

Structural and functional microbial diversity in deadwood respond to decomposition dynamics

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Abstract

We investigated the changes in microbial community diversities and functions in natural downed wood at different decay stages in a natural oak forest in the Italian Alps, through metagenomics analysis and *in vitro* analysis. Alfa diversity of bacterial communities was affected by the decay stage and log characteristics, while beta diversity was mainly driven by log diameter. Fungal and archaeal beta diversities were affected by the size of the sampled wood (log diameter), although, fungi were prominently driven by wood decay stage. The analysis of genes targeting cell wall degradation revealed higher abundances of cellulose and pectin-degrading enzymes in bacteria, while in fungi the enzymes targeting cellulose and hemicellulose were more abundant. The decay class affected the abundance of single enzymes, revealing a shift in complex hydrocarbons degradation pathways along the decay process. Moreover, we found that the genes related to Coenzyme M biosynthesis to be the most abundant especially at early stages of wood decomposition while the overall methanogenesis did not seem to be influenced by the decay stage. Intra- and inter-kingdom interactions between bacteria and fungi revealed complex pattern of community structure in response to decay stage possibly reflecting both direct and indirect interactions.

INTRODUCTION

Deadwood is a key component in forest ecosystems supporting biodiversity and ecosystem functioning (Harmon

et al., 1986). It provides habitat and substrate for a variety of organisms and serves as pool of organic carbon (C) which is slowly released during decomposition (Parisi et al., 2018). Therefore, the study of wood decomposition may reveal important patterns of accumulation and turnover of carbon and nutrients in forest ecosystems. Deadwood is characterized by low nitrogen (N) content, higher abundance of recalcitrant lignin, and limited physical

*In memory of prof. Giustino Tonon (1966-2021).

[Corrections added on 20 July 2023, after first online publication: Obituary footnote has been added in this version.]

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permeability resulting in a nutrient resource that is difficult to access and degrade for most organisms (Hoppe et al., 2016). Due to its properties, wood is primarily colonized and decomposed by fungi as they are able to produce a wide range of ligninolytic extracellular enzymes (Arnstadt et al., 2016) along with other non-enzymatic mechanisms that catalytically modify lignin and depolymerize cellulose (Goodell et al., 2017).

However, the enzymatic breakdown is initially promoted by the mechanical fragmentation of wood caused by abiotic damages and invertebrates' activity (Ulyshen, 2016) which can be more or less severe according to the climatic conditions (i.e., more intense in tropical ecosystems). The result is the increase of exposed surface that can be easily colonized and degraded by microbial communities. Besides fungi, recent findings pointed out the importance of bacteria at early stages of wood decay as they are thought to degrade easily accessed cellulose and other labile compounds with few bacteria also involved in lignin degradation (de Boer & van der Wal, 2008; Hervé et al., 2016; López-Mondéjar et al., 2020). Increase of bacterial biomass was also observed at the later stage of wood degradation due to bacterial growth at mycelia expenses (Štursová et al., 2020). Conversely, very little is known on the occurrence and role of archaea in degrading wood. It has been suggested that archaea might be an integral part of wood microbiota along with bacteria and that the labile fractions of C may be an important source for archaeal methanogenesis (Pastorelli et al., 2020; Rinta-Kanto et al., 2016). However, the broader patterns of archaeal diversity during decomposition, their role in the wood decay process and their interactions with wood-inhabiting microbial communities remain to be elucidated through genomic studies.

The diversity of wood microbial communities is affected by multiple biotic and abiotic factors. Biotic interactions with other wood-inhabiting organisms have been extensively investigated in the past years, especially between insects and fungi (Jacobsen et al., 2015). Such interactions involve a wide range of functional relationships including nutrition, dispersal, deactivation of toxic compounds, and protection (Birkemoe et al., 2018). Among wood traits, tree species, log size and type, and decay class have been shown to drive the structure of wood-inhabiting fungal and bacterial communities (Hoppe, Krüger, et al., 2015; Purahong et al., 2017, 2018; Uhl et al., 2022). As an example, Operational Taxonomic Unit (OTU) richness and community structure of bacteria, have been reported to differ between sapwood and deadwood, mainly because of changes in pH and water content (Moll et al., 2018). Moreover, during decomposition, changes in wood physico-chemical properties such as C/N ratio and lignin content were found to significantly explain the variation in microbial community structure and diversity (Hoppe, Krüger, et al., 2015; Pioli et al., 2018).

Previous research suggested that fungal community composition follows distinct successional changes during the decay process (Fukasawa et al., 2009; Lepinay et al., 2022; Ottosson et al., 2014; Ovaskainen et al., 2013) as generalist primary colonizers are replaced by less competitive specialist taxa with higher decomposition abilities (Kubartová et al., 2007). Therefore, fungal compositional shift determines reciprocal changes in the wood structure and stoichiometry during decomposition, altering the available niches for the other wood-inhabiting organisms.

Despite the evidence mentioned above, the identification of wood traits that influence the responses of the species to micro-habitat temporal and spatial heterogeneity remains an open question, especially for bacteria and archaea. Moreover, very few studies investigated the relationships between log characteristics, community structure, and microbial-mediated ecosystem functions during wood decomposition (Hoppe, Purahong, et al., 2015). Decay patterns, and thus, carbon and nutrient dynamics are determined by microbial communities' functional traits. Fungi and bacteria can degrade wood polymers by secreting an array of diverse plant cell wall extracellular hydrolytic and oxidative enzymes. These enzymes, most of which have been categorized in the database of Carbohydrate-Active EnZymes (CAZy; <http://www.cazy.org/>) (Lombard et al., 2014; Marinović et al., 2018), are known to target cellulose, cellobiose and xylobiose, fungal glucans, lignin, and chitin. A recent study found that a large proportion of the CAZymes is produced by fungi and mainly targeted lignin or cellulose, while only 1.4% of CAZymes produced by bacteria targeted these recalcitrant substrates. However, the bacterial contribution to biopolymers decomposition was greater for starch/glycogen and peptidoglycan (Tláškal et al., 2021). In contrast, the role of archaea in wood cell degradation has not been fully studied yet, although, they contribute to C cycling via methanogenesis (Rinta-Kanto et al., 2016). Methane is the second most important anthropogenic greenhouse gas and the recent discovery that woody debris might constitute a source of biogenic methane suggests that CH₄ metabolic pathways are likely to vary with factors influencing wood decomposition (Covey et al., 2016; Mukhin & Voronin, 2009).

Despite this new evidence, more data are needed to better depict how genes related to C cycle change across decomposition stages and in which proportion. The presence of active bacterial and fungal decomposers and their complementary role in wood suggest the microbial communities to be related by complex interactions. Microbial interactions are reported to range from competition to mutualism. Although combative interactions are well known between wood decay fungi (Boddy, 2000), facilitation and niche complementarity have also been observed (Ottosson et al., 2014; Tiunov & Scheu, 2005; Toljander et al., 2006). For

example, the succession of wood fungi with different enzymatic abilities may facilitate the colonization of more specialized taxa (Maynard et al., 2018). Similarly, nitrogen-fixing bacteria were shown to support other decomposers under N-limited conditions like in deadwood (Hoppe et al., 2014; Tláskal et al., 2021; Tláskal & Baldrian, 2021), suggesting that synergistic or facilitative interactions might play a greater role in structuring microbial communities in natural environments than competition (Wardle, 2006).

Many studies focused on the intra- and inter-kingdom interactions between bacteria and fungi in wood (Hervé et al., 2016; Johnston et al., 2016; Murray & Woodward, 2003), although often using in vitro assessment that hardly reflect the complexity of natural systems where multiple species interact with each other and under varying environmental conditions. Therefore, recent approaches involved the study of co-occurrences to reveal patterns of species combative/facilitative interactions using multivariate logistic models (Ovaskainen et al., 2010). However, there is an ongoing debate whether to consider co-occurrences as indicative of direct or indirect interactions as they can simply reveal groups of species having similar habitat requirements (Odriozola et al., 2021; Ovaskainen et al., 2010). Therefore, multiple approaches involving genomic analyses coupled with in vitro studies are necessary to reveal the overall importance and dynamics of species interactions during wood decomposition.

In this study, we used shotgun metagenomics to identify changes in alpha and beta diversity of bacteria, fungi, and archaea in natural logs in relation to decay stage and wood properties. We also wanted to determine the main metabolic pathways involved in the decomposition process, with focus on genes encoding for microbial extracellular enzymes. Finally, we investigated the intra- and inter-kingdom species co-occurrences across decay classes using both in vitro assays and molecular data. In particular, we addressed the following hypotheses: (1) fungi are affected by changes in wood decay stage and C/N content with a larger extent compared with bacteria and archaea; (2) the abundance of genes involved in plant cell wall degradation and C dynamics varies according to decay stage; (3) co-occurrences patterns reflect ecological interactions among species of the same/different kingdom.

EXPERIMENTAL PROCEDURES

Study site and experimental design

The study was conducted in a sessile oak (*Quercus petraea* [Matt.] Liebl.) dominated forest in the Monticolo area (Autonomous Province of Bolzano, Italy; 46°25'35"N; 11°17'55"E) at 550 m above sea level (a.s.l.). Other minor species in the upper layer are Scots

pine (*Pinus sylvestris* L.) and sweet chestnut (*Castanea sativa* Mill.). Brown soil originating from porphyritic quartz rock characterizes the forest stand. Climate is temperate continental with an average of 11.4°C annual temperature and 800 mm of average annual precipitation (Giammarchi et al., 2020). In 2014, nine circular plots (12 m radius) have been permanently established for multidisciplinary research purposes. Our experiment was carried on within six of these plots.

In November 2017, three lying dead logs of sessile oak at three different decay stages were randomly selected from each plot for wood sampling. Decay class was assessed following Hunter (1990) but only three main decay stages were considered for our analysis: early (decay Class 2), intermediate (decay Class 3), and late (decay Class 4). Samplings of sawdust for microbial characterization were performed at three sampling points at the top, centre, and base of each log (Figure 1). At each sampling point, two sawdust samples were collected using a sterilized electric drill with a 10 mm diameter drill bit (see Pioli et al., 2018 for details). All samples were transported on ice and frozen at -20°C within 3 h. An additional wood sample was collected from each log for C and N characterization and microbial isolation. Total C and N were determined as described by Pioli et al. (2018). Ultimately, all the main features of the log (i.e., length, minimum and maximum diameter) were assessed in the field and used for multivariate analyses.

DNA extraction and quantification

Sawdust of each sampling point was pooled resulting in three replicates per log. After pooling, a total of 54 samples were ground in liquid nitrogen using a sterile mortar and pestle and kept frozen until further processing. DNA extraction was performed using PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, California) on ~0.2 g wood powder following manufacturer's instruction. Total DNA concentration was assessed using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California) and the Qubit dsDNA HS Assay Kit. DNA extracts were then shipped to the facilities of the Sequencing and Bioinformatic Service of the FISABIO (Valencia, Spain) for shotgun metagenomics using the Illumina NexSeq500 platform with 150 bp paired-end chemistry.

Bioinformatics

The resulting raw sequence reads were quality checked using the PRINSEQ-lite program v0.20.4 (Schmieder & Edwards, 2011). Sequences were trimmed for a minimum mean Q30 from the right side in a window of 20 bp and reads shorter than 50 bp were

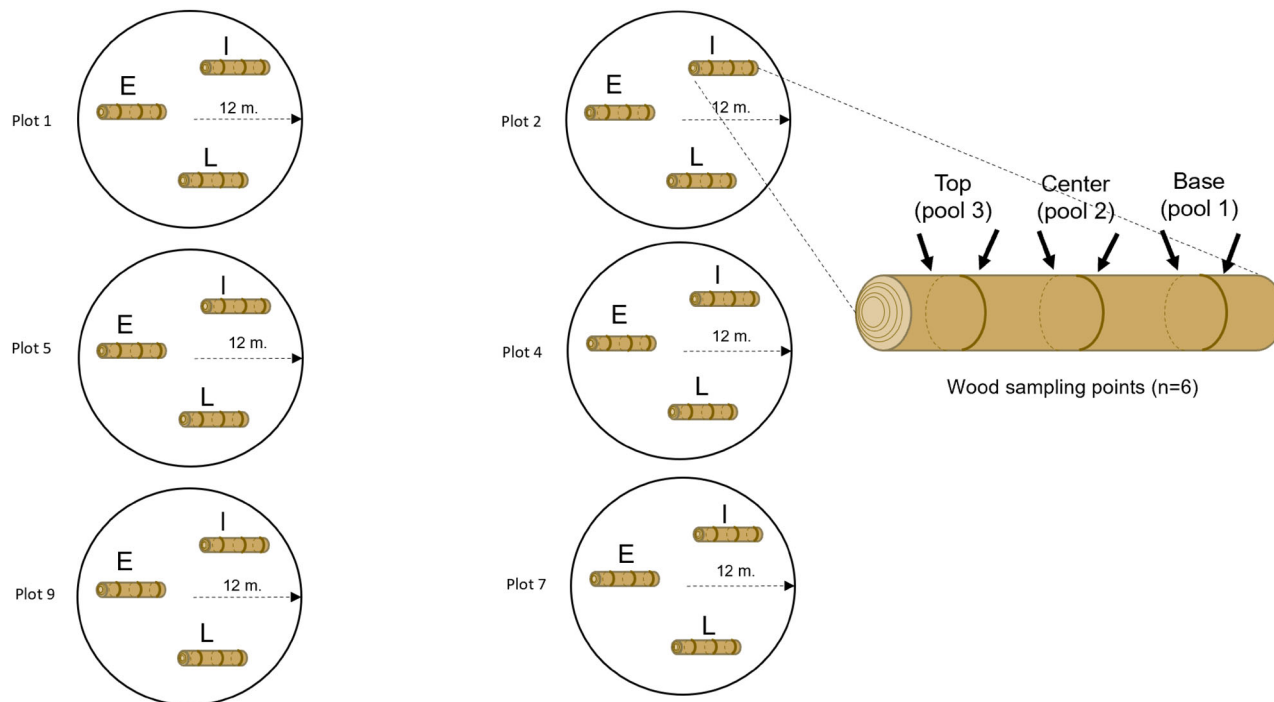


FIGURE 1 Schematic representation of the sampling design. Only lying dead logs of *Quercus petraea* were included (see text for details). E, early decomposition stage; I, intermediate decomposition stage; L, late decomposition stage.

removed. Residual adaptor-related sequences were eliminated using Trimmomatic v0.36 (Bolger et al., 2014). Forward and reverse sequences were joined with the FLASH program (Magoc & Salzberg, 2011), applying the default parameters.

Taxonomic classification was performed using Kraken (version 2.0.8-beta; Wood & Salzberg, 2014) which has been proven to ensure faster performances and comparable accuracy than any previously described tool for shotgun metagenomics (Lu & Salzberg, 2020). Kraken was run with default settings using the standard RefSeq database. Species abundance was estimated using Bracken (Lu et al., 2017). The Bracken's report files from each sample were then analysed using Pavian, a web tool for exploring metagenomics classification results (Breitwieser & Salzberg, 2020). The output tables from each sample obtained from Pavian were then combined and processed using ad hoc statistical tools.

Functional annotation of bacteria, fungi, and archaea was performed using MG-RAST server v4.0.3 (Meyer et al., 2008). Briefly, gene predictions were done using the FragGeneScan software, and the sequences were annotated based on BLASTX searches against the RefSeq and KO Subsystem using an e-value cutoff of $1e^{-5}$, a minimum alignment length of 15, and a minimum identity of 60% (default). Output tables were then filtered to identify specific metabolic pathways and genes predicted to encode CAZY. All uploaded metagenome datasets are publicly accessible on the MG-RAST server.

Microbial isolation, sequencing, and co-culturing

One fresh wood sample for each of the three decay classes was subsampled with sterilized scalpel and used for bacterial and fungal isolation following specific protocols. For bacteria, inner wood fragments (~1 g) were suspended in 100 mL of sterilized distilled water and homogenized by vortexing. Hundred millilitre of supernatant was serially diluted to 10^{-6} dilution using autoclaved distilled water. Dilution liquid was then spread over Luria Bertani agar plates amended with cycloheximide (Sigma). Plates were incubated at 25°C for 72 h, after which single colonies were selected according to their morphology and isolated. In total 40 isolates were obtained and stored at -80°C in 70% glycerol.

An adapted protocol from Lane et al. (2012) was used for the isolation of fungi from wood at different decay stages. Fresh surface-sterilized wood was plated on potato dextrose agar (PDA; three fragments per plate) and incubated in the dark at 25°C for 4–5 days. Developed colonies were then subcultured. In total, 28 fungal isolates were obtained and stored at -80°C in 10% glycerol.

A selection of isolated bacterial and fungal strains was further processed for rDNA amplification and sequencing. The bacterial 16S ribosomal DNA region was amplified via colony PCR method (Woodman, 2008) using primers 27F and 1494R (Lane, 1991) and previously reported PCR conditions (Marzorati et al., 2010). Fungal DNA was isolated from mycelium using the

protocol described by Penouilh-Suzette et al. (2020). The fungal ITS rDNA region was amplified and sequenced using primers ITS1F and ITS4R (Gardes & Bruns, 1993; White et al., 1990) with PCR conditions as in Carneiro and Baric (2021). All PCR products were shipped to Stabvida (Stabvida, Caparica, Portugal) for sequencing. For species-level identification, sequences were used as a query for a BLAST search in the NCBI database (<https://www.ncbi.nlm.nih.gov>) using a similarity cutoff value of 98%–100% for presumed species. Among bacterial and fungal isolates, four bacterial and three fungal strains were chosen for co-culturing after a preliminary taxonomic screening of shotgun molecular data. Isolated bacteria strains of *Luteibacter rhizovicinus*, *Bacillus simplex*, *Dermacoccus nishinomiyaensis*, and *Pseudomonas abietaniphila* were used for cross-species interaction as well as for bacterial–fungal interactions. In this latter case, all bacterial strains were cultured in a 100-mL Erlenmeyer flask containing 20 mL of potato dextrose broth (PDB) for 24 h at 25°C, with shaking at 120 rpm. Afterwards, 1 mL of the bacteria were collected by centrifugation. For bacterial–fungal interaction, three fungal strains were used, namely, *Trichoderma harzianum*, *Mucor plumbeus*, and *Absidia glauca*. The bacterium was inoculated first by placing 20 µL of the PDB culture at the centre of a PDA Petri dish. After 5–6 days at 25°C, the fungal strain was inoculated using two plugs (~5 mm of diameter) of a 3 days old actively growing mycelium and placed 2 cm from the centre. Plates with dual cultures and controls were incubated for 3–7 days at 25°C.

For bacterial–bacterial interactions, a modified cross-streak method was used as described by Kamat and Velho-Pereira (2012) where each strain was tested against one another. Briefly, the bacterial strain tested for inhibitory activity (tester) was single streaked in the centre of the Petri dish and incubated at 25°C for 3–7 days until clear colonies were formed. The plates were then seeded with target strains by streaking perpendicular to the line of test organism at fixed distances and plates were further incubated at 25°C for 14 days. The potential antagonistic interaction was determined by limited/inhibited growth of the target strains towards the test strain compared with the control plates where no test organism was inoculated.

Finally, for fungal–fungal interactions, mycelia disc plugs of two different fungal strains were placed on a PDA plate at a distance of 2 cm from the centre. All combinations of the aforementioned fungal strains were tested. Controls and dual cultures were incubated at 25°C for 3–7 days and checked daily.

Statistical analysis

Microbial gene counts as well as OTUs sequence reads were normalized to account for varying sequencing depth using cumulative sum scaling (Pereira et al., 2018). All statistics were run in R version 4.0.3 (R Core Team, 2020). Taxonomic alpha diversity of

bacteria, fungi, and archaea in different decay classes was assessed using OTUs richness (S), Shannon diversity (H'), and Pielou's Evenness (J) indices as implemented in the 'vegan' package (Oksanen et al., 2010; see Pioli et al., 2020 for indices formulas). Diversity indices were correlated with wood traits using Pearson's correlation (package 'Hmisc'; Harrell, 2014). Beta diversity of bacteria, fungi, and archaea was analysed using nonmetric multidimensional scaling (NMDS) based on Bray Curtis distance. Wood-related variables were fitted to the NMDS ordination plot using the *envfit* function, with *p*-values calculated on 999 permutations. To test for significant differences among decay classes for both diversity indices and gene abundances, we used one-way analysis of variance, followed by a Tukey's post hoc test ($p < 0.05$) for normally distributed data. Where assumptions of normality were not met, we used the non-parametric Kruskal–Wallis test, followed by Bonferroni correction for multiple comparisons (package 'agricolae'; De Mendiburu, 2015). Normality of data was checked using Shapiro–Wilk test. All graphs were created with the 'ggplot2' library (Wickham, 2016). Heatmaps showing relative abundances of microbial genes associated with decay classes were created using the 'ComplexHeatmap' package (Gu et al., 2016). Co-occurrence networks were performed using the CoNet (Faust & Raes, 2016) plugin for Cytoscape (Shannon et al., 2003; <http://www.cytoscape.org>). Correlation coefficients were calculated with both Pearson and Spearman correlation measures, Hellinger distance measure was used to infer synergistic and antagonistic relationships between taxa. The final network was generated from 1000 bootstraps iterations, and a *p*-value < 0.05 was considered to indicate statistical significance. The networks were visualized based on the type and number of relationships between OTUs. For bacteria, a simplified OTU table containing genus-level assignments was used to reduce computational issues.

RESULTS

After bioinformatics and quality filtering, a total of 14,268,414 raw reads and 4287 OTUs were found for bacteria. Fungi were represented by 1,409,339 reads and 57 OTUs, while for archaea 42,753 reads and 231 OTUs were found in total. The average number of OTUs per sample was 3377, 51 and 136 for bacteria, fungi, and archaea, respectively.

Diversity of microbial communities in wood

The most abundant orders among bacteria were Rhizobiales, Burkholderiales, Pseudomonadales, Sphingomonadales, and Corynebacteriales with this latter being

more represented in the advanced decay stages (Figure S1). The most abundant orders for fungi were Sordariales, Hypocreales, Glomerellales, Eurotiales, and Saccharomycetales. Decay Class 2 accounted for higher relative abundances of Hypocreales compared with the other decay classes, while in the advanced decay, we found higher abundances of Helotiales (Figure S1). Ultimately, we found Haloferocales, Halobacteriales, Natrialbales Methanosarcinales, and Thermococcales as representative of the most abundant orders among archaea. In this case, the relative abundance of the different orders did not change considerably according to decay stages except for Methanobacteriales that resulted higher than Thermococcales in decay Class 2 (Figure S1).

Bacterial alpha diversity resulted significantly affected by decay stage and wood characteristics (Table 1). Bacterial communities in well-decayed wood (decay Class 4) had higher Shannon and Evenness. Similarly, C/N ratio positively affected Shannon diversity and Evenness, whereas mean N was negatively related to these two indices for bacteria (Table 1[B]). Length of the log also positively affected bacterial alpha-diversity as increased length was related to Evenness. In contrast, diversity indices of fungal and archaea did not respond to variation in wood physical and chemical characteristics. Although the difference was not statistically significant, we observed increased species richness at decay Class 3 for all the microbial groups ($p > 0.05$; Table 1[A]).

The overall community structure of bacteria, fungi, and archaea in relation to wood traits was investigated via NMDS (Figure 2). As resulted from the envfit function, we observed that bacterial communities were mainly diversified because of different deadwood diameters ($p < 0.05$). Carbon content log length and diameter were significantly related to the structure of fungal communities, although, decay class resulted the main driver ($p < 0.01$). Also archaea were influenced by the diameter of log ($p < 0.05$), but in this case no significant effect of decay class was observed.

It is worthy to note that for the bacteria and fungi, each log hosted significantly different communities ($p < 0.05$ and $p < 0.01$, respectively) indicating that undetected microhabitat condition and possibly, biotic interactions at very small scales, may also influence the diversity and species assembly of microbial communities.

Microbial functional genes according to wood decay stage

Genes encoding CAZy targeted to plant cell wall degradation were proportionally different between bacteria and fungi (Figure S2). A total of 14 encoding genes were found for bacteria and fungi targeting pectin, starch,

TABLE 1 (A) Diversity indices of bacteria, fungi and archaea across decay stages (mean \pm SD). (B) Pearson's correlation coefficients between diversity indices and wood properties.

	Bacteria			Fungi			Archaea		
	S	H	J	S	H	J	S	H	J
A									
Decay Class 2	3300 \pm 700	5.99 \pm 0.88 b	0.74 \pm 0.12 b	50.8 \pm 1.42	3.02 \pm 0.24	0.77 \pm 0.06	131 \pm 45.1	4.32 \pm 0.45	0.91 \pm 0.02
Decay Class 3	3560 \pm 350	6.25 \pm 0.66 ab	0.76 \pm 0.08 b	51.7 \pm 1.08	3.11 \pm 0.16	0.79 \pm 0.04	144 \pm 28.5	4.46 \pm 0.20	0.90 \pm 0.02
Decay Class 4	3280 \pm 600	6.60 \pm 0.22 a	0.81 \pm 0.04 a	50.7 \pm 2.54	2.98 \pm 0.22	0.76 \pm 0.05	133 \pm 39.8	4.38 \pm 0.33	0.91 \pm 0.03
B									
Mean_CN	-0.17	0.33*	0.36**	-0.04	0.03	0.03	-0.07	-0.08	0.11
Cmean	-0.17	0.22	0.26	-0.23	-0.15	-0.13	-0.15	-0.17	0.15
Nmean	0.18	-0.33*	-0.36**	0.04	-0.13	-0.14	0.06	0.09	-0.09
Diam	-0.14	0.2	0.23	-0.17	-0.2	-0.18	-0.14	-0.21	0.01
Length	-0.2	0.26	0.35*	-0.24	-0.15	-0.12	-0.11	-0.21	0

Note: Cmean, average wood C content; Diam, average log diameter; H, Shannon diversity; J, Pielou's Evenness; length, log length; Mean_CN, average C/N ratio; Nmean, average wood N content; S, species richness. Different letters indicate significant differences between means (Kruskal–Wallis tested, followed by Bonferroni correction; $p < 0.05$). Asterisks denote significance (* $p < 0.05$; ** $p < 0.05$) levels.

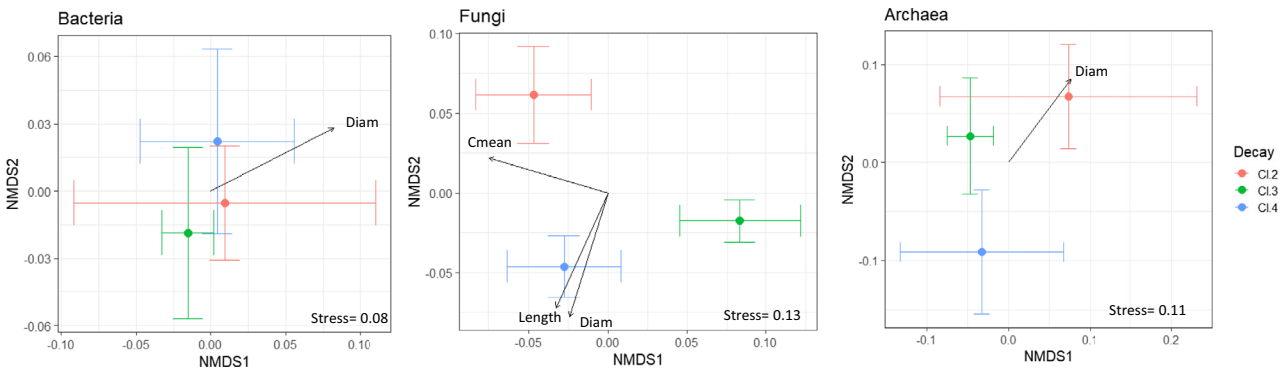


FIGURE 2 Nonmetric multidimensional scaling (NMDS) ordination of Bacteria, Fungi, and Archaea. Different colours indicate different decay class. Cmean, average wood C content; Nmean, average wood N content; Mean_CN, average C/N ratio; Diam, average log diameter; Length, log length.

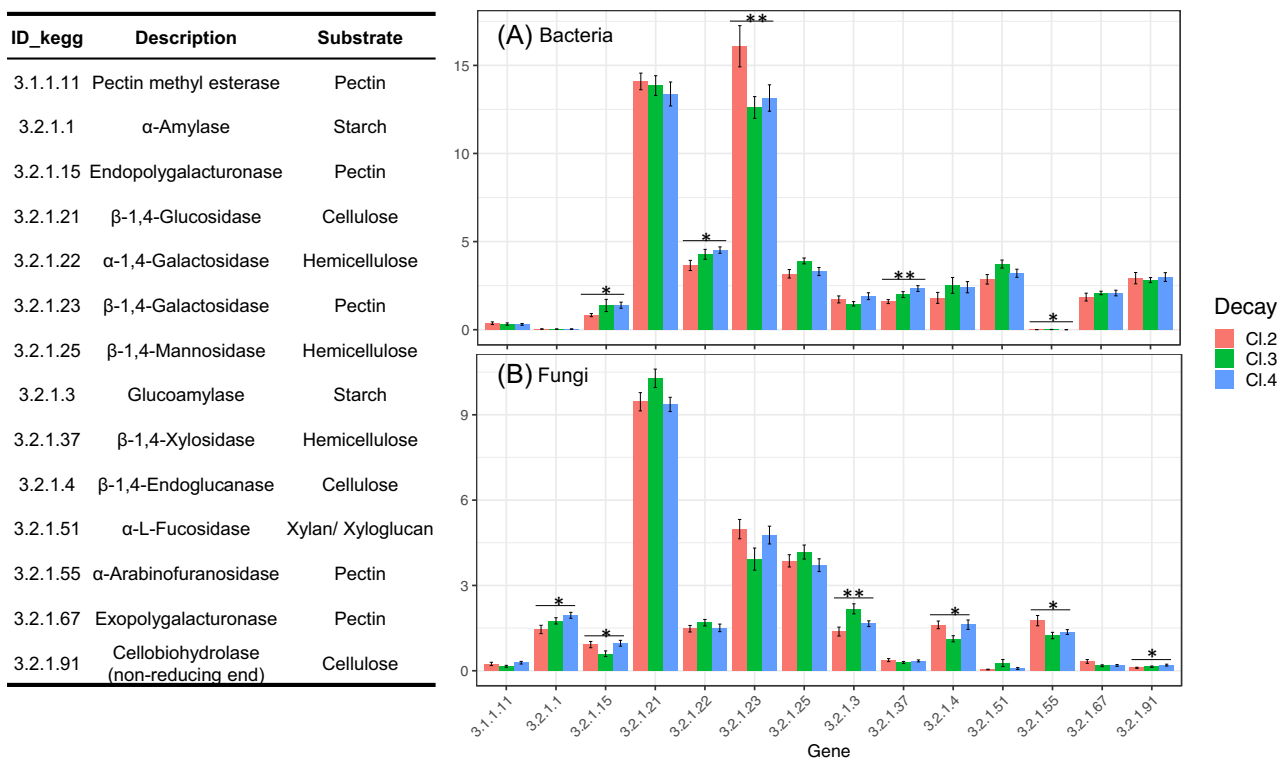


FIGURE 3 List of carbohydrate-active enzymes and their differences among decay classes in Bacteria (A) and Fungi (B). Asterisks denote significant differences between means (Kruskal–Wallis tested, followed by Bonferroni correction; * $p < 0.05$, ** $p < 0.01$).

cellulose, heteromannan, hemicellulose, and xylan substrates (Figures 3, S3, and S4). The β -1,4-Galactosidase involved in pectin degradation and the β -1,4-Glucosidase involved in cellulose degradation were the most abundant in bacteria and fungi, respectively. In bacteria, the reduced presence of β -1,4-Glucosidase targeted towards cellulose was compensated by higher abundances of Cellobiohydrolase. Other enzymes that differ between bacteria and fungi were those involved in xylan degradation that were more abundant in bacteria, and those responsible for starch degradation which were proportionally higher in fungi (Figure S5A). Several

enzymes resulted significantly different between decay classes in both bacteria and fungi (Figure 3). Wood in early stages of decomposition accounted for higher β -1,4-Galactosidase and lower Endopolygalacturonase in bacteria. Interestingly, the heteromannan depolymerizing enzymes were all higher in advanced decay stages (Figure S5B). For fungi, the intermediate class of wood decomposition was characterized by higher Glucoamylase and lower Endopolygalacturonase along with β -1,4-Endoglucanase (Figure 3). Higher levels of α -Amylase were observed in advanced decay stages, while α -Arabinofuranosidase was more abundant in early stages

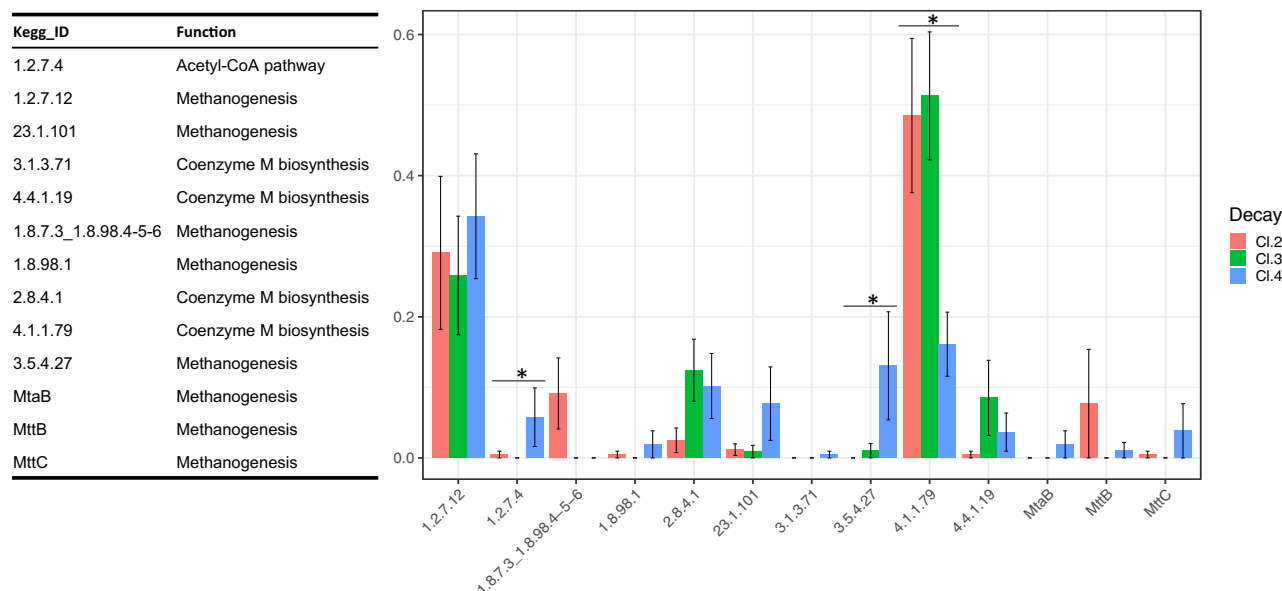


FIGURE 4 List of genes involved in methane cycling and their differences among decay classes in Archaea. Asterisks denote significant differences between means (Kruskal–Wallis tested, followed by Bonferroni correction; * $p < 0.05$).

of wood decomposition (Figure 3). In general, starch-degrading enzymes in fungi were significantly higher in intermediate and late decay stages (Figure S5B).

Other common pathways were investigated as well, such as nitrogen cycling and xylene degradation, however, since the genes involved were not statistically different between decay classes they were not further examined (Figures S6–S9).

Archaeal functional pathways were investigated by analysing the methane metabolism (Figures 4, S10, and S11). As reported in Figure 4, several genes involved in methanogenesis and coenzyme M biosynthesis were significantly different between decay classes. The most abundant genes were those related to Coenzyme M biosynthesis, which showed higher abundances at early stages of wood decomposition. On the other hand, genes regulating the methanogenesis were higher at both decay Classes 2 and 4 possibly indicating that despite the single genes changed during the decay process, the overall methanogenesis might not be influenced by decay stage.

Intra- and inter-kingdom co-occurrences of bacteria and fungi along the decay process

Co-occurrences networks revealed potential patterns of intra- and inter-kingdom interactions between bacteria and fungi in response to different decay stages (Figures S12–S14 and Tables S1 and S3). Bacterial networks in early decomposition classes were characterized by the dominance of positive co-occurrences with only scattered clusters of negative interactions, as for those including OTUs such as *Sneathia* (571) and *Kordia* (335) (Figure S12A and Table S1A,B). Interestingly,

Actinobacteria were almost entirely included in a single cluster where only few OTUs from other phyla were represented with negative co-occurrences (i.e., *Segniliparus* [560] *Paraoerskovia* [458], and *Cnui-bacter* [179]). Another important cluster was composed by a group of Proteobacteria almost entirely characterized by positive co-occurrences. As wood decomposition progresses (Class 3), we observed an increase in bacterial negative co-occurrences (Figure S12B and Table S1A,B) mainly determined by members of family *Flavobacteriaceae* (Bacteroidetes). Particularly evident are the networks sustained by: *Winogradskyella* (678), *Croceibacter* 190, *Polaribacter* (489) and, to a lesser extent, *Algibacter* sp. (40). In this decay, class is possible to observe a cluster grouping the majority of Actinobacteria interconnected by positive co-occurrences too. This cluster is less consistent in the last phase of decay process (Class 4) characterized by only few negative co-occurrences such as the ones related to *Candidatus Baumannia* (125) and *Cobetia* (180) (Figure S12C and Table S1A,B).

Networks of fungi were more simplified due to lower number of OTUs (Figure S13). Decay Class 2 is represented by small clusters interconnected by positive co-occurrences and one cluster with only negative co-occurrences, mostly determined by *Fusarium proliferatum* (23) (Figure S13A and Table S2). In this group, is worthy to mention the presence of *T. harzianum* (51), one of the fungal strains identified using the culture method. Fewer clusters were present in more advanced decomposition stages. At decay Class 3 we observed negative co-occurrences between *Neurospora crassa* (37), and other 6 species such as *Ustilago maydis* (52), *Cryptococcus gattii* (10), and *Malassezia restricta* (34) (Figure S13B and Table S2).

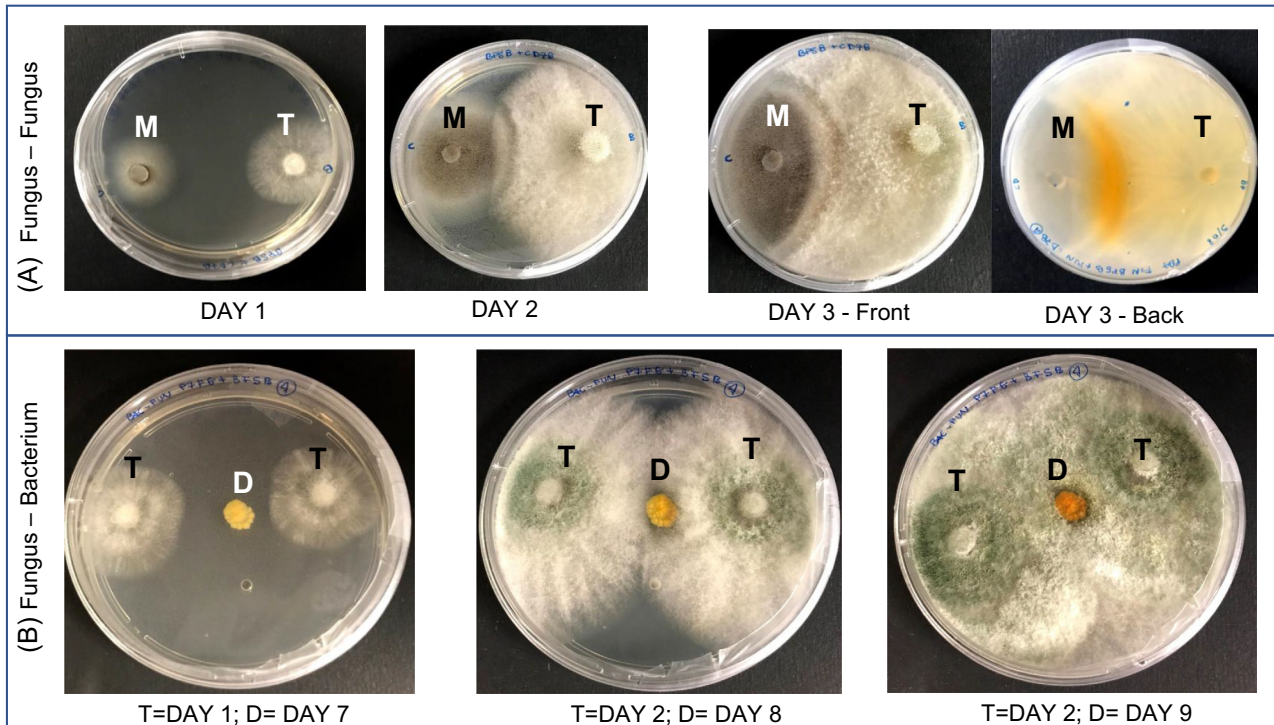


FIGURE 5 (A) Mycelia development of *Trichoderma harzianum* (T) in co-culture with *Mucor plumbeus* (M). (B) Mycelia development of *Trichoderma harzianum* (T) in co-culture with *Dermacoccus nishinomiyaensis* (D).

Complex networks characterized the co-occurrences patterns between bacteria and fungi especially at the end of the decomposition process (Figure S14 and Tables S1 and S2). Early stages of decay were characterized by the presence of few fungal taxa related by both positive and negative co-occurrences with groups of bacteria (Figure S14A and Tables S1 and S2). *Fusarium proliferatum* (23) was the fungal taxon accounting for the highest number of negative co-occurrences with bacteria ($n = 32$). A similar pattern was shown by *Pleurotus ostreatus* (39), and *T. harzianum* (51). Conversely, *Lachancea thermotolerans* (32) and *Naumovozyma dairenensis* (36) were some of the fungal taxa that positively co-occurred with a large number of bacteria (61 and 47, respectively). At middle decomposition stages, co-exclusion patterns were only determined by the presence of *P. ostreatus* (39) (Figure S14B and Tables S1 and S2). Positive co-occurrences at this stage were mainly supported by the presence of the genus *Clostridium* (178) for bacteria and the species *Komagataella phaffii* (31) for fungi. The complex network of negative and positive co-occurrences in advanced decomposition stage was mostly determined by *Yarrowia lipolytica* (53) from fungi, and bacteria of the genus *Streptococcus* (593) showing 35 and 33 co-occurrences, respectively (Figure S14C and Tables S1 and S2). Focusing on the positive inter-kingdom co-occurrences, *Streptococcus* (593) and *Fusobacterium* (265) accounted for the highest number among bacteria (33 and 26, respectively).

Among fungi, *Ogataea parapolyomorpha* (38) had the largest number of positive co-occurrences ($n = 28$). Ultimately, negative co-occurrences were dominated by a bacterium of the genus *Rhizobium* (521; $n = 19$) and the fungus *Sugiyamaella lignohabitans* (45; $n = 15$).

Microbial interactions were also tested using co-culturing methods and a selection of the resulting petri dishes is shown in Figures 5, S15, and S16. Fungal-fungal interaction involving *T. harzianum* were characterized by the dominance of this fast-growing fungus over the other species tested. As shown in Figure 5A, the development of *T. harzianum* mycelium over *M. plumbeus* resulted in a reduced radial growth of this latter compared with the control 3 days post-inoculation (Figure S15A). A common strategy in response to interspecific interaction is the formation of a barrage zone well visible from the back of the petri, where the growth of inhibitors and other metabolites may enhance the competitive potential of the two species involved (Luo et al., 2017). Competitive interactions were also reported between *T. harzianum* and *D. nishinomiyaensis*, a bacterium of the order Actinomycetales (Figure 5B). In this case, a slight antifungal activity was visible as a zone of fungal inhibition surrounding the bacterium. Moreover, a shift in bacterial pigmentation was evident after fungal inoculum compared with control (Figure S15B), possibly due to secondary metabolites produced by *T. harzianum*, which can be seen as another consequence of microbial physical interaction. Assays

between selected bacteria strains (i.e., *P. abietaniphila*, *Pseudomonas helmanticensis*, *D. nishinomiyaensis*, and *L. rhizovicinus*) are reported in Figure S16. We observed that the growth of all target bacteria strains was prevented by *B. simplex* which, in turn, was not affected by any of the strains, although a slight interaction was observed with *P. abietaniphila*. *Luteibacter rhizovicinus* only limited the growth of *D. nishinomiyaensis* while this latter prevented *P. abietaniphila* and *L. rhizovicinus* to develop. Finally, *P. abietaniphila* showed inhibitory effects on *D. nishinomiyaensis* and *L. rhizovicinus*.

DISCUSSION

In this study, we investigated the changes in microbial community structure and functions in natural downed wood at different decay stages. Moreover, we investigated intra- and inter-kingdom interactions between bacteria and fungi using co-occurrences networks and in vitro dual assays that revealed complex pattern of community structure in response to decay stage possibly reflecting both direct and indirect interactions.

Structural and functional microbial diversity across decomposition stages

Wood decomposition is a heterogeneous process leading to chemical and structural modifications at fine scales. The wood abiotic conditions at each decay step reflect the patterns of microbial assemblages that are composed by taxa with selective decaying and competitive abilities (Pastorelli et al., 2020). Therefore, the structure and diversity of communities involved in wood decomposition are affected by decay stage, as confirmed by our study. However, we observed that the extent of decay stage effect on microbial communities is different for each group of organisms. Bacteria respond to decay class in their diversity indices, fungi are affected by decay class only in their beta diversity, whereas archaea diversity indices and community structure did not respond to decay class. Other studies found higher bacterial diversity at late stages of wood decay suggesting that the greater availability of niche during this stage may support a more diverse community (Pastorelli et al., 2020). Similarly, we found Shannon and Evenness indices to increase as decomposition progress, although no significant change was observed for bacterial richness.

As in our study, fungal community structure has been widely reported to be influenced by deadwood decay class (Hoppe et al., 2016; Lepinay et al., 2022; Purahong et al., 2016) indicating successional shifts along the decomposition process, as primary decomposers are replaced by secondary and late colonizers that may be more competitive or more efficient in their

resource uptake. As a result, fungal decomposers alter the chemical and physical properties of wood which, in turn, determine shifts in fungal community (Boddy, 1992). For these reasons, we initially hypothesized that fungi could respond more directly to decay class than the other tested parameters and, in comparison, with bacteria and archaea.

We have mentioned that during wood decomposition, chemical and structural attributes change over time. In particular, C/N ratio decreases as a result of N accumulation. Even though, changes in the wood chemical composition may also reflect local condition such as the activity of the microbial community itself, which can determine differential rates of N immobilization, accumulation, and translocation during decomposition (Cornwell et al., 2009). Therefore, we tested the effect C and N content independently from decay class. Somehow surprisingly, we found a negative correlation between N and bacteria diversity indices. Increased N availability in certain logs may have altered bacterial diversity by selecting a more specialized community as reflected by the less balanced species composition (Dai et al., 2018; Liu et al., 2020; Wang et al., 2018).

Carbon content significantly affected community structure of all microbial groups. Carbon was reported to reflect in part the variation in lignin concentration in wood, which represents the recalcitrant compound limiting microbial decomposition. Therefore, despite C is considered an important macronutrient for microbial growth and reproduction, higher C abundance does not necessarily indicate a higher C availability (Purahong et al., 2018). Our finding corroborates results from recent studies demonstrating C content to drive alpha diversity of bacteria (Hoppe, Krüger, et al., 2015) and community composition of wood-inhabiting fungi (Hoppe, Purahong, et al., 2015; Rajala et al., 2011), respectively. Only few studies considered the effect of wood physico-chemical attributes on archaeal community (Pastorelli et al., 2020; Rinta-Kanto et al., 2016). In our case, C content affected neither the community structure nor the diversity indices of archaea, supporting the results from Pastorelli et al. (2020) who found no correlation between total C and archaeal abundance.

In our study, we also tested log characteristics, that is, length and diameter, that resulted to drive the beta diversity of bacteria and fungi. The role of log size for fungal diversity has been widely acknowledged in the literature based on evidence from fruit bodies inventories that showed positive correlations between wood size and fungal diversity (Kruys et al., 1999; Sippola et al., 2001; see Edman et al., 2004). Whereas the effect of log size on bacteria still needs to be elucidated. In general, deadwood of larger size represents a more heterogeneous resource with a variety of micro-niche available, therefore, sustaining more complex communities (Edman et al., 2004). Moreover, logs with

greater size may influence the microbial colonization pathways having larger surface in contact with air/soil (Edman et al., 2004).

Understanding the link between species diversity and ecosystem functions is one of the main goals of microbial ecology. Our study supported the idea that decomposition dynamics affect both structural and functional diversity and that bacteria and fungi both contribute to wood cell degradation. Wood fibres are composed mainly by cellulose, followed by hemicelluloses, pectin, and lignin with varying percentages depending on plant species, part, and age class (Schmidt, 2006). Other non-structural components include extractives (e.g., phenols and tannins), water-soluble compounds (e.g., sugars and starch), as well as proteins and ashes. We then examined the abundance of bacterial and fungal gene-encoding enzymes targeted to degrade the aforementioned polymers. Surprisingly, no peroxidases or laccase involved in lignin oxidation process were identified in the metagenomic annotation, possibly due to the relative low number of representative sequences available in the KEGG database or insufficient sequencing depth. However, we found an unexpected diversity of enzymes related to bacteria with some differences according to decay stage. Cellulose-targeting enzymes were found to be consistent across decomposition class for both bacteria and fungi while bacterial hemicellulolytic enzymes increased with decomposition. This pattern could indicate a stable or increasing resource availability; however, this is in contrast with several studies reporting a depletion of cellulose concentration in wood as decomposition progresses owing to a preferential degradation by brown-rot fungi, with a concomitant increase in the aromatic compounds (Gómez-Brandón et al., 2020; Lombardi et al., 2013; Petrillo et al., 2015; Strukelj et al., 2013). Yet, we have not measured the concentrations of wood organic fractions at different decay stages, which limit the possibility to infer changes in wood traits in relation to gene abundances. Moreover, metagenomics can provide information on the functional potential of microorganisms, but it does not imply that the predicted genotype is expressed.

Interestingly, starch-degrading enzymes in fungi were higher at middle decay stages. Starch is a very labile compound in wood and, thus, rapidly lost during the initial decay phases (Aneja et al., 2006). Therefore, higher gene encoding enzyme for starch may reflect a shift in fungal assemblages which we have seen to respond to C gradients. For example, *Aspergillus oryzae* (Eurotiales) is known to produce very high levels of solid starch-gel digesting amyloglucosidase that are widely used in food processing industries (Sivaramakrishnan et al., 2007). *Aspergillus oryzae* was found to be more abundant at decay Class 3 in our dataset, suggesting a possible role in the increased glucoamylase production. Similarly, members of Capnoidales (e.g., *Zymoseptoria*

tritici, *Cercospora beticola*) were more abundant at late decay stages. Although less studied compared with *Aspergillus*, the genome of *Z. tritici* was reported to encode at least 24 putative starch-degrading enzymes including α -amylases and a glucoamylase (Momeni et al., 2019), which may contribute to the observed increase of these enzymes at mid and late decay stages.

One of the key processes in forest ecosystems that impacts on global carbon cycle is the biological methane production. The most common pathway for the methane production in natural wood is the microbial methanogenesis by archaeal methanogens. Methanogenic archaea belong to the phylum Euryarchaeota and produce CH_4 as the end product of their anaerobic respiration. (Liu & Whitman, 2008) Although the effect of decay class on archaea has been only seldom assessed, their abundance has been found to increase with decomposition, while richness and Shannon diversity were reported to peak at middle decay stages (Pastorelli et al., 2020). We observed a similar trend in our study, with highest values of archaeal diversity at decay Class 3; however, our results were not significant. Nevertheless, we found two archaeal genes involved in methanogenesis to significantly change with decay class. Due to the complexity of the biochemical reactions required for the whole cycle, it is challenging to predict CH_4 dynamics based on decay class. Contrasting results have been reported in the emission of CH_4 rates along the decay process. Lagomarsino et al. (2021) and Pastorelli et al. (2017) found an increase of CH_4 emissions in the last classes of wood decay in black pine forests, while Covey et al. (2016) hypothesized that nonstructural labile C characterizing the early stages represent an important substrate for methanogenesis as it is more rapidly converted to CH_4 than recalcitrant structural compounds. Moreover, it has been proposed that mutualistic interactions between fungi (especially ectomycorrhizal fungi) and archaea may facilitate archaeal colonization (Mukhin & Voronin, 2009; Rinta-Kanto et al., 2016) and CH_4 production, as fungi may break down complex carbon compounds into smaller molecules for further reduction to CH_4 and CO_2 by archaeal methanogens (Covey et al., 2016). Since we have not explored the interactions between archaea and fungi, it is even tougher to draw conclusions on CH_4 dynamics. Nevertheless, our study represents a first attempt to track changes in methane pathways in natural decomposing wood using a metagenomic approach, suggesting an unexplored role of archaea in the deadwood nutrient cycling.

Microbial co-occurrences patterns in decaying wood

Microbial interactions in wood are thought to be a key factor controlling community structure and decay

processes. Although, to better understand the nature and extent of these interactions, field studies should be complemented by laboratory assays that directly address species combative traits. On the other hand, controlled microcosms could not fully reflect competitive dynamics and microbial succession occurring in natural environments under the influence of environmental variables (Song et al., 2012). In our study, we combined the results from the metagenomic analysis with *in vitro* bioassays. Unfortunately, only few cultivated strains of both fungi and bacteria matched the metagenomic database for a reliable comparison of intra- and inter-kingdom co-occurrences.

In general, we have observed that intra- and inter-kingdom interactions at all decay stages were mainly determined by positive co-occurrences, which is in line with previous studies on leaf litter (Pioli et al., 2020) and deadwood (Odriozola et al., 2021). Positive interactions including mutualism and facilitation are well known to occur during organic matter decomposition as most fungal and bacterial strains relies on the activity of strong decomposers that target lignin and other recalcitrant compounds with a resulting release of the more labile C fraction (McGuire & Treseder, 2010). Other synergistic interactions were observed between cellulolytic fungal species and fungi that were not able to degrade cellulose suggesting a predominance of facilitative over competitive interactions when large carbon sources are available (Tiunov & Scheu, 2005). Therefore, most of the facilitative interactions observed in deadwood are related to the ability of decomposers to create available niches for other decomposers of similar trophic position (Tiunov & Scheu, 2005). In our study, we have observed that actinobacteria were grouped apart from the other bacteria and were related by positive co-occurrences. Actinobacteria are known to produce bioactive metabolites that could potentially lead to combative interactions with other microbial organisms as observed with, for example, *Sneathia*, *Allofrancisella*, and *Arthrospira* at decay Class 2. However, a recent study on soil revealed that the actinobacteria generally tend to have neutral interactions with other co-occurring microbes (Yan et al., 2021) which could explain the isolation of the actinobacteria cluster from the others. These neutral processes may play an important role in maintaining the co-existence in highly diverse microbial communities, especially under oligotrophic conditions, such as in our study. Moreover, it has been suggested that bacteria with close phylogenetic distance have similar growth requirements and share similar niches (Yan et al., 2021), therefore, positive co-occurrences may indicate similar resource requirements, as for our actinobacteria cluster.

The intermediate decay stages (Class 3) were characterized by increased number of negative co-occurrences. It is possible that the loss of the bark and the shift in the microclimatic conditions at this stage

may affect the competition of microorganisms with different moisture and temperature optima (Song et al., 2012).

Negative co-occurrences may reflect competitive interactions. Competition for a limiting resource may either maintain microbial diversity via niche differentiation or determine a reduction in diversity through extinction of the inferior competitor. In this latter case, ecosystem functioning and, specifically, decomposition could be affected as the superior competitor might not be the most efficient decomposer (McGuire & Treseder, 2010). Suggested mechanisms leading to negative co-occurrences include priority effects, the production of inhibitory compounds as well as historical and phylogenetic processes (Pan & May, 2009). As an example, we found many Flavobacteriales negatively co-occurring with multiple bacterial strains at all decay stages. It is possible that these interactions are related to the production of (unidentified) inhibitory compounds as seen for *Flavobacterium* sp. in a study on *Arabidopsis* leaf microbiome (Helfrich et al., 2018).

As emerged from the comparison between co-occurrences network and co-culturing assays, species of the same genus may show different patterns from each other. In fact, we have reported a high antimicrobial activity of *B. simplex* against target strains that were not able to grow under lab conditions in dual essays. In contrast, *Bacillus* in our networks, positively co-occur with other bacteria and fungi at decay stage 2. However, none of the target strains involved in the *in vitro* assays was also found in the metagenomic dataset, limiting a direct comparison and the possibility to draw reliable conclusions.

Direct interactions between competing fungi in natural environments are controlled by several mechanisms, including the production of non-volatile and volatile toxins, extracellular enzymes, mycoparasitism, and hyphal interference (van der Wal et al., 2013). In contrast, indirect interactions may involve the presence of certain fungal species that, through changes in the substrate quality, can affect the decomposition activity of other co-occurring species (Osono, 2007). We have observed *T. harzianum* interactions to be consistent for both networks and bioassays and largely reflecting results from earlier studies. This fungus, like other species of the genus, is known to produce non-volatile antibiotics that, along with the faster metabolic rates that we also have observed, and its higher spatial and nutrient competition are key factors, which contribute to the successful antagonism of this fungus against a range of wood-decaying fungi and bacteria (Dennis & Webster, 1971; Schoeman et al., 1996; Verma et al., 2007). Moreover, antagonistic interactions were directly observed between *T. harzianum* and a species of the genus *Fusarium* (Verna 2007) which is consistent with our networks showing *F. proliferatum* to negatively co-occur with *T. harzianum* at early decay stages.

Fusarium proliferatum is a lignin-degrading fungus able to produce fusaric acid, which also inhibits the production of antifungal metabolites by bacteria (Boddy, 2016), explaining in part the negative inter-kingdom co-occurrences observed between *F. proliferatum* and other bacteria. Therefore, in these cases, the negative co-occurrence in our network was very likely to indicate direct antagonistic properties between taxa.

Concerning inter-kingdom interactions, recent studies on leaf litter suggested that bacteria may facilitate the decomposition processes by mediating other members of the decomposer communities, especially fungi (Romani et al., 2006). As an example, Purahong et al. (2016) found that the potential lignocellulose fungal decomposers in leaf litter such as *Clitocybe* spp. and *Mycena* spp. co-occurred with many bacterial genera able to fix nitrogen (e.g., *Bradyrhizobium*, *Mesorhizobium*, *Pseudomonas*, and *Rhizobium*). This is in contrast with our results showing *Rhizobium* to be negatively related with fungi, possibly indicating that wood in advanced decay stage is a more selective environment for potential N-fixing bacteria compared with leaf litter due to modifications of wood chemistry by the fungal activity. In fact, competition between bacteria and fungi has been most frequently reported during the early stages of wood decay characterized by a higher availability of more labile C fractions enhancing the occurrence of non-specialized decomposers (de Boer & van der Wal, 2008). Therefore, the larger number of negative co-occurrences at later decay stages that we have observed, are more likely determined by indirect interactions than direct competition or simply reflect different habitat requirements.

Understanding the direction and extent of microbial interactions is of key importance for predicting the patterns of ecosystem processes under varying environmental pressures. Experiments on litter decomposition dynamics have shown that positive interactions may enhance leaf litter degradation (Bani et al., 2019) while competitive interactions among multi-species microbial consortia were reported to negatively affect litter and wood decomposition (Boddy, 2000; Bonanomi et al., 2015; Cox et al., 2001). However, positive co-occurrences may just reflect similar habitat requirements of the species involved with no effect on process rates and/or a lack of competitive exclusion (Odriozola et al., 2021).

CONCLUSIONS

To conclude, the log itself should be considered as a unique and independent environment where the interaction between abiotic factors and biotic communities determines the patterns of microbial diversity. Our results also suggest re-evaluating the role of bacteria in

wood decomposition especially in relation to the more labile fractions and served as a basis for further investigation on archaeal functional pathways in wood.

We provided evidence that co-occurrences networks are very powerful tools for assessing species interactions within large microbial communities; however, it is difficult to draw general patterns as we have proved they can indicate either direct competitive/facilitative relationships as well as indirect interactions and differential niche preferences. Therefore, genomic studies coupled with in vitro co-culturing are recommended for disentangling the co-occurrences underlying nature. Finally, future research should address the effect of species interactions on ecosystem functioning by including decomposition rates and nutrients dynamics under varying environmental conditions.

AUTHOR CONTRIBUTIONS

Silvia Pioli: Investigation (lead); writing—original draft (lead); writing—review and editing (lead). **Elisa Clagnan:** Investigation (supporting). **Atif Aziz Chowdhury:** Investigation (supporting). **Alessia Bani:** Investigation (supporting). **Luigimaria Borruso:** Investigation (supporting). **Maurizio Ventura:** Investigation (supporting). **Giustino Tonon:** Conceptualization (supporting). **Lorenzo Brusetti:** Conceptualization (lead); funding acquisition (lead); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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