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ENVIRONMENTAL MICROBIOLOGY

Changes in the Microbial Community of *Pinus arizonica* Saplings After Being Colonized by the Bark Beetle *Dendroctonus rhizophagus* (Curculionidae: Scolytinae)

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Abstract

The death of trees is an ecological process that promotes regeneration, organic matter recycling, and the structure of communities. However, diverse biotic and abiotic factors can disturb this process. *Dendroctonus* bark beetles (Curculionidae: Scolytinae) are natural inhabitants of pine forests, some of which produce periodic outbreaks, killing thousands of trees in the process. These insects spend almost their entire life cycle under tree bark, where they reproduce and feed on phloem. Tunneling and feeding of the beetles result in the death of the tree and an alteration of the resident microbiota as well as the introduction of microbes that the beetles vector. To understand how microbial communities in subcortical tissues of pines change after they are colonized by the bark beetle *Dendroctonus rhizophagus*, we compare both the bacterial and fungal community structures in two colonization stages of *Pinus arizonica* (Arizona pine) employing Illumina MiSeq. Our findings showed significant differences in diversity and the dominance of bacterial community in the two colonization stages with Shannon ($P = 0.004$) and Simpson ($P = 0.0006$) indices, respectively, but not in species richness with Chao1 ($P = 0.19$). In contrast, fungal communities in both stages showed significant differences in species richness with Chao1 ($P = 0.0003$) and a diversity with Shannon index ($P = 0.038$), but not in the dominance with the Simpson index ($P = 0.12$). The β-diversity also showed significant changes in the structure of bacterial and fungal communities along the colonization stages, maintaining the dominant members in both cases. Our results suggest that microbial communities present in the Arizona pine at the tree early colonization stage by bark beetle change predictably over time.

Keywords Microbial structure · Bacteria · Fungi · Bark beetle · Arizona pine

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Introduction

The death of trees is a natural and complex ecological process that promotes the regeneration, organic matter recycling, and the community structure within forests [1]. Several biotic (e.g., insects, diseases) and abiotic (e.g., changes in temperature, levels of precipitation, fire) factors can lead to disturbances and the loss of biodiversity [1–3]. *Dendroctonus* bark beetles (Curculionidae: Scolytinae) are natural inhabitants of coniferous forests (Pinaceae) that play an important role in the process of forest recycling and regeneration [4].

The life cycle consists of adults emerging from host trees and dispersing in the forest to locate and colonize new trees. Females attract mates, and together, they build galleries where females oviposit, and then larvae develop, feeding

on phloem tissue [5]. Some species use secondary chemicals as cues for host-recognition, or for synergizing aggregation pheromones employed by bark beetles to carry out mass attacks and overcoming the host tree's defenses [6]. Populations of some species undergo periodic outbreaks during which they can kill thousands of healthy trees, causing significant disturbance in woodlands and economical losses to the forestry industry [5, 7].

Significant changes in the tree physiology occur as result of host-colonization by *Dendroctonus* bark beetles. Firstly, boring and feeding of bark beetles induce resinosis, and the allocation of terpenes, phenolic compounds, carbohydrates, and minerals, as well as the formation of traumatic resin ducts. In addition, severe damage occurs in vascular tissues due to the introduction of microbes that penetrate phloem, as well as cavitation caused by a water column breakage of the plant. This promotes foliar moisture declination, as photosynthesis ceases, along with needles desiccation [8–12].

From colonization to death, many microorganisms (bacteria, yeasts, archaea, and filamentous fungi) coexist in the subcortical habitat. These microbes come from several sources, some are endophytes or epiphytes [13–15], while others are vectored into the tree by bark beetle in their guts [16–19]; on their exoskeletons [17]; or, for some, in specialized structures (e.g., mycangium) [20, 21], as well as of other contamination sources, such as the frass of other insects or environmental contamination [22, 23].

Despite several studies characterizing the microbial communities in healthy tissues of coniferous trees [24–28], it is to our knowledge that there have been no approaches done that evaluate how these communities present in subcortical tissues change after colonization by insects. Given that bark beetles and the associated microbes are a strong selective pressure that triggers severe physiological changes in trees [29], we hypothesize that microbial communities change as they die. Arizona pine is a preferential host of *Dendroctonus rhizophagus* in the Sierra Madre Occidental in Mexico, which is an aggressive species that does not produce massive attacks on mature pine trees, but a pair or two—in some cases—colonizes and kills saplings < 10 cm diameter and < 3 m high from 11 pine species, including the Arizona pine [30]. Because *D. rhizophagus* is a species with a univoltine (i.e., from egg to adult is completed within a year) and synchronous (i.e., the same developmental stage occurs at the same time throughout its distribution range) life cycle, we characterized and compared the changes in the microbial community (bacterial, archaeal, and fungal) present in the subcortical tissue of Arizona pine saplings in two well-defined colonization stages: when paired female and male are present into tree prior to oviposition (early stage) and when their offspring is found in pupa stage (late stage).

Materials and Methods

Site Location, Collection, and Processing of Samples

The bacterial, archaeal, and fungal communities were recovered from subcortical tissues of Arizona pines saplings (approx. 10 years) attacked by *D. rhizophagus* in San Juanito, Bocoyna Municipality, Chihuahua State (27° 45′ 11″ N 107° 38′ 06″ W, 2288 masl). The phloem was collected near to insect gallery just prior to oviposition and while the color of the sapling foliage was green (early colonization stage; early August) (Supplementary Fig. 1A-C). It was also collected near to pupal chamber when broods of the couple reached the pupa stage, other arthropods were present into trees, foliage ranged from yellow to red, and trees were nearly completely dead (late colonization stage; early April) (Supplementary Fig. 1D-G). The phloem was carefully scraped using sterile fine forceps and scalpels, placed in sterile 50-ml tubes, and stored at 4 °C for transport to the laboratory, where they were immediately processed. Three replicates were prepared for each stage, and three pines for each replicate were selected and pooled.

The tissues of each replicate were rinsed for 30 s with 70% ethanol. A volume of 30 ml of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and 0.1% Triton X-100 [17] was added to tubes. The samples were shaken overnight (180 rpm), and then the pooled microbial suspensions were centrifuged for three mins at 8000×*g* in sterile 2-ml screw-cap tubes to concentrate the biomass.

Metagenomic DNA Extraction, Library Preparation, and Sequencing

Metagenomic DNA was extracted using the method described by Gonzalez-Escobedo et al. [28]. DNA was quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE) and was observed in an agarose gel 1.0%. The extracted DNA samples were amplified for the V3-V4 region of 16S rRNA gene using the primer pairs Bakt_341F (5′-CCTACGGGNGGCWGCAG-3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′) for characterizing the bacterial community [31], and the internal transcriber spacer (ITS) region, using ITS1 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primer pairs for characterizing the fungal community [32]. The 16S rRNA and ITS amplicons were sequenced using paired-end 2 × 300 bp on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA) at Macrogen Inc. (Seoul, South Korea).

Bioinformatic Analysis

Illumina-paired reads were merged using FLASH v 1.2.11 [33] with default settings. Sequence files were then

converted from fastq to fasta. Merged reads were processed with VSEARCH v 2.4.3 [34] and Quantitative Insights Into Microbial Ecology (QIIME) v 1.8 [35]. Bacterial/archaeal and fungal reads were each pooled, de-replicated, and checked for chimeras. Chimeras, singletons, and doubletons were discarded, and then, reads were clustered into operational taxonomic units (OTUs) at 97% similarity and an OTU table was constructed for each. Taxonomy was assigned to each OTU using the RDP naïve Bayesian classifier (bacterial/archaeal) [36] and UNITE (fungal) [37] databases and corroborated manually against the EzBioCloud (bacterial/archaeal) [38] and GenBank (bacterial/archaeal/fungal) [39]. Sequences that matched chloroplasts were manually removed, and then, for each OTU table (bacteria, archaea, and fungi), a heat map at the genus level of samples of colonization stages was constructed, with the information of the relative abundance presented as a percentage. In addition, the bacterial and fungal OTUs and assigned genera distributions across two stages were presented in Venn diagrams that were generated with the web application Venny [40]. Unfortunately, due to the extremely low number of OTUs for the archaeal community, diversity analyses were not carried out for it.

Bacterial and fungal communities were homogenized with respect to the sample with the lowest reads. To calculate α-diversity within these communities in both stages, we estimated species richness, using the observed OTU number and Chao1 [41], species diversity with Shannon [42], and the dominance with Simpson index [43] in QIIME. The diversity indices of early and late colonization stages were compared, using the Mann-Whitney U test to evaluate statistical significance between the samples ($P < 0.05$). To calculate the sequence coverage obtained for the 16S rRNA and ITS region datasets, Good's coverage estimator was used [44], along with rarefaction curves that were generated with QIIME to compare relative levels of bacterial and fungal OTU diversity across all samples.

To measure the degree of differentiation of the bacterial and fungal communities in the two colonization stages, we estimated β-diversity using the Bray-Curtis dissimilarity index. We performed, using the Bray-Curtis matrix, a hierarchical cluster analysis with the unweighted paired group method with arithmetic mean (UPGMA) and a principal coordinate analysis (PCoA) to assess the dissimilarities or distances among communities during the ordination solution. The dendrogram and plots corresponding were visualized in NTSYSpc v 2.02 [45]. Significant differences in the community composition of bacterial and fungal of the two colonization stages were tested by mean of a permutational multivariate analysis of variance (PERMANOVA), after 999 random permutations in PAST v 3.11 [46].

Sequence Accession Numbers

The Illumina sequence data reported here were deposited in the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP149017.

Results

Sequencing Results

A total of 1,111,959 (bacteria), 1716 (archaea), and 1,122,557 (fungi) high-quality reads were obtained from all samples at the two colonization stages. The $OTU^{97\%}$ number represented in these reads was 682 for bacteria, 9 for archaea, and 634 for fungi (Table 1). Of these, 92%, 100%, and 72% were assigned to the taxonomic category of genus of these microbial groups, respectively; the remaining $OTUs^{97\%}$ (8% bacteria and 28% fungi) were classified into taxonomic categories above the genus level (from family to phylum).

Microbial Community Composition

A total of 20 distinct phyla, 40 orders, 54 families, and 60 bacterial genera were identified from total $OTUs^{97\%}$ in all samples of two stages. The phylum Proteobacteria comprised 93.80% of the relative abundance of all $OTUs^{97\%}$, while 19 phyla represented the remaining 6.20% (Supplementary Fig. 2A). At the family level, Enterobacteriaceae was the most abundant (69.10%) taxonomic group, followed by Pseudomonadaceae (12.29%), Xanthomonadaceae (6.13%), Acetobacteraceae (3.25%), Sphingomonadaceae (2.60%), and Acidobacteriaceae (1.18%), and 48 families with abundances comprising < 1.00% of total reads each (Supplementary Fig. 3A).

At the genus level, *Rahnella* was the dominant taxa (63.23%) in all the samples tested, followed by *Pseudomonas* (13.05%), *Pseudoxanthomonas* (5.90%), *Enterobacter* (4.24%), *Kosakonia* (1.92%), *Sphingomonas* (1.69%), *Pantoea* (1.22%), *Burkholderia* (1.12%) (Fig. 1a), and other low-frequency genera (< 1.0%). Substantial changes in relative abundance were shown in the most abundant bacterial genera between colonization stages (Supplementary Fig. 4A). Only two archaeal genera, *Methanoculleus* and *Methanosaeta*, belonging to the Euryarchaeota phylum, were detected in both stages (Fig. 1b).

On the other hand, a total of five phyla, 33 orders, 54 families, and 83 genera were identified from total $OTUs^{97\%}$ in all samples of the fungal of both colonization stages. Ascomycota was the most abundant phylum (92.12%), followed by Basidiomycota (7.28%), Zygomycota (0.54%), Glomeromycota (0.025%), and

Table 1 Summary of Illumina data and α-diversity indices

	Replicate	No. of high-quality reads	Observed OTUs$^{97\%}$	Good's coverage (%)	Diversity index		
					Chao1	Simpson	Shannon
Bacterial/archaeal							
Early stage	S1Bac1	144,799/0	277/0	99.99	287.5	0.75	3.04
	S1Bac2	217,083/951	481/9	99.99	498.27	0.77	3.44
	S1Bac3	162,968/362	455/7	99.98	473.10	0.72	2.73
Late stage	S2Bac1	168,612/81	484/7	99.97	516.22	0.97	5.82
	S2Bac2	201,122/132	528/8	99.99	543.83	0.95	5.52
	S2Bac3	217,375/190	490/7	99.98	515.5	0.92	4.72
Fungal							
Early stage	S1Fun1	181,436	264	99.98	281	0.89	4.47
	S1Fun2	205,390	266	99.99	269	0.94	5.7
	S1Fun3	158,578	232	99.99	244	0.84	4.18
Late stage	S2Fun1	227,256	115	99.99	116.5	0.86	3.45
	S2Fun2	166,828	130	99.99	131.5	0.8	3.43
	S2Fun3	183,069	128	99.99	129.2	0.73	2.99

Chytridiomycota (0.02%) (Supplementary Fig. 2B). At the family level, Saccharomycetaceae (38.89%), Pichiaceae (18.34%), Trichocomaceae (10.10%), Helotiaceae (6.61), Ophiostomataceae (5.26%), Dothioraceae (3.82%), Mycosphaerellaceae (2.79%), Herpotrichiellaceae (2.58%), Sporobolomycetaceae (2.58%), Clavicipitaceae (1.58%), Rhizopogonaceae (1.58%), and Venturiaceae (1.10%) were the most abundant groups, followed by the 42 families present in low frequencies (< 1.00% of relative abundance) (Supplementary Fig. 3A).

At the genus level, *Cyberlindnera* (40.51%), *Candida* (19.1%), *Penicillium* (10.52%), *Ceratocystiopsis* (4.44%), *Chalara* (3.89%), *Sydowia* (3.81%), *Cladosporium* (2.91%), *Rhodotorula* (1.94%), *Phaeomoniella* (1.74%), *Rhizopogon* (1.65%), *Cordyceps* (1.64%), and *Ophiostoma* (1.04%) were the most abundant taxa (Fig. 1c), while the remaining genera were present in frequencies of < 0.1% of total reads each. Significant changes were observed in the relative abundance of fungal community (Supplementary Fig. 4B).

The Venn diagrams displayed the bacterial and fungal OTUs/genera shared and unique between both colonization stages (Fig. 2). In bacteria, 473 OTUs/50 genera were shared between two stages, and 72 OTUs/4 genera and 137 OTUs/6 genera were exclusive of early and late colonization stages, respectively (detailed information on bacterial genera available in Supplementary Table 1). On the other hand, in fungi, 91 OTUs/34 genera were shared between both stages, and 379 OTUs/33 genera and 164 OTUs/16 genera were exclusive of early and late colonization stages, respectively (detailed information on the fungal genera available in Supplementary Table 2).

α- and *β*-diversity

Rarefaction curves of bacterial and fungal samples all tended to approach the saturation plateau (Fig. 3), while Good's coverage values were also > 99.9% also for all samples (Table 1), indicating that the sequencing effort was enough to recover most of the bacterial and fungal diversity.

No significant differences were found in the species richness of bacterial communities between colonization stages with Chao1, because the richness was almost similar ($P_{Chao1} = 0.19$). However, significant differences were observed in the diversity ($P_{Shannon} = 0.004$) and dominance ($P_{Simpson} = 0.0006$), with *Rahnella* being the most abundant and dominant in the early stage, and this genus together with *Pseudomonas* and *Pseudoxanthomonas* in the late stage. On the other hand, the fungal α-diversity showed significant differences between colonization stages in the species richness ($P_{Chao1} = 0.0003$) and diversity ($P_{Shannon} = 0.038$), given that the taxa number ranged from 244 to 281 in the early stage and 116.5 to 131.5 in the late stage. The dominance did not show differences ($P_{Simpson} = 0.12$), being *Cyberlindnera*, *Candida*, and *Penicillium* the dominant genera in both stages.

The three first coordinates of PCoA analysis, using the Bray-Curtis dissimilarity matrix, explained 95.49 and 91.62% of the total observed variation in the analyses of bacterial (Fig. 4a) and fungal (Fig. 4c) communities, respectively. PCoA analyses showed significant differences in the β-diversity of bacterial (PERMANOVA; $P = 0.02$) and fungal (PERMANOVA; $P = 0.0001$) communities between colonization stages. In addition, the cluster analysis, also based also in the Bray-Curtis dissimilarity matrix, showed likewise that the composition of bacterial and fungal communities was different depending on the colonization stage (Fig. 4b, d).

a

Taxa	Early stage			Late stage		
	S1Bac1	S1Bac2	S1Bac3	S2Bac1	S2Bac2	S2Bac3
Rahnella	90.81	90.77	95.52	29.01	28.68	50.23
Pseudomonas	0.09	0.11	0.13	22.50	25.10	25.68
Pseudoxanthomonas	0.19	0.12	0.15	17.70	5.17	11.82
Enterobacter	1.25	2.35	0.01	2.18	14.40	3.29
Kosakonia	0.00	0.00	0.00	0.05	10.43	0.00
Sphingomonas	0.56	0.52	0.37	8.06	1.77	0.11
Pantoea	4.84	0.70	0.05	1.14	0.60	0.83
Burkholderia	0.19	0.33	0.05	3.72	1.69	0.96
Acidocella	0.24	0.12	0.03	4.35	1.31	0.04
Lactobacillus	0.04	1.22	0.94	0.36	1.42	0.80
Novosphingobium	0.24	0.22	0.40	0.95	2.59	0.18
Serratia	0.07	0.00	0.00	1.00	1.99	1.08
Prevotella	0.00	1.28	0.71	0.33	1.05	0.72
Acidisoma	0.19	0.16	0.07	2.23	0.28	0.12
Raoultella	0.01	0.01	0.00	0.61	0.21	1.53

b

Taxa	Early stage			Late stage		
	S1Arc1	S1Arc2	S1Arc3	S2Arc1	S2Arc2	S2Arc3
Methanoculleus	0.00	82.23	38.07	100.00	76.52	100.00
Methanosaeta	0.00	17.77	0.00	0.00	23.48	0.00

c

Taxa	Early stage			Late stage		
	S1Fun1	S1Fun2	S1Fun3	S2Fun1	S2Fun2	S2Fun3
Cyberlindnera	23.21	33.11	37.20	36.84	59.83	54.59
Candida	0.10	6.35	15.08	37.18	21.49	31.55
Penicillium	32.08	1.74	27.38	0.05	0.37	0.03
Ceratocystiopsis	0.00	0.00	0.00	12.81	7.92	5.55
Chalara	3.23	5.87	3.64	10.18	0.00	0.08
Sydowia	13.87	5.80	2.46	0.32	0.14	0.07
Cladosporium	7.59	6.43	3.51	0.00	0.00	0.11
Rhodotorula	0.90	9.99	0.72	0.39	0.48	0.13
Phaeomoniella	2.38	4.73	3.67	0.00	0.00	0.00
Rhizopogon	0.01	5.42	0.05	0.10	2.16	2.64
Cordyceps	4.24	5.75	0.00	0.02	0.00	0.14
Ophiostoma	0.01	0.23	0.15	1.17	3.04	1.81
Oidiodendron	2.92	1.20	0.06	0.07	0.03	0.00
Rhizosphaera	3.22	0.67	0.03	0.00	0.00	0.00
Mortierella	0.04	2.22	0.48	0.03	0.00	0.31

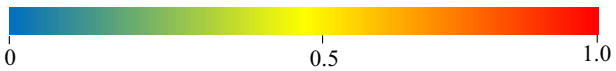

Fig. 1 Heat maps showing the most abundant bacterial (**a**), archaeal (**b**), and fungal (**c**) genera, all with their relative abundance as percentages

Discussion

The present study analyzes the structure of microbial communities in subcortical tissues of Arizona pine saplings and the changes produced in them as a result of the attack and colonization carried out by the bark beetle *D. rhizophagus*. Our findings showed that the number of bacterial (20) and fungal (5) phyla detected in this study were almost constant across of the two colonization stages analyzed. However, the bacterial phyla number was over three times that of those detected (7) in a previous study that analyzed tissues of healthy sapling of Arizona pine [28]; whereas the fungal phyla number could not be compared because they have not been studied in healthy saplings.

Proteobacteria was the dominant bacterial phylum in the two colonization stages analyzed, which was also found to be the dominant group in decaying wood of conifers and other trees (e.g., *Keteleeria evelyniana*, *Picea abies*, *Fagus sylvatica*). This was done using both conventional molecular methods and next-generation sequencing (NGS) technologies [47–50]. The dominance of

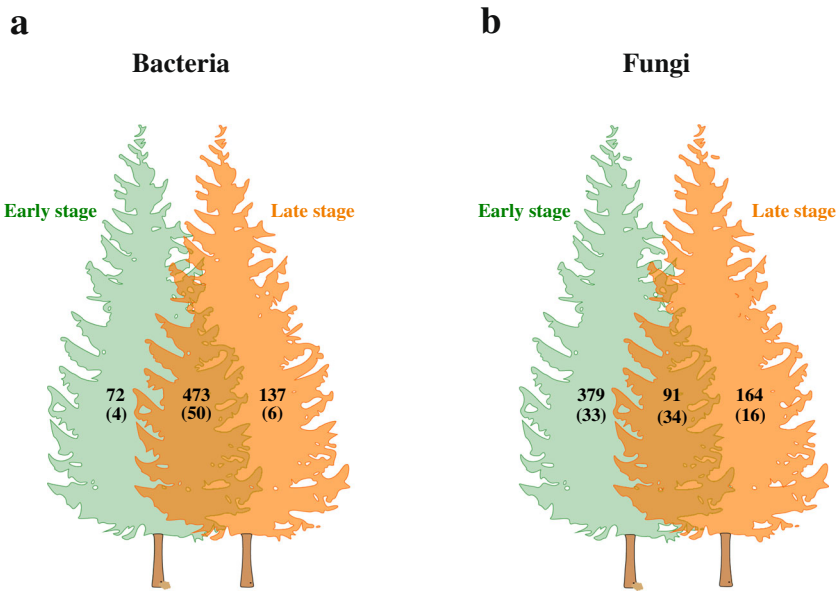

Fig. 2 Venn diagrams showing the distribution of (**a**) bacterial and (**b**) fungal $OTUs^{97\%}$ associated with Arizona pine in early and late colonization stages by *Dendroctonus rhizophagus*. The number in parentheses represents the bacterial and fungal genera shared and unique, defined from taxonomic assignment of $OTUs^{97\%}$, between colonization stages

this phylum in wood samples under an advanced decaying state is assumed to be a reflection of their capacities to degrade available nutrients in acidic environments and with oxidative stress caused by the activity of wood-decay fungi [51, 52]. In fact, the presence of Archaea belonging to the Euryarchaeota phylum, mainly of the genera *Methanoculleus* and *Methanosaeta*, suggests a favorable environment for these members. In addition, these Archaea have also been detected in association with mycorrhizal fungi in *Picea abies*, *Alnus glutinosa*, and *Pinus sylvestris* [53, 54]. Other studies have reported Euryarchaeota from soil, which have the capacity to produce methane and act as decomposers of organic matter [55, 56], which may be a possible contribution to these Archaea in attacked pines by bark beetles.

The high abundance of Ascomycota members (e.g., *Cyberlindnera*, *Candida*, and *Penicillium*) in tissues of Arizona pines attacked by *D. rhizophagus* could be explained by their natural presence in healthy trees as endophytes [57, 58] and due to a number of them being pioneer species that can rapidly colonize new substrates [59], such as recently colonized pines and its death by the action of these insects. Whereas the Ascomycota was the most abundant in the two stages analyzed; no studies have reported this phylum in the tissues of trees colonized by insects. Previous studies have reported Basidiomycota as the main fungi in decaying tissues of fallen trunks, branches, stumps, littered leaves, and needles of pines and other conifers [60, 61]. However, all these studies were done in advanced stages of natural tree death, not in trees colonized by insects.

As such, it is not possible to make direct comparisons on α-diversity with previous studies, as, to our knowledge, no studies have characterized bacterial and fungal communities in tissues of pines attacked by other bark beetles. Our findings show that the diversity of bacterial and fungal genera increases as the state of decomposition of subcortical tissues in Arizona pine progresses (Table 1), favoring the presence of both bacteria (e.g., *Azospirillum*, *Anaeromyxobacter*) and fungi (e.g., *Phoma*, *Neonectria*) opportunists that produce enzymes that degrade the wood polymers, such as cellulose, lignin, and hemicellulose [62].

The presence of *Rahnella* as the dominant member in the early stage, and of this genus, together with *Pseudomonas* and *Pseudoxanthomonas* in the late stage, suggests that from the beginning of the attack of the bark beetle, an alteration of the endophytic microbial communities occurs in the subcortical tissue of these pine trees. In fact, it is well-known that during tree colonization, bark beetles alter the physical and mechanical defenses, along with the physiology of the tree and introduce microorganisms that might modify the microbiome of healthy pines [63–65]. Unfortunately, there are not studies about endophytic fungi in healthy Arizona pine tissues. Hence, it is not possible to know whether the dominance of the fungal genera (*Candida*, *Cyberlindnera*, and *Penicillium*) found in this study was a result from the action of this bark beetle.

An interesting aspect that should be noted is that dominant bacterial genera in the early and late stages of colonization of the subcortical tissues of Arizona pine are not the most frequent members in the endophytic bacterial community of healthy pine saplings. In contrast, they are the dominant bacteria in the *D. rhizophagus* gut across their different developmental stages [18, 66, 67],

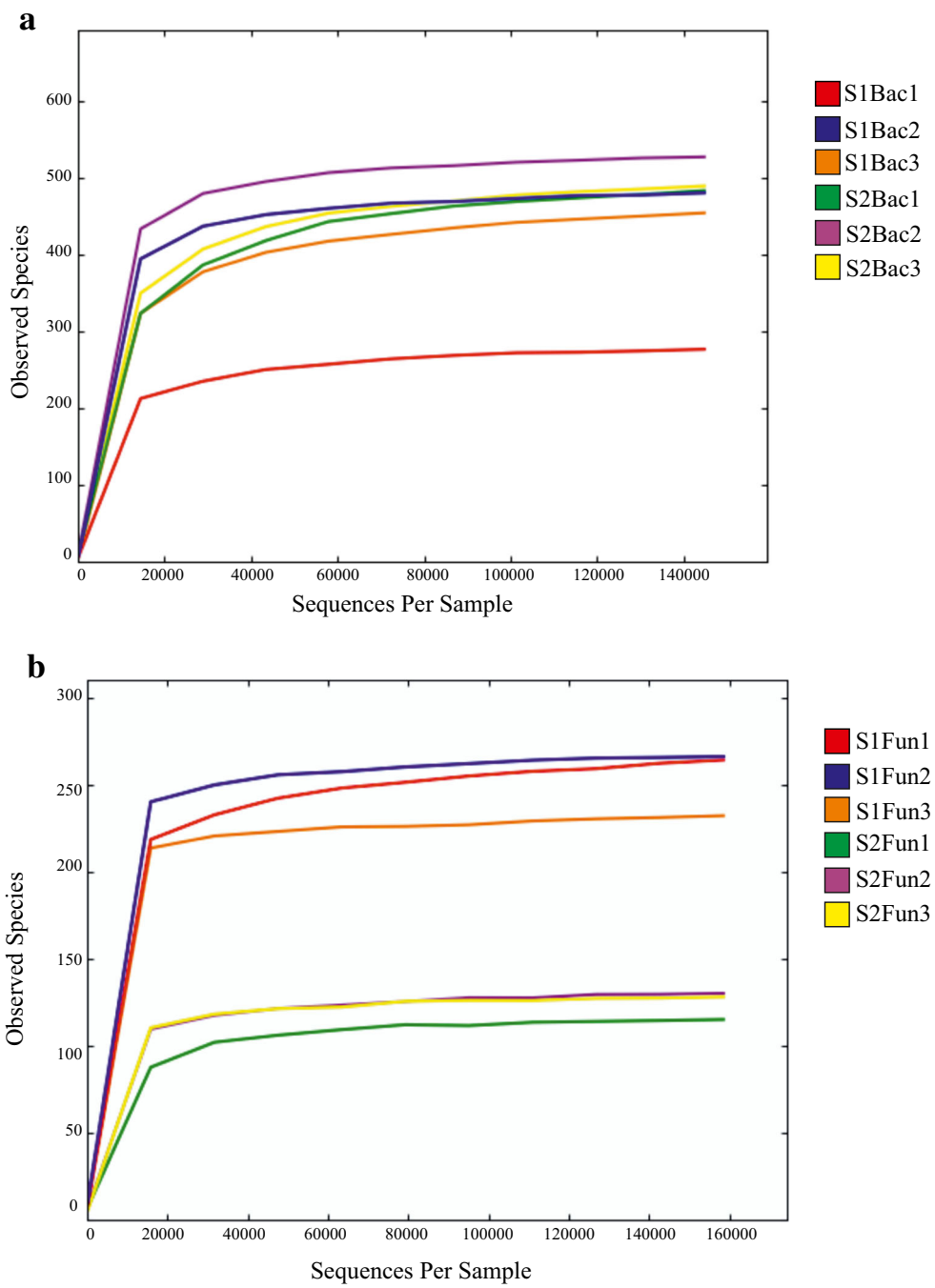

Fig. 3 Rarefaction curves of bacterial (**a**) and fungal (**b**) samples

and they also constitute the gut core bacteriome of several species of this genus [19]. In addition, they have also been frequently recovered from other species of these bark beetles (e.g., *D. ponderosae* and *D. simplex*) [17, 63]. These aspects might be applicable to fungal dominant genera, *Cyberlindnera* and *Candida*, which have also been recovered from the gut and other parts of the body of *Dendroctonus* species [16, 67], despite of their frequency in subcortical tissues of healthy tree saplings being unknown.

Overall, results of β-diversity show that bacterial and fungal communities are segregated according to the colonization stage, resulting in significant differences in the community structure of these microbes (Figs. 3 and 4). This change may be due to physiological state of the tree [68, 69], or changes in different physicochemical factors, such as the temperature, pH, moisture, and organic matter content [70, 71], as well as regulatory processes (quorum sensing) and competition with other microbial groups [72], which may compromise the presence of some genera

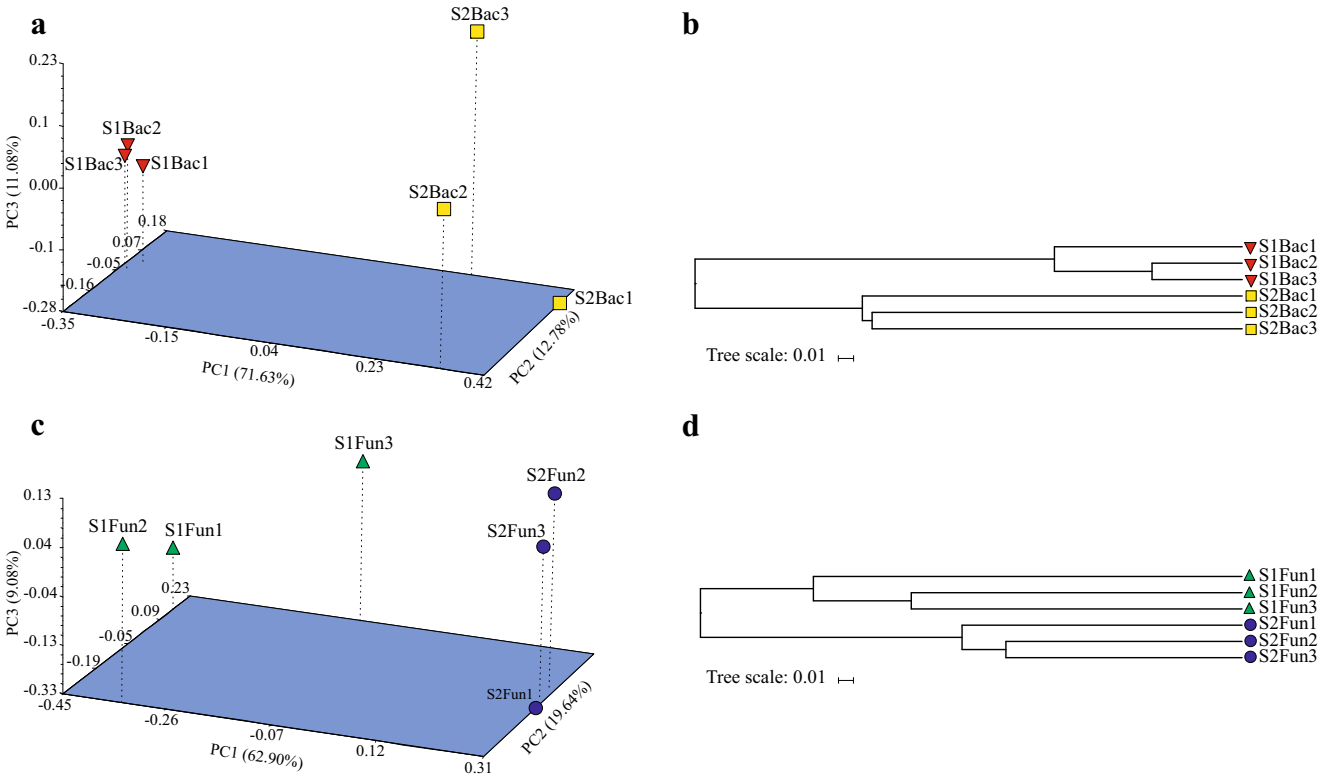

Fig. 4 PCoAs and dendrograms by the UPGMA method built with pairwise Bray-Curtis dissimilarity matrices of bacterial (**a** and **b**) and fungal (**c** and **d**) communities at two colonization stages

and favor the presence of others in this changing environment. However, despite this situation, the dominant bacteria and fungi maintain their prevalence, not only in the subcortical environment, but also in the gut, apparently performing specific metabolic functions for the benefit of both microorganisms and insects, as it has been well-demonstrated in other *Dendroctonus* species [20, 67, 73].

In summary, our findings provide evidence that microbial communities in subcortical tissues of Arizona pine are strongly influenced by the colonization stage of *D. rhizophagus*, resulting in a change in the structure of microbial communities as the decomposition state progresses. Several dominant members of these communities seem to be resident of trees as endophytes and are well-adapted to micro-environmental conditions in the subcortical tissues. The structure of whole microbial community varies as tree decomposition increases, apparently as a result of the shift in physicochemical conditions that can affect the microbiota composition, as well as microbial interactions. Finally, this research provides the basis for further studies to test metabolic functions in vivo of particular members of these microbial communities.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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