be a crucial factor for *Boletus* species development (Keizer and Arnolds, 1993; Eberhart et al., 1996). However, Peintner et al. (2007) suggested that *Boletus* biology possibly also depends on other fungal or bacterial species in the soil. Only three studies have analyzed the microbial community associated with *B. edulis* by using DNA metabarcoding: Mediavilla et al. (2019) investigated plant-fungus-bacteria symbiosis involved in *Cistus ladanifer-Boletus edulis* systems; Qin et al. (2016) studied bacterial diversity of surrounding soil of *B. edulis* mycelium; and Koskinen et al. (2019) investigated the communities of bacteria, fungi and arthropods in *Picea* stands.

A greater understanding of bacterial communities in forest soils is essential also because they are a crucial component of the tri-trophic interaction plant–fungi–bacteria (Bonfante and Anca, 2009). Of particular interest are those bacteria involved in the mycorrhizal symbiosis, referred as "mycorrhiza helper bacteria" (MHB), who interact with fungi reducing mycelium stress, stimulating its growth, and increasing the contact points between roots and fungi (Garbaye, 1994; Frey-Klett et al., 2007; Deveau and Labbé, 2016). These biotic interactions are not casual and may be essential to ensure a stable infection of ectomycorrhizal mushrooms on the host plant. The MHB strains that have been identified to date belong to gram-negative *Proteobacteria* (*Agrobacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas*, *Klebsiella* and *Rhizobium*), gram-positive *Firmicutes* (*Bacillus*, *Brevibacillus*, and *Paenibacillus*) and gram-positive *Actinomycetes* (*Arthrobacter*, *Rhodococcus*, and *Streptomyces*) (Frey-Klett et al., 2007). Literature offers several examples of MHB strains isolated from both ectomycorrhizal and arbuscular mycorrhizal fungi, under different environmental conditions, whose reported effects ranged from increasing of mycorrhizal rate and mycorrhizal infection to promoting lateral root development (see Frey-Klett et al., 2007 for a review). On the other hand, bacterial genera such as *Conexibacter*, *Ferruginibacter*, *Gemmatimonas*, *Opitutus*, *Mucilaginibacter*, *Stella* and *Terriglobus* were considered to be significant indicators of highly productive site of *B. edulis* sporocarps (Mediavilla et al., 2019). At present interesting results have been obtained in vitro with *Laccaria bicolor*, whose growth was significantly promoted by *Paenibacillus* sp. isolates (Deveau et al., 2007), and with *Lactarius rufus* inoculated with *Burkholderia* sp. (Poole et al., 2001) and *Paenibacillus* sp. (Aspray et al., 2013). *Boletus edulis* mycorrhization was promoted by *Pseudomonas fluorescens* on *Cistus ladanifer* (Mediavilla et al., 2016), and by *Bacillus cereus* on *Pinus thunbergii* (Wu et al., 2012).

Many abiotic factors shape microbial community, but especially soil pH, C:N ratio and water content have been shown to be the most important (Marschner et al., 2001; Wu et al., 2013; Li et al., 2016; Liu et al., 2018). However, in forest ecosystems, season changes (Du et al., 2018), forest disturbance (Holden and Treseder, 2013), soil type and plant species (Marschner et al., 2001; Kuske et al., 2002) may significantly alter the circulation of soil nutrient and consequently the microbiota assemblages in the rhizosphere. In addition, it has been reported that plant age significantly impacts soil microbial communities in *Pinus tabuliformis* (Dang et al., 2017), *Robinia pseudoacacia* (Liu et al., 2018), *Eucalyptus* spp. (Wu et al., 2013; Qu et al., 2020) *Juglans regia* (Gao et al., 2019), and *Pinus elliottii* (Wu et al., 2015) plantations, being higher the abundance of microbial biomass with increasing plant age. This effect could depend by the increase of canopy density that may affect photosynthesis rate, rhizosphere exudates, nutrient balance, leaf litter accumulation and soil water content (Badri and Vivanco, 2009; Liu et al., 2018; Mushinski et al., 2019).

To investigate the biotic factors that influenced frequency and concentration of *B. edulis* mycelium, the DNA metabarcoding approach to describe soil microbiota in chestnut orchards of different ages, was used. It was hypothesized that mature plants (40-years-old) have a greater soil microbiota associated at the rhizosphere, in comparison with young plants (10- and 3-years-old). Consequently, it was predicted that *B. edulis* mycelium should be more frequently found and should have a higher concentration in mature orchards, because of the higher number of beneficial interactions with bacterial and fungal taxa. The study was performed in 2018 and replicated in 2020, to assess whether bacterial and fungal assemblages in each orchard have the same characteristics along the time, and whether *B. edulis* mycelium concentration was significantly correlated with the same microbial taxa.

As far as we know, this is the first time that the tri-trophic interactions plant-fungi-bacteria in a *Castanea* agro-ecosystem are investigated to disentangle their role in *B. edulis* mycelium development.

## 2. Material and methods

### 2.1. Study site and experimental orchards

The study was carried out in Galicia (NW Spain), which is the second most important Spanish forestry region (MAPAMA, 2013). It is characterized by an Atlantic humid climate without long frost periods and with a uniformly distributed annual precipitation (Martínez-Cortizas and Pérez-Alberti, 2000). The average annual temperature ranges between 8 and 14 °C. Annual rainfall ranges from 600 to 2500 mm, with some sites over 3000 mm per year. Galicia exhibits a wide geological diversity where granites (about 45 %) and low-grade metamorphic rocks, like slates and schists prevail. These two types of parent material are associated with soils of sandy-loam textures overlying granites and loamy or silty-loam soils overlying slates and schists. The high precipitation of the region results in markedly acidic soils, containing abundant organic matter and low levels of nutrients (Rodríguez-Alleres et al., 2012). The soils are typically classified as Leptosols, Umbrisols, Cambisols and Regosols (IUSS-WRB, 2007, Carballas et al., 2016). The experimental chestnut orchards are located at Bora, Pontevedra province (42° 25′ 56.5″ N–8° 34′ 41″ W) and belongs to Hifas Foresta company. The orchards are in the same area (from 200 to 800 m apart from each other) and thus all share identical climate conditions. They are surrounded by pastures, fruit orchards, mature chestnuts, and oak trees (*Quercus robur*).

To characterize the soils of the experimental orchards, samplings were carried out in October 2018 and September 2020. For each experimental orchard, twelve soil samples 5 × 5 cm were taken in the first horizon (0–5 cm deep) with a soil corer, and another twelve soil samples at 20 cm deep. Chemical parameters (pH, organic C, available soil N, C:N, Ca, K, Mg, Na, Al) were assessed at the Plant Biology and Soil Sciences Department of the University of Vigo. Soil properties were analyzed following the methods described by Guitian and Carballas (1976). Results of chemical soil parameters are summarized in Table S1.

### 2.2. Experimental design and soil sampling for molecular study

In 2018, three orchards of the chestnut hybrid *Castanea* × *coudercii* (*C. sativa* × *C. crenata*) of three age classes (40-, 10- and 3 years-old), with two repetition each, were designed as A40, A10 and A3. In 2020, the study was replicated in the same experimental area (in this year the plots were named B40, B10 and B3, respectively), to assess the existence of changes of microbiota patterns.

Soil samples were taken in mid-September, mid-October and mid-November of 2018 and 2020. Every month, five soil samples were extracted per orchard using a 250 $cm^3$ soil extractor (3.5 cm diameter and 20 cm deep), for a total of 90 samples (6 experimental plots × 3 sampling months × 5 soil samples). Samples were taken next to the angles and in the center of the square plots with a maximum distance of 30 cm apart from the tree trunk, according to De la Varga et al. (2012) procedure. Soil samples were individually introduced in marked plastics bags and transported to the laboratory, where they were stored at 4 °C until processed.

### 2.3. Metabarcoding analysis for bacterial and fungal identification

Soil samples from 2018 (N = 90) and 2020 (N = 90) surveys were individually processed. Soil DNA extractions were carried out with the DNeasy® PowerSoil® Kit (QIAGEN Group), from 0.25 g of soil (fresh weight) per sample according to the manufacturer's instruction. The extracted DNA was stored at −20 °C until used. The DNA samples were used for assessing bacterial and fungal community through DNA metabarcoding by Illumina platform. To this end, the samples were sent to Macrogen laboratory (Macrogen Inc., Seoul, Korea, www.macrogen.com). For each experimental plot and sampling date, a bulk of 25 μl (5 μl of DNA from each of five soil samples) was used. The sequencing libraries were prepared according to the Illumina 16S Metagenomic.

Sequencing Library protocols to amplify the V3 and V4 region of bacterial 16S rDNA, and the internal transcriber spacer ITS2, who lies between the 5.8S and the 28S genes of fungal rDNA. The genomic DNA (2 ng) was PCR amplified with 5× reaction buffer, 1 mM of dNTP mix, 500 nM each of the universal PCR primers, and Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA). The cycle condition for the first PCR was 3 min at 95 °C for heat activation, and 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, followed by a 5-min final extension at 72 °C.

The universal primer pair with Illumina adapter overhang sequences used for the first amplifications were as follows: Bakt-341F (CCTACGGGNGGCWGCAG) and Bakt-805R (GACTACHVGGGTATCTAATCC) for V3-V4 region, and ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) for ITS2 of fungal rDNA.

The first PCR product was purified with AMPure beads (Agencourt Bioscience, Beverly, MA). Following purification, 2 μl of first PCR product was PCR amplified for final library construction containing the index using NexteraXT Indexed Primer. The cycle condition for second PCR was same as the first PCR condition except for 10 cycles. The PCR product was purified with AMPure beads. The final purified product was then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantificatoin kits for Illumina Sequecing platforms) and qualified using the Tape Station D1000 Screen Tape (Agilent Technologies, Waldbronn, Germany). Finally, they were sequenced using the MiSeq™ platform (Illumina, San Diego, USA).

Bioinformatic analyses was performed by Macrogen laboratory. The clean-up of the raw sequences was performed by using the fastp program (a FASTQ data pre-processing tool) (Chen et al., 2018), adapter sequences were removed and error-correction was performed for areas where the two reads overlap. By assembling paired-end sequences created by sequencing both directions of library, the original library and single long reads were obtained by using FLASH (v.1.2.11) (Magoč and Salzberg, 2011). For precise Operational Taxonomic Unit (OTU) analysis, data containing sequence error, ambiguous base, and chimeric sequence were removed. After this process, clustering was performed based on sequence similarity. OTUs of the remained reads were created by cluster cut-off value of 97 %, by using CD-HIT-OTU program (Li et al., 2012). For bacterial species, among the assembled reads, reads shorter than 400 bp or longer than 500 bp were removed. QIIME (v1.9.0) (Caporaso et al., 2010) was used for OTU analysis and obtaining taxonomy information. Bacterial OTU taxonomic identity was ascertained by using NCBI 16S Microbial database with BLASTN (v2.9.0) algorithm. For fungal species, among the assembled reads, reads shorter than 300 bp or longer than 500 bp were removed. For taxonomic identity, the UNITE Fungi database and UCLUST (v.1.2.22) algorithm have been used.

### 2.4. Molecular detection and quantification of Boletus edulis mycelium

The frequency (i.e. the number of samples that resulted positive for mycelium presence/total samples per plot and sampling month in each year), and the concentration (mg mycelium/g soil) of extraradical soil mycelium of *B. edulis* of the 180 soil samples were both assessed by qPCR.

To this end, all the DNA samples were shipped to the AllGenetics laboratories (AllGenetics & Biology SL, www.allgenetics.eu). Mycelium quantification was performed targeting the ITS genomic region using the primers FWD-Bedu (CTGTCGCCGGCAACGT) and RVS-Bedu (TGCACAGGTGGATAAGGAAACTAG), and TaqMan® probe STQBedu (6FAM-CCCTTTCTCTTTCGTGGAACCTCCCC-BHQ1) designed by De la Varga et al. (2012). The dye 6-carboxy-fluorescein (6-FAM) and the Black Hole Quencher (BHQ1) were attached to the primers' 5′ and 3′ ends, respectively. The qPCRs were carried out in a final volume of 20 μL, containing 10 μL of NZY qPCR Probe Master Mix ROX plus (NZYTech), 0.25 μM of the probe, 0.9 μM of the amplification primers, 2 μL of template DNA, and ultrapure water up to 20 μL. The reaction mixture was incubated as follows: an initial incubation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 1 min, extension at 60 °C for 1 min; and a final extension step at 60 °C for 30 s. Negative qPCR controls that contained no DNA were included to check for cross-contamination. A total of 9 plates were analyzed in this study, with the qPCR reactions performed in triplicate on each sample and controls. Five-fold dilution series (performed in triplicate) of known ITS copy numbers from identified *B. edulis* sporocarp samples ranging from 5.08E-02 ng to 5.08E-06 ng, to establish the standard curve and to assess the reaction efficiency, were carried out. A total of nine standard curves were generated, one for each plate. StepOne (Applied Biosystems) to register the intensity of the fluorescence emitted by the probe at each cycle of the qPCR reaction for each sample and to estimate their Cq value (Quantification cycle) was used.

### 2.5. Soil microbiota diversity

To describe soil microbiota composition in the experimental orchards, three diversity parameters were adopted. Bacterial and fungal species richness was expressed as the number of the observed OTUs. Chao1 estimates the potential richness based on the relationship of singleton observations (species represented by only one individual) to the observed richness. Shannon-Wiener (H′) index was used to compare bacterial and fungal diversity between the soil samples. The Shannon-Wiener index increases when the number of taxa increase, or the distribution of the species becomes more even. These parameters were calculated by using the software EstimateS 9.1.0 (Colwell, 2006). Alpha rarefaction curve, based on Chao 1 index, shows whether the number of reads used in the analysis was sufficient in identifying OTUs.

### 2.6. Data analyses

The impact of the experimental orchards with different age (plots), the sampling years (2018 and 2020), the sampling month (September, October, November), and the interaction between them on OTU abundance (species richness), Chao 1 and Shannon-Wiener diversity index was assessed by using two-way ANOVA. Count data were log (x + 1) transformed to meet ANOVA assumption. Pairwise comparisons were done with Least Significant Difference (LSD) Fisher's test. Significance level was declared at $\alpha = 0.05$.

Changing of mycelium frequency and concentration between plots, years and sampling month was calculated by two-way ANOVA. Mycelium data were log (x + 1) transformed to assume equal variance conditions.

Correlations between mycelium concentration and bacterial and fungal taxa depending on the plot have been assessed by Spearman correlations analysis. These analyses were performed with GenStat release 10.2 (VSN International, Hemel Hempstead, UK).

To test for differences in bacterial and fungal species composition between the plots and to estimate the components of variation, we used the permutational multivariate analysis of variance (PERMANOVA), with the number of OTUs per group as the dependent variable. Data were square-root transformed prior de analysis. The plot, the sampling

month and the interaction between them were used as fixed factors. PERMANOVA analyses were based on Bray–Curtis similarities. The analysis was performed using 9999 permutations, with permutation of residuals under a reduced model, fixed effects sum set to zero and type III sums of squares.

The submodule SIMPER (Percentage Similarity Analysis) was also run to examines the contribution of each taxon to average resemblances between groups. For Bray-Curtis similarities it determines the contributions to the average Bray-Curtis dissimilarity between pairs of groups and within a group. Two factors (plot and sampling month) were used. Only similarities and dissimilarities between samples within the same level of the second factor (sampling month) were considered. The cumulative percentage cut-off point after which rarer species are ignored was 70 %.

To compare bacterial and fungal community composition across the experimental plots, we used non-metric multidimensional scaling (NMDS) analysis, after Hellinger standardization of the OTU table. Then, we performed an analysis of similarities (ANOSIM) with a 2-way crossed layout for bacterial and fungal communities in 2018 and 2020, using transformed data, with 9999 permutations, to test for difference between plots and between sampling date. The ANOSIM test is a comparative measure of the degree of dissimilarity between groups, calculated from resemblances. R value is a ratio of the between groups variation to the within group variation. R = 1 implies that all replicates within groups are more similar to each other than between groups, while R ≅ 0 implies little or no differences within and between the groups (Somerfield et al., 2021). Significance was declared at α = 0.05. The software PRIMER 7.0.17 with the PERMANOVA + 1 add was used for these analyses.

## 3. Results

### 3.1. Bacterial community

In both years, at phylum level, the 99.8 % of the 16S rRNA reads belonged to *Bacteria*, with the remaining 0.2 % belonging to *Archaea*. All the OTUs have been identified at species level. In 2018, a total of 825,673 bacterial reads sorted into 20 phyla, 47 classes, 105 orders, 207 families, 552 genera, and 995 species, has been generated. In plot A40, 8672 OTUs were identified, 10,909 OTUs in plot A10, and 10,636 OTUs in plot A3. In 2020, 855,838 bacterial reads were obtained. The identified OTUs were sorted among 21 phyla, 51 classes, 112 orders, 217 families, 585genera and 1034 species. In plot B40, 9235 OTUs were determined, 11,135OTUs in plot B10, and 11,166 OTUs in plot B3. Total

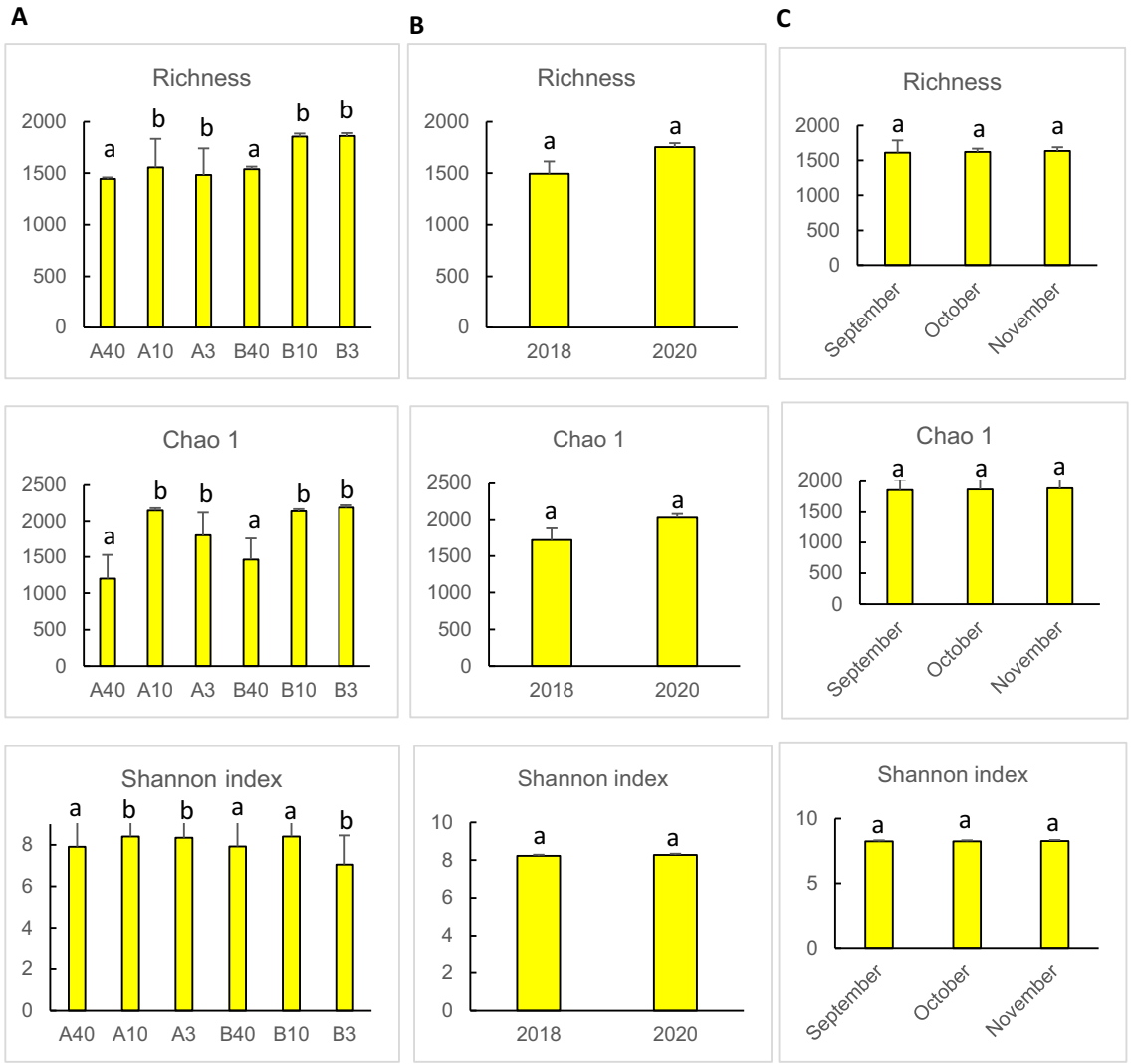



**Fig. 1.** A-B-C. Mean (±SD) of species richness, Chao 1, and Shannon-Wiener (H′) diversity index relative to bacterial taxa sorted per plot (A), year (B), and sampling month (C), are shown. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. Columns marked with different lowercase letters were significantly different (α = 0.05) by one-way ANOVA.

read bases, read count, OTU number (species richness) and ecological parameters per plot of both years are shown in Table S2. Rarefaction analysis of the OTUs shown in Fig. S1, indicates that bacterial sequence abundance was higher for plots A10 and A3 in 2018, and for plots B10 and B3 in 2020. In both years, the curve becomes flatter to the right, which indicates that a reasonable number of reads have been used in the analysis, thus additional sequencing was not necessary.

In 2018, ANOVA analysis of the impact of plant age (plot) on the three diversity parameters showed a significant difference ($\alpha = 0.05$) between the plots A40/A10 and A40/A3, but were not significant between the plots A10/A3 (species richness: $P = 0.267$; Chao 1: $P = 0.165$; Shannon-Wiener index: $P = 0.384$) (Fig. 1). In 2020, again, significant differences were found between plots B40/B10, with the exception of Shannon-Wiener index ($P = 0.177$). All the parameters showed significant differences between the plots B40/B3, although between the plots B10/B3 only Shannon-Wiener index was significant, whereas the other two parameters did not (species richness: $P = 0.391$; Chao 1: $P = 0.421$) (Fig. 1). On the other hand, the year (Fig. 1B), and the sampling month (Fig. 1C) have not any effect on species richness, Chao 1 and Shannon-Wiener index. The interactions plot/year, plot/sampling month and year/sampling month for the three diversity parameters were also not significant (data not shown).

In 2018, the general taxonomic composition assessment revealed the dominance of the phylum *Proteobacteria* (33 % of the total OTUs), followed by *Acidobacteria* (28 %), *Actinobacteria* (10 %), *Firmicutes* (7 %), *Verrucomicrobia* (7 %), *Choloflexi* (4 %) and *Bacteroidetes* (4 %). The other phyla had an abundance lower than 4 %. Similarly, in 2020, bacterial community was composed by *Proteobacteria* (33 %), *Acidobacteria* (30 %), *Actinobacteria* (10 %), *Firmicutes* (7 %) *Verrucomicrobia* (6 %), and *Chloroflexi* (4 %). OTU mean sequence abundance of the phylum *Proteobacteria* did not vary depending on the plots neither in 2018 ($P = 0.958$) (Fig. 2A), nor in 2020 ($P = 0.110$) (Fig. 2B). The main species representing *Proteobacteria* was *Acidibacter ferrireducens*, who had the12% of the total sequences in 2018, and the13% in 2020, although it was equally sorted among the plots in both 2018 ($P = 0.166$) (Fig. S2), and 2020 ($P = 0.392$) (Fig. 3B). For *Acidobacteria* phylum, mean sequence abundance was affected by the plots in both 2018 ($P < 0.001$) (Fig. 2A). and 2020 ($P = 0.046$) (Fig. 2B). Inside this phylum, in 2018 *Acidobacterium ailaaui* (34 % of the total OTUs) and *Paludibaculum fermentans* (25 %), were found in all the plots and had a significant

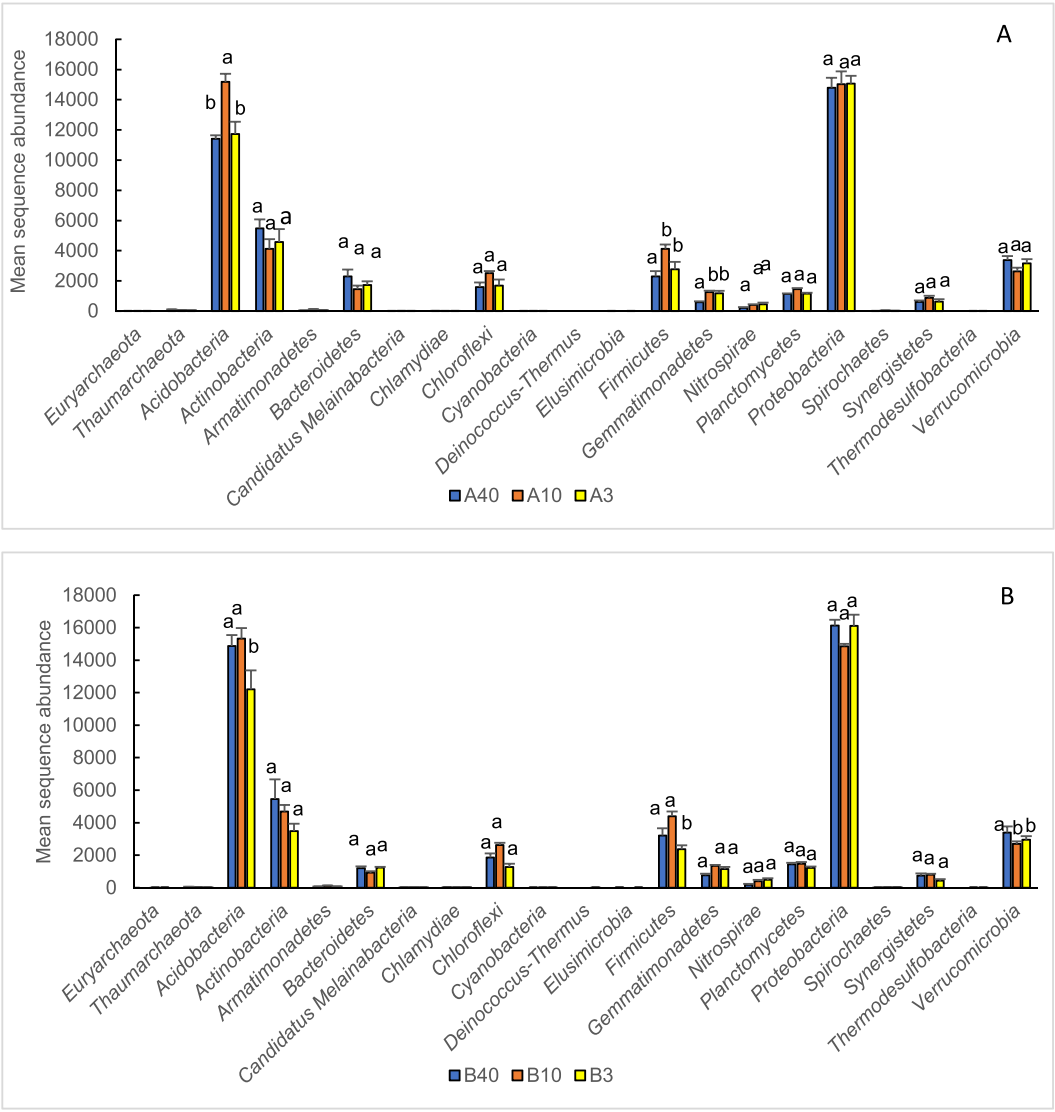
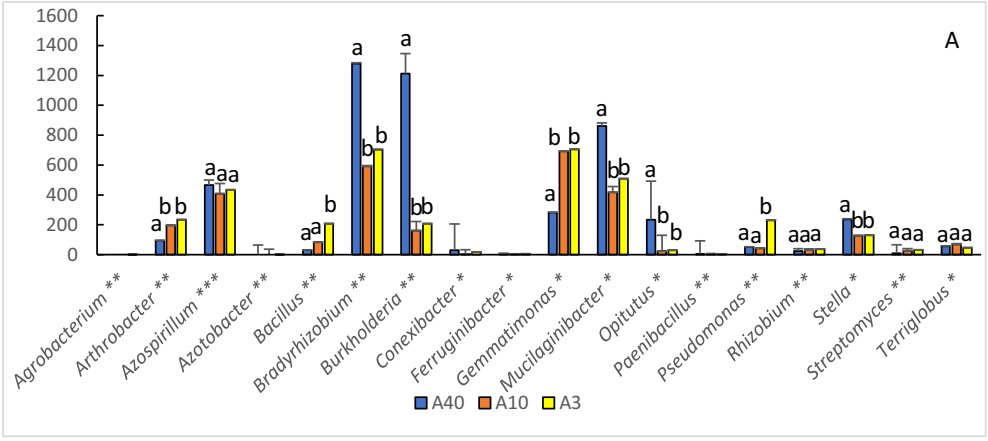



**Fig. 2.** A-B. Classification of bacterial mean sequence abundance (± SD) at the phylum level, sorted between the experimental orchards sampled in 2018 (A) and 2020 (B). Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. Columns marked with different lowercase letters were significantly different ($\alpha = 0.05$) between the experimental plots by one-way ANOVA.



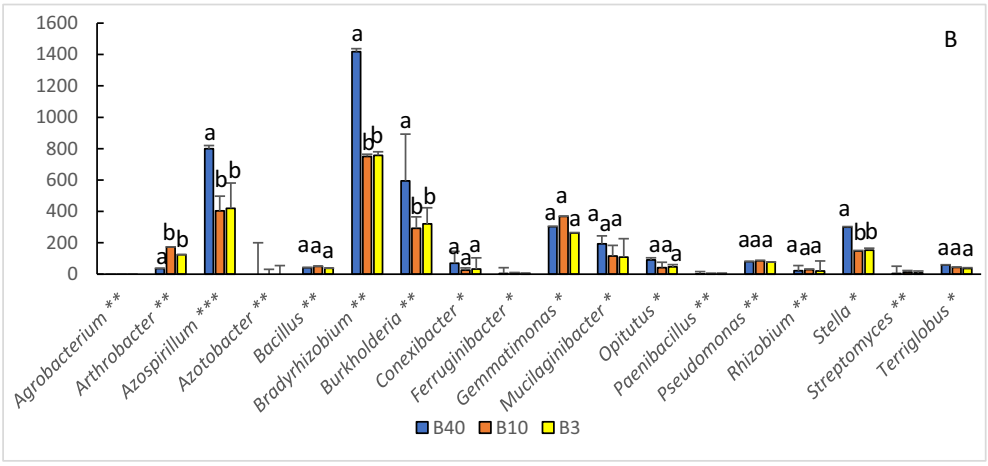




**Fig. 3.** Mean sequence abundance (± SD) of bacterial taxa considered indicators of productive sites of *B. edulis* sporocarps (marked with *) by Mediavilla et al., 2019, and of mycorrhizal helper bacteria (marked with **) by Frey-Klett et al., 2007, sampled in 2018 (A) and 2020 (B). The genus *Azospirillum* (marked with ***) has been cited by both authors. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. Columns marked with different lowercase letters were significantly different ($\alpha = 0.05$) between the experimental plots by one-way ANOVA.

higher representation in plot A10 (*A. ailaaui*: $P < 0.0001$; *P. fermentans*: $P = 0.049$) (Fig. S2-A). In 2020, relative sequence abundance of *Acidobacterium ailaaui* (34 %) did not change across the plots ($P = 0.072$), whereas *Paludibaculum fermentans* (26 %) increased in plot B40 ($P = 0.011$), (Fig. S2-B). For *Actinobacteria* phylum, mean sequence abundance did not change with the plots neither in 2018 ($P = 0.399$) (Fig. 2A) nor in 2020 ($P = 0.367$) (Fig. 2B), and the same occurred for its most abundant taxon *Aciditerrimonas ferrireducens* in 2018 ($P = 0.084$) (Fig. 3A), and 2020 ($P = 0.448$) (Fig. S2-B).

The mean sequence abundance of seven bacterial taxa considered indicators of *B. edulis* productive sites, and eleven taxa known as MBH is shown in Fig. 3A–B. The genera *Bradyrizhobium*, *Burkholderia*, *Mucilaginibacter* and *Stella* were significantly more abundant in both plots A40 and B40, whereas *Mucilaginibacter* and *Opitutus* only in plot A40, and *Azospirillum* only in plot B40. *Arthrobacter* was the dominant taxon in plots A10 and A3, and B10 and B3. The abundance of the genera *Gemmatimonas* and *Pseudomonas* was significantly higher only in plots A10 and A3.

PERMANOVA analysis detected the existence of significant difference of the bacterial community structure between plots A40/A10 and A40/A3 in 2018, and between all the plots in 2020 (Table 1). The interaction between the experimental orchard (plot) and sampling month (time) had no effect, whereas the sampling month (September) had a significant effect on bacterial assemblages when comparing plots A40/A10 and B40/B10 (Table 1).

In 2018, SIMPER analysis showed that the major contribution to dissimilarity between plots A40/A10 and A40/A3 was due in both cases to *Burkholderia pseudomultivorans*, and by *Rhodanobacter glycinis* between plots A10/A3 (Table 2). In 2020, *Sphingomonas limnosediminicola* accounted for dissimilarity between plots B40/B10, and B40/B3, and by *Massilia eurypsychrophila* between plots B10/B3 (Table 2).

The NMDS analysis resulted in a two-dimensional ordination of the samples with a final stress value of 0.07 for the sampling done in 2018 (Fig. 4A), and 0.08 for the sampling in 2020 (Fig. 4B). The ordination plot for both years showed a well separated assemblages of the bacterial community according to the experimental orchards. The 2-way ANOSIM, however, confirmed these separations only between plots A40/A10 ($R = 1$, $P = 0.037$), A40/A3 ($R = 0.667$, $P = 0.037$), sampled in 2018, and between plots B40/B10 ($R = 1$, $P = 0.037$), B40/B3 ($R = 0.917$, $P = 0.037$) and B10/B3 ($R = 0.337$, $P = 0.037$), sampled in 2020.

### 3.2. Fungal community

In 2018, a total of 1,710,316 reads have been obtained, of which only 3508 referred to unidentified taxa. The OTUs were sorted among 9 phyla, 32 classes, 79 orders, 180 families, 258 genera and 148 species. In plot A40, 1453 OTUs were identified, 1473 in plot A10, and 1718 plot A3. In 2020, 1,659,342 reads were obtained, of which 2224 read remained unidentified. The identified OTUs were sorted among 9 phyla, 33 classes, 77 orders, 182 families, 250 genera and 135 species. In plot B40, 1243 OTUs were identified, 1422 in plot B10, and 1517 in plot B3. Total base number, raw read count, OTU number (species richness) and ecological parameters per plot are shown in Table S3. Rarefaction analysis of the OTUs shown in Fig. S3, indicates that there is a higher diversity for plots A10 and A3, and for plots B10 and B3.

In 2018, only Shannon-Wiener index significantly differs ($\alpha = 0.05$) when comparing plots A40/A10 and A40/A3, whereas the other ecological parameters had no effects (species richness: $P = 0.090$; Chao 1: $P = 0.168$) (Fig. 6A). Similarly, in 2020, significant difference was found only for Shannon-Wiener index between plots B40/B3 and B10/

**Table 1**
Results of PERMANOVA analysis to test for significant changes of bacterial and fungal community structure between the experimental orchards in 2018 and 2020. Plant age (plot), sampling month (time) and the interaction between them were used as fixed factors. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. Significance was declared at $\alpha = 0.05$.

| Bacteria 2018 | | | | | Fungi 2018 | | | |
|---|---|---|---|---|---|---|---|---|
| A40/A10 | SS | MS | Pseudo-F | P (perm) | SS | MS | Pseudo-F | P (perm) |
| Plot | 1702.4 | 1702.4 | 13.102 | 0.0025 | 9775.1 | 9775.1 | 12.376 | 0.006 |
| Time | 622.83 | 311.41 | 2.3968 | 0.015 | 1990.7 | 995.34 | 1.2602 | 0.209 |
| Plot × time | 213.72 | 106.86 | 0.82245 | 0.6723 | 1345.2 | 672.62 | 0.85161 | 0.711 |
| Residual | 779.57 | 129.93 | | | 4738.9 | 789.82 | | |
| Total | 3318.5 | | | | 17,850 | | | |
| A10/A3 | SS | MS | Pseudo-F | P (perm) | SS | MS | Pseudo-F | P (perm) |
| Plot | 575.73 | 575.73 | 2.3754 | 0.1297 | 2482.3 | 2482.3 | 2.6717 | 0.0354 |
| Time | 558.4 | 279.2 | 1.1519 | 0.3565 | 1402.5 | 701.26 | 0.75476 | 0.714 |
| Plot × time | 258.63 | 129.32 | 0.53354 | 0.8272 | 1156.2 | 578.09 | 0.62219 | 0.8453 |
| Residual | 1454.2 | 242.37 | | | 5574.7 | 929.11 | | |
| Total | 2847 | | | | 10,616 | | | |
| A40/A3 | SS | MS | Pseudo-F | P (perm) | SS | MS | Pseudo-F | P (perm) |
| Plot | 1426.9 | 1426.9 | 5.3064 | 0.0105 | 7061.9 | 7061.9 | 6.9107 | 0.0016 |
| Time | 616.23 | 308.12 | 1.1458 | 0.3468 | 1286.1 | 643.03 | 0.62926 | 0.8529 |
| Plot × time | 391.65 | 195.83 | 0.72824 | 0.6678 | 1143.3 | 571.64 | 0.5594 | 0.9037 |
| Residual | 1613.4 | 268.9 | | | 6131.3 | 1021.9 | | |
| Total | 4048 | | | | 15,622 | | | |

| Bacteria 2020 | | | | | Fungi 2020 | | | |
|---|---|---|---|---|---|---|---|---|
| B40/B10 | SS | MS | Pseudo-F | P (perm) | SS | MS | Pseudo-F | P (perm) |
| Plot | 1095.9 | 1095.9 | 9.7106 | 0.0016 | 6392.3 | 6392.3 | 7.1817 | 0.002 |
| Time | 505.72 | 252.86 | 2.2405 | 0.0231 | 1342 | 671.01 | 0.75387 | 0.7703 |
| Plot × time | 217.23 | 108.62 | 0.96244 | 0.5193 | 1231.9 | 615.93 | 0.69199 | 0.8507 |
| Residual | 677.13 | 112.86 | | | 5340.5 | 890.09 | | |
| Total | 2496 | | | | 14,307 | | | |
| B10/B3 | SS | MS | Pseudo-F | P (perm) | SS | MS | Pseudo-F | P (perm) |
| Plot | 721.89 | 721.89 | 3.8105 | 0.029 | 5729.6 | 5729.6 | 5.0631 | 0.0031 |
| Time | 482.49 | 241.25 | 1.2734 | 0.2822 | 1224 | 612 | 0.54081 | 0.926 |
| Plot × time | 184.01 | 92.003 | 0.48564 | 0.8713 | 1295.7 | 647.85 | 0.57249 | 0.908 |
| Residual | 1136.7 | 189.45 | | | 6789.8 | 1131.6 | | |
| Total | 2525.1 | | | | 15,039 | | | |
| B40/B3 | SS | MS | Pseudo-F | P (perm) | SS | MS | Pseudo-F | P (perm) |
| Plot | 1724.2 | 1724.2 | 8.2597 | 0.0016 | 12,136 | 12,136 | 10.024 | 0.0024 |
| Time | 569.54 | 284.77 | 1.3642 | 0.2388 | 1309.6 | 654.82 | 0.54085 | 0.9298 |
| Plot × time | 249.02 | 124.51 | 0.59645 | 0.7996 | 1438.1 | 719.06 | 0,5939 | 0.8968 |
| Residual | 1252.5 | 208.75 | | | 7264.4 | 1210.7 | | |
| Total | 3795.3 | | | | 22,148 | | | |

B3, being the other parameters not significant (species richness: $P = 0.102$; Chao 1: $P = 0.299$) (Fig. 5A). On the other hand, the year had not any effect on the diversity parameters (species richness: $P = 0.345$; Chao 1: $P = 0.211$; Shannon-Wiener index: $P = 0.088$) (Fig. 5B), whereas the sampling month significantly influenced species richness and Chao1, but not Shannon-Wiener index ($P = 0.077$) (Fig. 5C). The interaction plot/year, plot/sampling month and year/sampling month for the three diversity parameters were not significant (data not shown).

In 2018, taxonomic composition of sequences revealed the dominance of Basidiomycota, whose OTU abundance accounted for the 50 % of the total sequences, followed by Ascomycota (25 %), Mortierellomycota (17 %), and Mucoromycota (6 %) (Fig. 7A). Similar results were obtained in 2020 with Basidiomycota (51 %), Ascomycota (12 %), Morteriellomycota (11 %) and Mucoromycota (2 %) (Fig. 7B). In both years, the other phyla had a representation below the 1 %.

The phylum Basidiomycota dominated the fungal community of both plot A40 ($P = 0.012$) and B40 ($P < 0.001$) (Fig. 6A–B). In 2018, Russula grata was the most abundant species (19 %), and also the most represented taxon in plot A40 (25 %), whereas it was absent in plot A10 and rare in plot A3 (Fig. S4-A). *R. parazurea*, was the second most abundant species across the plots (15 %), also being the second most abundant species in plot A10 (19 %), but rare in plots A40 and A3 (Fig. S4-A). In 2020, *R. parazurea* represented the 19 % of OTU abundance, followed by an unidentified *Russula* species (13 %), *R. violeipes* (10 %) and *R. grata* (9 %). In this year, *Russula* species were dominant in plots B40 and B10, although absent in plot B3 (Fig. S4-B).

In 2018, the phylum Ascomycota ($P = 0.014$) and Mortierellomycota ($P = 0.0001$) were highly represented in plots A10 and A3, as it occurred in 2020 in plots B10 and B3 (Ascomycota: $P < 0.001$; Mortierellomycota $P < 0.001$) (Fig. 6A–B). Inside this phylum, the endophytic fungus *Acidomelania panicicola* was the best represented taxon (7 %), reaching the 61 % of the OTUs in plot A10, whereas in 2020, *Trichoderma* sp. was its most represented taxon (10 %) in plot B3 (Fig. S4A–B). The distribution of the phylum Mortierellomycota was significantly affected by the plots ($P < 0.001$). It was represented by the genus *Mortierella* who reached the highest abundance in plot A10 (56 %) and A3 (41%) in 2018, and in plot B3 (60%) in 2020 (Fig. S4A–B). The phylum Mucoromycota was significantly affected by the plot ($P = 0.0001$). It was represented by the genus *Umbelopsis* (99 %), who was mainly detected in plot A40 (79 %) in 2018 (Fig. 8A), and in plot B40 (35 %) in 2020 (Fig. S4-B).

PERMANOVA analysis showed the existence of significant differences of fungal community structure between all plots in both years (Table 1). SIMPER analysis showed that the major contribution to dissimilarity between plots A40/B3 and plots A40/A3 in 2018 was due to *R. grata* (Table 2). In 2020, the major dissimilaty was due to *R. parazurea* between plots B40/B10 and B10/B3, and to *R. grata* between plots B40/B3 (Table 2).

In both 2018 (Fig. 7A) and 2020 (Fig. 7B), the NMDS analysis resulted in two-dimensional solution with a final stress value of 0.05 and 0.06, respectively. The ordination plot showed a well separated assemblages of the fungal community in both years, although in 2018 fungal

**Table 2**
SIMPER results for dissimilarity (%) of bacterial and fungal taxa between the experimental chestnut orchards in 2018 and 2020. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. Contribution for dissimilarity (Contrib.%) of the first six taxa is shown.

| Bacteria | | | | | |
|---|---|---|---|---|---|
| A40/A10 (31.71 %) | | A40/A3 (33.46 %) | | A10/A3 (25.01 %) | |
| Species | Contrib.% | Species | Contrib.% | Species | Contrib.% |
| *Burkholderia pseudomultivorans* | 3.13 | *Burkholderia pseudomultivorans* | 2.85 | *Rhodanobacter glycinis* | 2.52 |
| *Sphingomonas limnosediminicola* | 2.45 | *Rhodanobacter glycinis* | 2.74 | *Collimonas pratensis* | 1.58 |
| *'Massilia aquatica'* | 2.17 | *Povalibacter uvarum* | 1.93 | *Methyloversatilis thermotolerans* | 1.45 |
| *Rhodanobacter glycinis* | 1.94 | *Sphingomonas limnosediminicola* | 1.38 | *Labilithrix luteola* | 1.26 |
| *Povalibacter uvarum* | 1.93 | *'Massilia aquatica'* | 1.35 | *Nitrosospira multiformis* | 1.25 |
| *Bradirhizobium lupini* | 1.63 | *Undibacterium arcticum* | 1.33 | *Massilia eurypsychrophila* | 1.24 |
| B40/B10 (24.36 %) | | B40/B3 (29.96 %) | | B10/B3 (23.04 %) | |
| Species | Contrib. % | Species | Contrib. % | Species | Contrib. % |
| *Sphingomonas limnosediminicola* | 1.21 | *Sphingomonas limnosediminicola* | 1.07 | *Massilia eurypsychrophila* | 1.08 |
| *'Massilia aquatica'* | 0.94 | Rhodoplanes tepidicaeni | 0.89 | *Thermanaerovibrio velox* | 1.04 |
| *Edaphobacter acidisoli* | 0.91 | *Massilia eurypsychrophila* | 0.86 | *Edaphobacter acidisoli* | 0.99 |
| *Burkholderia pseudomultivorans* | 0.84 | *Terrimicrobium sacchariphilum* | 0.81 | *Desulfofundulus thermocisternus* | 0.94 |
| *Edaphobacter lichenicola* | 0.83 | *Edaphobacter acidisoli* | 0.79 | *Rhodanobacter glycinis* | 0.81 |
| *Terrimicrobium sacchariphilum* | 0.83 | *Rhodanobacter glycinis* | 0.76 | *Dictyobacter aurantiacus* | 0.78 |

| Fungi | | | | | |
|---|---|---|---|---|---|
| A40/A10(86.83 %) | | A40/A3 (84.83 %) | | A10/A3 (67.11 %) | |
| Species | Contrib.% | Species | Contrib.% | Species | Contrib.% |
| *Russula grata* | 14.76 | *Russula grata* | 18.92 | *Russula parazurea* | 17.01 |
| *Russula parazurea* | 11.89 | *Inocybe renispora* | 10.06 | *Scleroderma citrinum* | 9.53 |
| *Inocybe renispora* | 8.02 | *Russula violeipes* | 6.06 | *Russula violeipes* | 5.61 |
| *Scleroderma citrinum* | 7.37 | *Metarhizium anisopliae* | 3.93 | *Inocybe calospora* | 4.90 |
| *Acidomelania panicicola* | 4.13 | *Russula virescens* | 3.63 | *Scleroderma polyrhizium* | 4.85 |
| *Inocybe calospora* | 3.38 | *Acidomelania panicicola* | 3,42 | *Metapochonia bulbillosa* | 3.51 |
| B40/B10 (70.96 %) | | B40/B3 (90.17 %) | | B10/B3 (71.62 %) | |
| Species | Contrib.% | Species | Contrib.% | Species | Contrib.% |
| *Russula parazurea* | 14.19 | *Russula grata* | 13.23 | *Russula parazurea* | 21.45 |
| *Russula grata* | 13.34 | *Russula violeipes* | 12.05 | *Scleroderma citrinum* | 12.02 |
| *Inocybe renispora* | 11.43 | *Inocybe renispora* | 11.15 | *Russula violeipes* | 10.57 |
| *Scleroderma citrinum* | 10.95 | *Russula parazurea* | 6.87 | *Scleroderma polyrhizium* | 4.92 |
| *Russula violeipes* | 5.32 | *Acidomelania panicicola* | 3.68 | *Tomentella stuposa* | 3.67 |
| *Scleroderma polyrhizium* | 5.13 | *Tomentella stuposa* | 3.56 | *Nadsonia starkeyi-henricii.* | 2.92 |

species of plots A10 and A3 resulted to be close to each other (Fig. 7A–B). The 2-way ANOSIM confirmed these separations only for plots A40/A10 (R = 1, P = 0.037) and A40/A3 (R = 1, P = 0.037) sampled in 2018, and for plots B40/B10 (R = 0.917, P = 0.037) and B40/B3 (R = 1, P = 0.037) and B10/B3 (R = 1, P = 0.045) sampled in 2020.

### 3.3. *Boletus edulis* mycelium frequency and concentration

The qPCR found *B. edulis* mycelium in the 19 % of the samples in 2018, and in the 97 % of the samples in 2020 (Table 3). The standard curve for quantification of *B. edulis* mycelium in the soil fulfilled the requirements for qPCR in terms of efficiency ($R^2 = 0.99$) (Fig. S5).

Mycelium frequency showed similar percentages in A40 (10 %), A10 (27 %) and A3 (20%) plots, in 2018. Similarly, in 2020, mycelium frequency was equally sorted in plots B40 (100 %), B10 (93 %) and B3 (97%). Globally, mycelium frequency was significantly higher in 2020 (P < 0.001) and depending on the sampling month (P < 0.001), whereas the plot was not significant (P = 0.812). The effect of the month, however, depended on the fact that mycelium frequency in all the months in 2020 was significantly higher than in all the months in 2018. In addition, the interaction year/plot (P = 0.294), plot/sampling month (P = 0.992) and year/sampling month (P = 0.143) did not have any effect. When considering the years separately, mycelium frequency did not change between the plots neither in 2018 (P = 0.442) nor in 2020 (P = 0.554), being the sampling month not significant neither in 2018 (P = 0.177) nor in 2020 (P = 0.554).

In general, the concentration of *B. edulis* mycelium (Table 3) significantly changed by the year (P = 0.001), being higher in 2020, and in September (P = 0.036), however it did not vary between the plots (P = 0.462). The interactions year/plot (P = 0.079), plot/sampling month (P = 0.100) and year/sampling month (P = 0.266) did not have any effect. In 2018, the impact of the plots on mycelium concentration (P = 0.372) and of the sampling month (P = 0.372) was not significant. In 2020, the impact of the plots on mycelium concentration was not significant (P = 0.475), whereas the concentration was higher in September (P = 0.022).

### 3.4. Correlations with soil microbiota

The analyses of the correlations between *B. edulis* mycelium concentration and bacterial phyla, indicated a significant impact of all the groups, with the exception of *Bacteroidetes*, with an increasing number of significant correlations from 40-years-old to 3-years-old plots in both years (Table 4). Including those phyla poorly represented, such as *Armatimonadetes*, *Synergistetes*, and *Thaumarchaeota* showed a strong although negative correlation with *B. edulis* mycelium in 10- and 3-years-old orchards. A totality of 173 bacterial species (only the main taxa above 4000 reads were considered) belonging to thirteen phyla have been tested for the existence of significant correlation with *B. edulis* mycelium concentration (Table S4). In 2018, the 5 % of the taxa had a significant correlation with the mycelium in plot A40, the 10 % in plot A10 and the 50 % in plot A3. In 2020, the percentage of significant correlations was the 6 % in plot B40, the 43 % in plot B10 and the 32 % in plot B3. Then, in general, the 40-, 10- and 3-years-old orchards had the 12 %, the 54 % and the 82 % of the significant correlations, respectively.

Among the bacterial taxa involved in significant correlations, there were nine MHB and seven bacterial species known as indictors of high

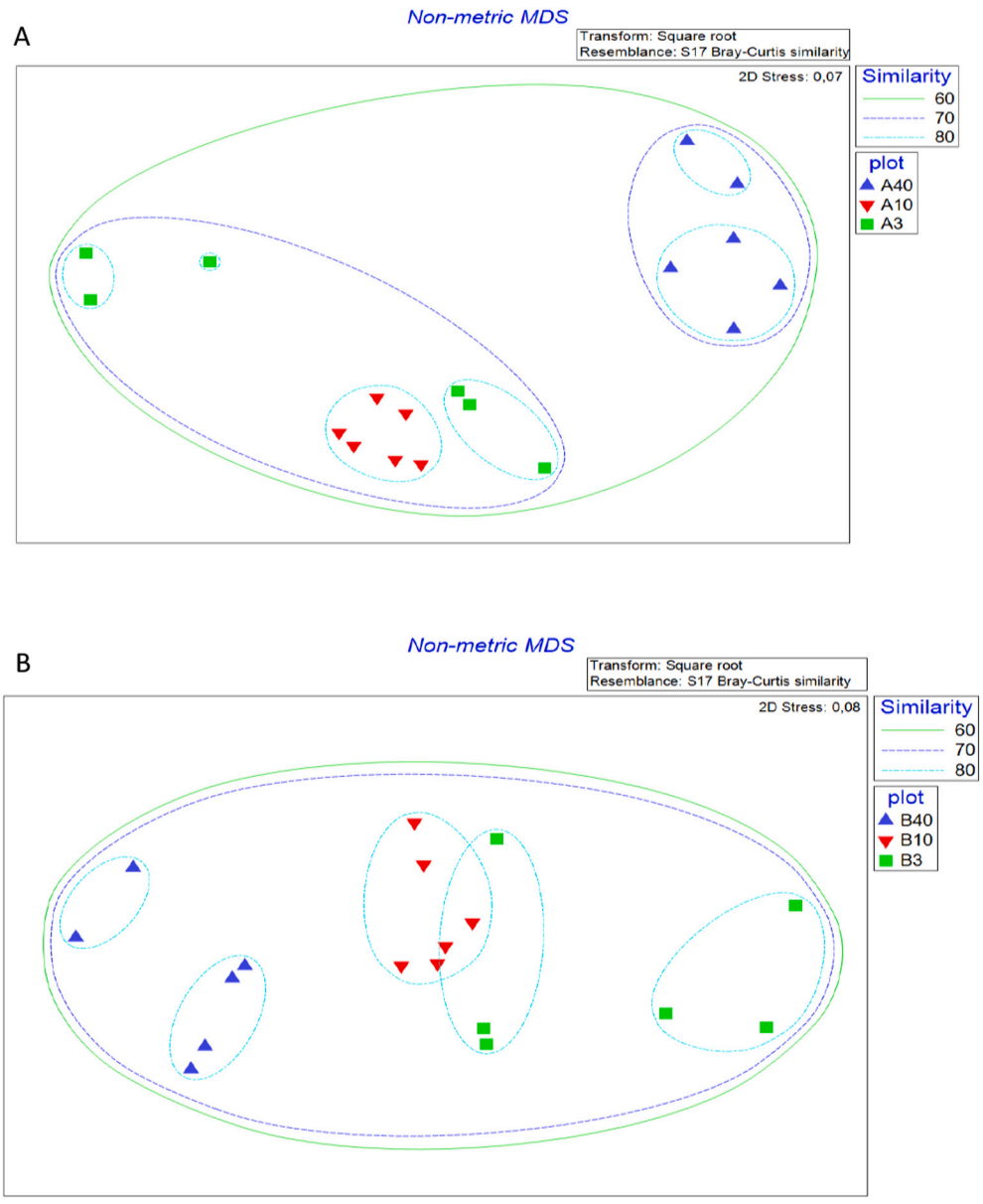



**Fig. 4.** Two-dimensional non-metric multi-dimensional scaling (NMDS) ordination depicting the similarity between the samples collected in 2018 (A) and 2020 (B), regarding bacterial taxa. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. For each orchard, 30 soil samples have been analyzed (2 repetition plots × 5 soil samples × 3 sampling dates). The cluster similarity contours (bubbles) at 60 %, 70 % and 80 % similarity are also presented.

productive site of *B. edulis* mushrooms (Table S4). The phylum *Acidobacteria*, *Actinobacteria* and *Proteobacteria*, the three most prominent groups, had the 43 %, the 45 % and the 54 % of positive correlations, respectively. The 93 % of the totality of such positive correlations occurred in 10- and 3-years-old orchards.

Regarding the fungal community, at phylum level, it was found that Ascomycota were not involved in significant correlations with *B. edulis* mycelium concentration, whereas Basidiomycota had significant correlation in 40- and 10-years-old orchards (Table 4). At species level, a total of 180 fungal taxa (above 4000 reads), sorted among seven phyla have been tested (Tables S5, S6, S7). In 2018, the 5 % of these taxa had a significant correlation with the *B. edulis* mycelium in plot A40, the 8 % in plot A10 and the 23 % in plot A3. In 2020, the percentage of significant correlations were the 7 % in plot B40, the 17 % in plot B10 and the 19 % in plot B3. In general, the 40-, 10- and 3-years-old orchards had the 12 %, the 24 % and the 42 % of the significant correlations, respectively. Inside Basidiomycota phylum, thirty species had significant correlations with *B. edulis* mycelium, being positive the 54 % of them (Table S5). In the 68 % of the cases, the significant correlations occurred in 10- and 3-years-old orchards (Table S5). Among the genera involved in positive correlations, there were some important macrofungi such as *Amanita*, *Cortinarius*, *Lepiota*, *Pleurotus*, and *Russula* (Table S5). Although *R. grata* was the most abundant taxon in 40-years-old orchards in both years, it did not have a significant correlation with *B. edulis* mycelium concentration (Table S5). On the other hand, *R. parazurea*, the most abundant taxon in 10-years-old plots, was significantly and positively correlated with the mycelium (Table S5).

Eighty-seven ascomycetes were involved in significant correlations with *B. edulis* mycelium concentration, having the 38 % of them positive correlations (Table S6). The 14 % of the significant correlations occurred in 40-years-old orchards, whereas the 91 % in 10- and 3-years-old orchards, where Ascomycota phylum was more represented.

In addition, ten fungal taxa belonging to the less represented phyla Chytridiomycota, Glomeromycota, Mortierellomycota, Mucoromycota, and Rozellomycota had 20 significant correlations with *B. edulis* mycelium concentration, being the 95 % of them in 10- and 3-years-old orchards (Table S7).

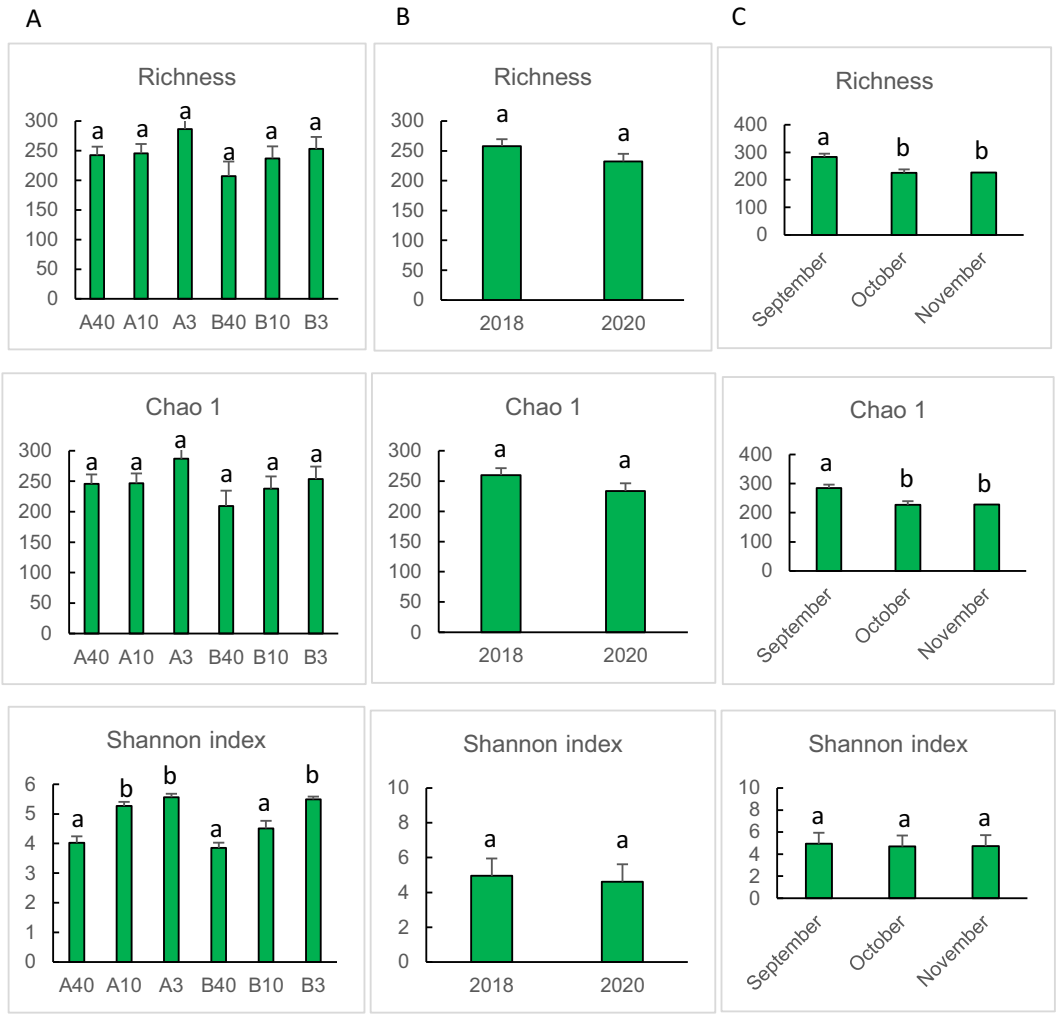



**Fig. 5.** A-B-C. Mean (±SD) of species richness, Chao 1, and Shannon diversity index relative to fungal taxa sorted per plot (A), year (B), and sampling month (C) are shown. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. Columns marked with different lowercase letters were significantly different ($\alpha = 0.05$) by one-way ANOVA.

## 4. Discussion

Previous investigations have shown that plant with different age have significantly distinct soil microbial communities (Wu et al., 2013; Bai et al., 2015; Wu et al., 2015; Dang et al., 2017; Liu et al., 2018; Gao et al., 2019; Qu et al., 2020). The density of the canopy increases with increasing plant age, reducing the availability of light and increasing the soil water content, thus resulting in changes in microbial community structure (Qu et al., 2020). In addition, the accumulation and decomposition of leaf litter, more abundant under aged plants, and changes in rhizosphere exudates may increase the input of soil nutrients, such as C and N, which can promote bacterial development (Liu et al., 2018; Mushinski et al., 2019). According to that, the 40-years-old orchards had a higher C:N content, whereas pH, Ca, K and Mg content were higher in 3-years-old orchards. Soil pH is a common predictor of bacterial composition (Gao et al., 2019). Generally, bacterial phyla prefer acid soil, consequently, a higher pH would not represent a more favorable environment for the microbiota. However, certain bacterial phylum, such as *Firmicutes*, are abundant also in post-fire soils, where pH increases (Mediavilla et al., 2019). Considering that *B. edulis* mycelium frequency and concentration did not change with plant age, the existence of a slight increase of pH in 3-years-old orchards, does not seem to be relevant for this fungal species.

Generally, there is a positive correlation between plant age and soil microbiota richness and diversity, and the distribution of the bacterial taxa across plant chronosequence also reflects their ecological function in the rhizosphere (Qu et al., 2020). In this study, a significant shift in bacterial and fungal richness and diversity across plant age, as well as different community assemblages, has been detected in both years. However, in contrast to our hypothesis, it was found that microbial community was larger in orchards with 10- and 3-years-old chestnuts and experienced higher percentages of significant correlations with *B. edulis* mycelium concentration. A possible explication of this finding is that the rhizosphere of young plants is rapidly colonized by fast growing, opportunistic bacteria, due to accumulation of organic acids, sugar, amino acids and other roots exudates (Badri and Vivanco, 2009). Root exudates mediate positive interactions including symbiotic associations with beneficial microbes, such as mycorrhizae, rhizobia and plant growth-promoting rhizobacteria (Hayat et al., 2010).

### 4.1. Bacterial community

*Proteobacteria*, *Acidobacteria* and *Actinobacteria* were the most represented phyla in both years. The acidic soils, as those that characterized the region, are known to be important drivers for bacterial development, especially *Alphaproteobacteria* and *Acidobacteria* (Shen et al., 2013). This bacterial community composition was in agreement with the results reported by Mediavilla et al. (2019) for *Cistus ladanifer* scrubland with

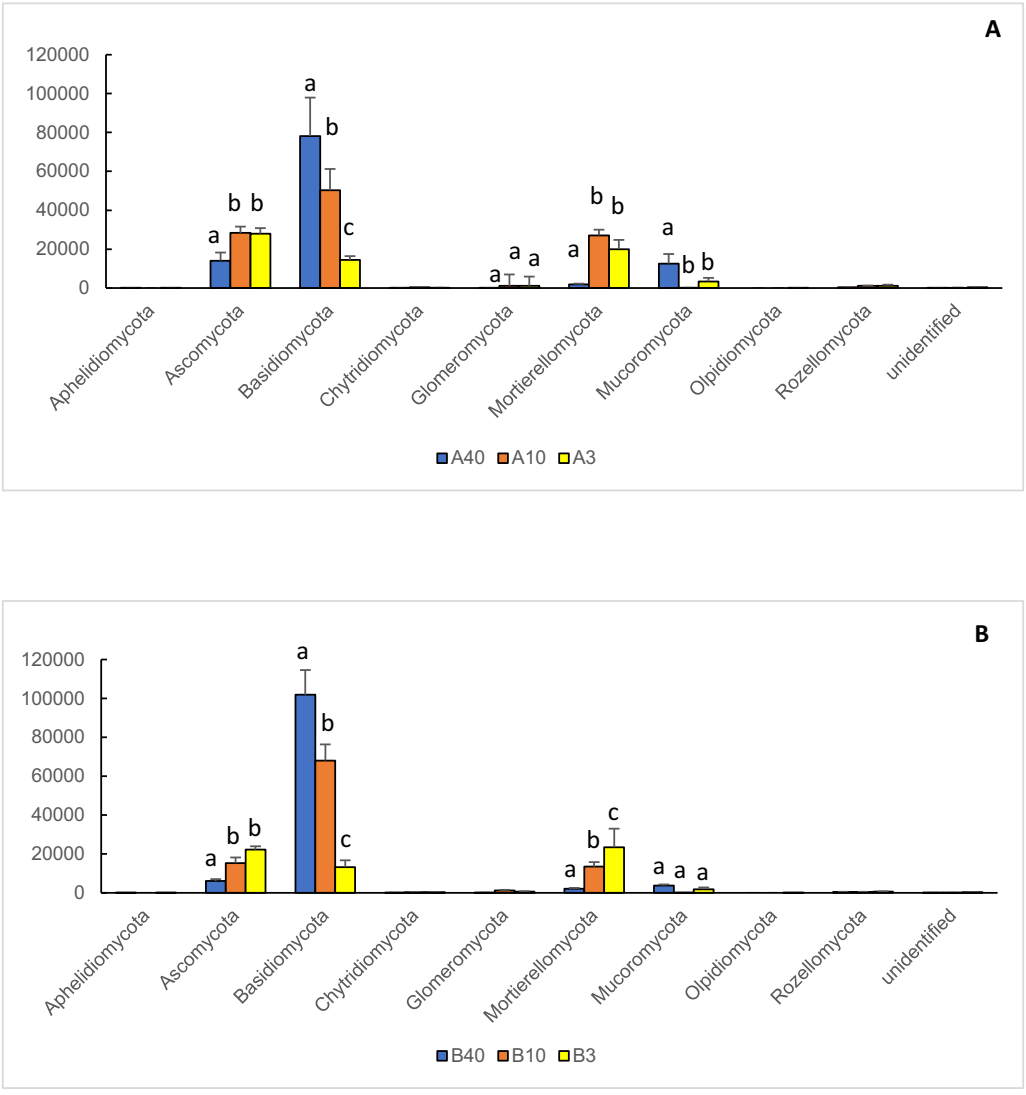



**Fig. 6.** A-B. Classification of fungal mean sequence abundance (± SD) at the phylum level sorted between the experimental orchards, in 2018 (A) and 2020 (B). Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. Columns marked with different lowercase letters were significantly different (α = 0.05) between the experimental plots by one-way ANOVA.

*Boletus* production, but also in other forest ecosystems (Sun et al., 2017; Qu et al., 2020), which highlights their functional importance for forest soils.

*Ferruginibacter*, *Azospirillum*, *Conexibacter*, *Gemmatimonas*, *Mucilaginibacter*, *Opitutus*, *Stella* and *Terriglobulus*, cited by Mediavilla et al. (2019) as significant indicators of highly productive sites of *B. edulis* sporocarps, have also been detected in the present study. *Azospirillum*, *Ferruginibacter*, *Mucilaginibacter* and *Stella* were significantly correlated with *B. edulis* mycelium concentration, in both years, in 3-years old orchards. This pattern of correlations suggests a role of such bacteria for *B. edulis* mycelium development. It has been shown in the literature that some bacterial metabolites stimulate hyphal growth, as it occurs for *Streptomyces* sp. AcH505, which interaction with *Amanita muscaria* enhanced the production of the secondary metabolite auxofuran, promoting the extension of the fungal mycelium (Frey-Klett et al., 2011). The genus *Opitutus* had a positive correlation with *B. edulis* mycelium in 10- and 3-years-old orchards. This genus was highlighted by Mediavilla et al. (2019), suggesting an influence on *B. edulis* sporocarp production, possibly due to its role in C cycle. *Azospirillum* belongs to the non-symbiotic nitrogen fixing bacteria (Hayat et al., 2010). This genus contains several rhizobacteria that promote plant growth by increasing the number of lateral roots and root hairs and consequently also the mycorrhizal roots (Hayat et al., 2010). Also, the genus *Gemmatimonas*, considered a phosphate solubilizing bacterium, was involved in significant correlations with *B. edulis* mycelium in 10 and 3-years old orchards.

The mycorrhiza helper bacteria (MBH) *Arthrobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Pseudomonas*, *Paenibacillus*, *Rhizobium* and *Streptomyces* had significant correlations with *B. edulis* mycelium concentration. *Bacillus* and *Paenibacillus* are among the most powerful phosphate solubilizing bacteria, useful to enhance phosphorous status of the plants (Hayat et al., 2010). They are members of the phylum *Firmicutes*, that had a good representation in all the orchards, especially in those of 10- and 3-years old. This phylum is known to be resistant to extreme conditions, including post-fire soils (Mediavilla et al., 2019). *Pseudomonas fluorescens* and *Bacillus cereus* have been successfully used to promote *B. edulis* mycorrhization under laboratory conditions on *Cistus ladanifer* (Mediavilla et al., 2016), and *Pinus thunbergii* (Wu et al., 2012), respectively. The *Proteobacteria Burkholderia pesudomultivorans* was one of the main responsible of the difference of bacterial community assemblage between orchards, being more abundant in 40-years-old orchards in both years. This bacterium, however, was significantly

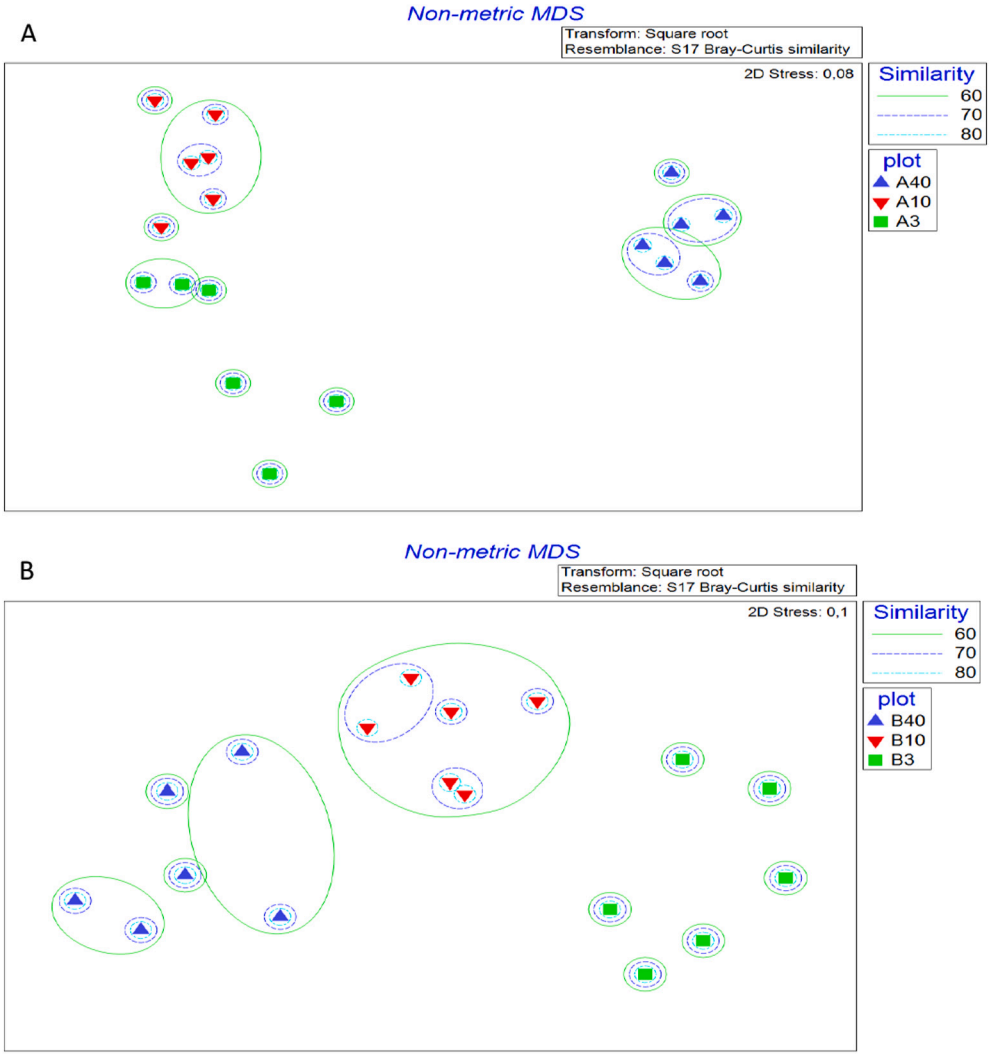



**Fig. 7.** A-B. Two-dimensional non-metric multidimensional scaling (NMDS) ordination depicting the similarity between the samples collected in 2018 (A) and in 20,202 (B), regarding fungal taxa. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. For each orchard, 30 soil samples have been analyzed (2 repetitions × 5 soil samples × 3 sampling dates). The cluster similarity contours (bubbles) at 60 %, 70 % and 80 % similarity are also presented.

correlated in both years with *B. edulis* mycelium in 3-years-old orchards. *Burkholderia* has been recognized as MHB, because it may promote the establishments of plant-fungus symbiosis by stimulating mycelial extension, increasing root-fungus colonization and reducing the impact of adverse conditions on mycelium spread (Frey-Klett et al., 2007). In particular, this bacterium may facilitate ectomycorrhization by stimulating flavonoid or hormone production, which attracts the mycorrhizal symbiont, alters host innate immune responses, or modifies fungal gene regulation, shifting mycelium from free-living to pre-symbiotic states (Poole et al., 2001; Kataoka and Futai, 2009). *Bradyrhizobium lupini* is a symbiotic bacterium able to produce molecules that promote plant growth (Hayat et al., 2010). As occurred for *B. pseudomultivorans*, *B. lupini* was a dominant taxon in both years especially in 40-years old orchards, although a significant correlation with *B. edulis* mycelium was find only in 3-years old orchards, where it was less represented. This finding suggests that the existence of significant correlations did not strictly depend on the concentration of bacteria into the soil.

*Acidobacterium ailauii* and *Paludibaculum fermentas*, the most prominent taxa of *Acidobacteria* phylum, were dominant in all the experimental orchards, and had positive correlations with *B. edulis* mycelium concentration mycelium in 10- and 3-years-old orchards. *P. fermentans*, commonly inhabits wetlands and groundwater bodies (Kulichevskaya et al., 2014), whereas *A. ailauii* has been described for the first time from geothermally heated microbial mats, but is also present in croplands, being implicated in the decomposition of organic material, soil C and N transformation, and plant growth promotion (Chen et al., 2019).

Bacteria belonging to *Actinobacteria* have a heliophilous vocation and tend to increase after forest clear-cutting (Mediavilla et al., 2019), however, a significant increase of species abundance in the soil of younger plants, which have a smaller canopy, was not detected. *Actinobacteria* are more active at high pH levels, and are especially important in degrading recalcitrant compounds, such as chitin, lignin, keratin, cellulose fungal, and animal polymers, forming stable humus (Hayat et al., 2010).

### 4.2. Fungal community

Fungal taxa showed different assemblages depending on chestnut age. It is well known that fungal species composition tends to change as stands mature, and older trees generally support a greater number of fungal symbionts than younger trees (Nara et al., 2003; Twieg et al., 2007). Mycobiota may be categorized as “early-stage”, “multi-stage” (occurring in all stages) and “late-stage” depending on the host plant age (Twieg et al., 2007). The phylum Ascomycota and Mortierellomycota dominated 10- and 3-years-old orchards, whereas Basidiomycota were clearly more abundant in the 40-years-old orchards, which reflected the successional patterns of fungal communities during the development of the plants. Ascomycetes are known to have a high plasticity in their lifestyles and metabolic capacities (Whiteway et al., 2015), which may explain why up to eighty-seven ascomycetes were involved in significant

**Table 3**
Detection of *B. edulis* mycelia in soil samples by qPCR in mg mycelium/g soil. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020. For each plot, 30 samples have been analyzed (2 repetitions × 5 soil samples × 3 sampling dates).

| Plot | Sampling point | September | October | November |
|---|---|---|---|---|
| A40 | 1 | 0.0000000 | 0.000000768 | 0.0000000 |
| A40 | 2 | 0.0000000 | 0.0000000 | 0.0000000 |
| A40 | 3 | 0.0000000 | 0.0000000 | 0.0000000 |
| A40 | 4 | 0.0000000 | 0.0000000 | 0.0000000 |
| A40 | 5 | 0.0000000 | 0.0000000 | 0.0000000 |
| A40 | 1 | 0.0000000 | 0.0000000 | 1.56E−10 |
| A40 | 2 | 0.0000000 | 0.0000000 | 0.0000000 |
| A40 | 3 | 4.4E−09 | 0.0000000 | 0.0000000 |
| A40 | 4 | 0.0000000 | 0.0000000 | 0.0000000 |
| A40 | 5 | 0.0000000 | 0.0000000 | 0.0000000 |
| A10 | 1 | 0.0115 | 0.0000000 | 0.0000000 |
| A10 | 2 | 0.0000000 | 0.0000000 | 0.0000000 |
| A10 | 3 | 0.0000000 | 0.0000000 | 0.0000000 |
| A10 | 4 | 0.000000026 | 0.0000000 | 0.00000092 |
| A10 | 5 | 0.0000000 | 0.0000000 | 0.000000712 |
| A10 | 1 | 0.0000000 | 0.0000000 | 0.000000777 |
| A10 | 2 | 2.02E−09 | 0.0000000 | 0.00000169 |
| A10 | 3 | 0.0000000 | 0.0000000 | 0.0000000 |
| A10 | 4 | 0.0000000 | 0.0000000 | 0.000000318 |
| A10 | 5 | 0.0000000 | 0.0000000 | 0.0000000 |
| A3 | 1 | 0.0000000 | 0.0000000 | 0.0000000 |
| A3 | 2 | 0.0000000 | 0.0000000 | 0.0000000 |
| A3 | 3 | 0.0000000 | 0.0000000 | 0.0000000 |
| A3 | 4 | 0.0000000 | 0.0000000 | 0.0000000 |
| A3 | 5 | 0.0000000 | 0.0000000 | 0.0000000 |
| A3 | 1 | 0.0000000 | 0.0000000 | 0.000000224 |
| A3 | 2 | 7.9E−09 | 0.0000000 | 0.000000137 |
| A3 | 3 | 0.0000000 | 0.0000000 | 0.0000000 |
| A3 | 4 | 8.59E−09 | 4.93E−11 | 0.000000585 |
| A3 | 5 | 0.0000000 | 0.0000000 | 0.0000000 |
| B40 | 1 | 0.000000185 | 0.000000727 | 0.00000079 |
| B40 | 2 | 0.000000397 | 0.000000597 | 0.00000122 |
| B40 | 3 | 0.000000856 | 0.000000395 | 0.00000114 |
| B40 | 4 | 1.66 | 0.000000843 | 0.000000493 |
| B40 | 5 | 0.000000144 | 0.000000753 | 9.45E−08 |
| B40 | 1 | 0.000000888 | 0.00000152 | 2.58E−10 |
| B40 | 2 | 0.000000462 | 0.00000209 | 0.0000363 |
| B40 | 3 | 0.000000477 | 0.00000859 | 0.00000445 |
| B40 | 4 | 0.000000409 | 0.00000221 | 0.00000101 |
| B40 | 5 | 0.000000869 | 0.00000157 | 0.00000102 |
| B10 | 1 | 0.00000584 | 0.00000174 | 0.00000222 |
| B10 | 2 | 0.000000343 | 0.0000022 | 0.000000538 |
| B10 | 3 | 0.000000105 | 0.00000102 | 0.00000444 |
| B10 | 4 | 0.000000276 | 0.00000205 | 0.00000594 |
| B10 | 5 | 0.000000982 | 0.00000392 | 0.00000804 |
| B10 | 1 | 0.000000545 | 0.00000114 | 0.0000000 |
| B10 | 2 | 0.000000892 | 0.000000528 | 0.00000333 |
| B10 | 3 | 0.000000567 | 0.00000201 | 0.0000000 |
| B10 | 4 | 0.000000406 | 0.00000563 | 0.00000781 |
| B10 | 5 | 0.000000439 | 0.00000183 | 0.00000412 |
| B3 | 1 | 0.000000291 | 0.00000201 | 0.00000294 |
| B3 | 2 | 1.09E−10 | 0.0000036 | 0.000000819 |
| B3 | 3 | 0.000000674 | 0.00000189 | 0.00000302 |
| B3 | 4 | 0.753 | 6.56E−08 | 0.00000168 |
| B3 | 5 | 0.00000121 | 0.0000000 | 0.00000175 |
| B3 | 1 | 1.91 | 5.35E−08 | 0.00000189 |
| B3 | 2 | 4.61E−09 | 0.00000171 | 0.00000139 |
| B3 | 3 | 0.000000823 | 0.000000854 | 0.00000342 |
| B3 | 4 | 0.00000152 | 0.00000288 | 0.00000361 |
| B3 | 5 | 0.000000364 | 0.00000185 | 0.00000391 |

correlations with *B. edulis* mycelium. On the other hand, thirty basidiomycetes had significant correlations with *B. edulis* mycelium concentration. Inside the Basidiomycota phylum, the genus *Amanita*, *Cortinarius*, *Inocybe* and *Russula* characterized the mycobiota frequently associated with *B. edulis* productive sites (Peintner et al., 2007; Baptista et al., 2010; Ambrosio and Zotti, 2015). A possible cooperation of these taxa with *B. edulis* development could be hypothesized, considering that *C. purpurascens*, *C. scaurus*, *C. torvus*, *A. citrina. I. ericetorum and R. parazurea* were involved in significant and positive correlations with *B. edulis* mycelium concentration. On the other hand, *Scleroderma citrinum* and *S. polyrhizum* were both significantly but negatively correlated with mycelium abundance, which is not surprising considering the antagonistic role that *Scleroderma* exerts against *Boletus* mycorrhizae in the field (Meotto et al., 1999). Previous study on the ectomycorrhizal fungal species associated with the root tips of 3-years old chestnuts, showed the dominance of generalist, early-stage fungi, such as *S. citrinum*, *Cenococcum geophilum* and *Laccaria* (Santolamazza-Carbone et al., 2021). In the present study, however, the fungal assemblage in the rhizosphere of 3-years-old chestnuts were dominated by the genus *Mortierella*, *Umbelopsis* and *Trichoderma*, which suggests that the taxonomic diversity of the fungal species hosted in the mycorrhizosphere and in the rhizosphere, also involves a diverse function.

### 4.3. *B. edulis mycelium*

The assessment of the distribution of extraradical mycelium of mycorrhizal fungi in the soil is still difficult, although recent adapting of qPCR provides a species-specific measure for mycelial biomass estimations (Parladé et al., 2007; De la Varga et al., 2012). In agreement with De la Varga et al. (2012), our results confirmed that qPCR may detect presence of the mycelium below 0.001 mg/g of soil. In the present study, the concentration of *B. edulis* mycelium per soil sample was low in both years, however, it satisfactory allowed to obtain significant results with the Spearman correlation test. In 2018 only the 19 % of the soil samples contained *B. edulis* mycelium, whereas in 2020 it was found in the 97 % of samples, with a higher concentration in September 2020 in comparison with October and November. Possibly, this difference between years could be due to the higher precipitations occurred in 2020 from August to November, whereas in 2018 rainfalls from July to September were very scarce (Paramos Sías, 2021). This hypothesis agrees with the results obtained by De la Varga et al. (2013), who found that *B. edulis* mycelium concentration was positively correlated with rainfall, although negatively with the temperature. On the other hand, monthly precipitation was negatively correlated with mycelium biomass and no correlation was found with monthly mean temperature (Parladé et al., 2017). The reduction of the mycelium biomass before or at the same time of sporocarp production (from October to December), possibly depend on the fact that the great part of the fungal resources is allocated for fructification (De la Varga et al., 2013).

In Italy, it was shown that in high productive sites *B. edulis* mycelia could not be found underneath the sporocarps, was rare in the soil and had a scattered distribution (Peintner et al., 2007). *Boletus* mycorrhizae have long distance exploration type, having the extraradical mycelia concentrated as rhizomorphs with a high degree of spatial heterogeneity (Agerer, 2001), which may explain its irregular distribution in the soil. *Boletus edulis* is generally considered a late-stage fungus that produces sporocarps in mature stands and should require a high level of carbon supply (Mediavilla et al., 2017; Martínez-Peña et al., 2012), then, it was predicted the presence of a higher amount of extraradical mycelium biomass in mature orchards. Our results, however, did not agree with this finding because frequency and concentration of *B. edulis* mycelium did not increase in mature orchards, and, in general, the mycelium was equally spread irrespective of plant age. The extraradical mycelium is the most dynamic component of the mycorrhizal symbiosis, and the fastest indicator of how mycorrhizal fungi respond to the environmental variations (Johnson et al., 2005). It has been shown that stand productivity of forest ecosystems decreases at mature ages by about 65 %, resulting in a reduction of the demand for nutrient and water from fine roots and their mycorrhizal fungi (Gower et al., 1996). On the other hand, higher rates of mycorrhizal mycelia production have been detected in the younger stands along an age gradient of *Pinus sylvestris* stands (Hagenbo et al., 2019).

There is just one other study on the impact of plant age on *B. edulis* in *Pinus sylvestris* habitat (Martínez-Peña et al., 2012), in which it is

**Table 4**
Correlations between *B. edulis* mycelium concentration and the sequence abundance of the bacterial and fungal phyla, calculated with Spearman correlation coefficient analysis (significance level at $\alpha = 0.05$). Results in bold show significant correlation. Letters A40, A10 and A3 refer to 40-, 10- and 3-years old chestnut orchards, respectively, sampled in 2018. Letters B40, B10 and B3 refer to the same chestnut orchards as above, sampled in 2020.

| Bacteria | | | | | | |
|---|---|---|---|---|---|---|
| Phylum | A40 | A10 | A3 | B40 | B10 | B3 |
| *Acidobacteria* | −0.334 | −0.232 | **−0.880** | 0.086 | 0.143 | −0.143 |
| *Actinobacteria* | 0.030 | −0.232 | 0.273 | 0.371 | **−0.600** | **0.600** |
| *Armatimonadetes* | 0.516 | **0.928** | **−0.941** | 0.143 | −0.257 | −0.086 |
| *Bacteroidetes* | −0.152 | −0.029 | 0.577 | −0.029 | −0.543 | 0.086 |
| *Candidatus_Melainabacteria* | −0.273 | −0.103 | **0.770** | 0.086 | −0.371 | −0.371 |
| *Chlamydiae* | 0.563 | 0.580 | **−0.880** | 0.377 | 0.058 | 0.371 |
| *Chloroflexi* | 0.213 | −0.435 | **−0.698** | 0.143 | −0.429 | **0.600** |
| *Cyanobacteria* | **0.696** | 0.279 | 0.154 | −0.370 | 0.464 | 0.290 |
| *Deinococcus Thermus* | **–** | – | – | – | – | **0.655** |
| *Elusimicrobia* | **0.696** | – | 0.417 | −0.131 | – | **−0.655** |
| *Firmicutes* | 0.152 | −0.029 | **−0.880** | 0.200 | **−0.657** | 0.543 |
| *Euryarchaeota* | **0.696** | −0.235 | **−0.677** | – | **0.886** | −0.213 |
| *Gemmatimonadetes* | −0.152 | 0.086 | **0.880** | 0.600 | 0.371 | −0.486 |
| *Nitrospirae* | 0.152 | −0.232 | **0.759** | −0.086 | **0.657** | **−0.714** |
| *Planctomycetes* | −0.152 | 0.319 | 0.030 | −0.029 | **−0.600** | 0.543 |
| *Proteobacteria* | 0.030 | −0.493 | −0.213 | 0.257 | 0.200 | **0.771** |
| *Spirochaetes* | 0.273 | **0.735** | **−0.647** | −0.232 | 0.486 | 0.029 |
| *Synergistetes* | 0.516 | **−0.725** | **−0.941** | −0.029 | 0.200 | **0.600** |
| *Thaumarchaeota* | 0.152 | −0.029 | **−0.941** | −0.058 | **0.783** | −0.086 |
| *Thermodesulfobacteria* | – | −0.105 | **0.844** | – | 0.031 | −0.273 |
| *Verrucomicrobia* | −0.395 | 0.319 | **0.759** | −0.543 | 0.086 | −0.543 |

| Fungi | | | | | | |
|---|---|---|---|---|---|---|
| Phylum | A40 | A10 | A3 | B40 | B10 | B3 |
| Aphelidiomycota | 0.215 | – | 0.139 | 0.393 | – | 0.507 |
| Ascomycota | 0.359 | 0.377 | −0.455 | 0.086 | −0.200 | 0.371 |
| Basidiomycota | **0.616** | **0.754** | 0.030 | **−0.771** | 0.086 | −0.257 |
| Chytridiomycota | 0.344 | 0.176 | 0.092 | −0.429 | −0.143 | **−0.657** |
| Glomeromycota | −0.154 | 0.029 | **0.759** | **−0.657** | −0.486 | −0.257 |
| Mortierellomycota | 0.205 | **−0.986** | **0.941** | 0.086 | **−0.771** | **−0.657** |
| Mucoromycota | −0.103 | **−0.493** | **−0.759** | −0.486 | −0.143 | **0.600** |
| Olpidiomycota | – | – | **0.826** | – | – | −0.372 |
| Rozellomycota | −0.205 | 0.580 | **−0.832** | −0.086 | **−0.657** | **0.600** |

reported that fungal productivity was higher in stands of 60–70 years-old. At present, *B. edulis* has been investigated only in *Pinus sylvestris* (De la Varga et al., 2012, 2013; Martínez-Peña et al., 2012; Parladé et al., 2017) and *Cystus ladanifer* habitats (Alonso Ponce et al., 2011; Mediavilla et al., 2017). Consequently, our investigation represents the first report about the impact of plant age on *B. edulis* mycelium dynamic in chestnut orchards. Further studies about *B. edulis* mycelium spread in *Quercus robur* forests are ongoing, to unveil whether mycelium pattern changes depending on host plant species.

According to Parladé et al. (2017), the mean *B. edulis* mycelium biomass in autumn was significantly correlated with sporocarp number and weight. Likewise, Mediavilla et al. (2017), found a statistically significant and positive correlation between *B. edulis* fresh weight production and the amount of mycelium in the soil. On the other hand, no correlations between sporocarp production and *B. edulis* mycelium were found by De la Varga et al. (2013). Sporocarp sampling was not included in the experimental design of this study, however, this parameter will be considered in future investigations to assess their relationship with mycelium dynamics during Autumn in chestnut orchards.

## 5. Conclusions

Analysis of the multiple interactions established by *B. edulis* mycelium with soil microbiota, assessed by DNA metabarcoding technique, offers a new understanding of the scenario of the organisms that interact around a mycorrhizal plant. This work highlights that the plant age has an impact on soil microbiota because soil of young chestnut orchards has a larger microbial assemblage. We also obtained clear evidence that, although *B. edulis* mycelium frequency and concentration did not change with plant age, and despite the low concentration of mycelia found in both years, the orchards with younger plants hosted a higher number of significant correlations between *B. edulis* mycelium and soil microbiota. In addition, several bacterial taxa had the same correlation pattern with *B. edulis* mycelium in two different years, suggesting a possible role in mycelium development. Indeed, the next step of the study will be to assess the significance of the interactions found with microbial partners, especially the MHB, for the development of *B. edulis* mycelium, ectomycorrhizae and sporocarps, taking into account also the role of soil and climatic parameters on mycelium yield.

## Funding

This research was funded by Spanish Ministry of Science, Innovation and Universities (grant number RTI2018-095568-B-I00) and Xunta de Galicia through “Red de Uso Sostenible de los Recursos Naturales y Agroalimentarios” (REDUSO, grant number ED431D 2017/18) and “Cluster of Agricultural Research and Development” (CITACA Strategic Partnership, grant numbered ED431E 2018/07).

## CRediT authorship contribution statement

Conceptualization, S.S-C.; methodology, S.S-C. and L. I-B; formal analysis, S. S-C.; investigation, S.S-C. and L. I-B.; resources, E.S-S. and P. P.G.; data curation, S.S-C. and L. I-B.; writing - original draft preparation, S.S-C.; writing-review and editing, S.S-C., L. I-B. and P.P.G.; supervision, P.P.G.; funding acquisition, E.S-S. and P.P.G. All authors have

read and agreed to the published version of the manuscript.

## Declaration of competing interest

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

## Data availability

Data will be made available on request.

## Acknowledgments

The authors acknowledge to the Spanish Ministry of Science, Innovation and Universities (grant number RTI2018-095568-B-I00) for the Postdoctoral Contract to Serena Santolamazza-Carbone.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2022.104790.

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