Coniferous Tree Species Identity and Leaf Aging Alter the Composition of Phyllosphere Communities Through Changes in Leaf Traits

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RESEARCH

Coniferous Tree Species Identity and Leaf Aging Alter the Composition of Phyllosphere Communities Through Changes in Leaf Traits

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Abstract

Phyllosphere microorganisms are essential for plant growth and health. Although there are an increasing number of studies showing that the composition of phyllosphere communities varies among different plant species, it remains unclear whether and how their bacterial and fungal community composition predictably varies with plant traits and leaf age. In this study, we used high-throughput sequencing to explore the diversity and composition of phyllosphere communities in needles of different ages (originating from different cohorts) for three evergreen coniferous species (*Pinus koraiensis*, *Picea koraiensis*, and *Abies nephrolepis*). Our results indicated that Gammaproteobacteria (bacteria) and Dothideomycetes (fungi) were dominant in newly formed needles, whereas Actinobacteria (bacteria) and Eurotiomycetes (fungi) were dominant in perennial needles. Tree species identity and needle age were the main factors explaining the variations of the α diversity (species richness of phyllosphere communities) and β diversity (dissimilarity among phyllosphere communities). In particular, we found that leaf dry matter content, leaf mass per area, and total phosphorus content emerged as key predictors of composition and diversity of phyllosphere microbial communities, underscoring the major influence of tree species identity and needle age on phyllosphere communities through changes in plant functional traits. Finally, we found that the interaction between tree species identity and needle age also contributed significantly to explaining the diversity and composition of phyllosphere communities, probably because differences in plant functional traits or environmental conditions between new and perennial needles depend on tree growth rates and resource acquisition strategies. These findings provide new insights into the mechanisms of community assembly among different evergreen tree species and offer a better understanding of the interactions between plant traits and phyllosphere microorganisms during needle aging.

Keywords Community structure · Diversity · Evergreen coniferous species · Needle age · Needle traits · Phyllosphere microorganisms

Introduction

Plants can be considered holobionts, meaning that living plants are associated with a variety of microorganisms [1]. Although the majority of studies have focused on the role of microbes associated with roots, an increasing number of investigations suggest that the phyllosphere microbiome (the community of microorganisms living on plant leaf surfaces) can also play a significant role in plant growth and survival [2–5]. Phyllosphere microorganisms can interact directly with the plant host, impacting plant nutrition through processes like nitrogen fixation [6–9], or influencing adaptability to environmental changes, particularly through effects on water absorption and nutrient use efficiency [5, 10–12]. While there is an increasing recognition that micro-environmental conditions on the leaf surface (ultraviolet radiation,

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water or nutrient availability) can affect microbial community composition or even prevent microbial colonization [13–15], microbial dispersal due to neighbor species can also be an important factor influencing the composition of phyllosphere communities [5, 16, 17]. For instance, a recent study demonstrated that microbial communities on the upper leaf surface were heavily colonized by bacteria from surrounding trees or the environment, whereas microbes on the lower leaf surfaces were more subject to host selection [17]. Although these studies have considerably improved our understanding of community assembly in aboveground plant parts, most studies are limited to broadleaved plants or a single kingdom of microbes (either bacteria or fungi) [18, 19]. As such, more effort is necessary to assess whether and how the composition and diversity of both bacterial and fungal communities vary during leaf aging in coniferous plants, notably because coniferous tree species have persistent needles characterized by a smaller surface area.

Plant species and host plant species genotype are important factors influencing the phyllosphere microbiome [20–23]. Such variability in the composition of phyllosphere communities can be related to leaf traits associated with morphology, chemistry, and physiology [18, 24, 25]. For example, leaf mass per area (LMA) is often considered one of the main drivers influencing the composition of microbial communities, notably because LMA, as part of the leaf economics spectrum, is closely related to photosynthetic resource utilization, with high LMA being related to low nutrient exudation and resource utilization efficiency [26, 27]. Leaf water content can also change the pH at the leaf surface, with further consequences for the diversity and abundance of bacteria [28]. For example, low pH may inhibit the growth of microorganisms and reduce microbial diversity, whereas it may promote the growth of specific bacterial groups such as Acidobacteria [29–31]. Furthermore, it has been shown that leaves characterized by a thick wax layer impede moisture seepage, whereas low photosynthetic rate may significantly affect the nutrition of certain microorganisms (such as fungal pathogens) in thick leaves [19, 32]. Therefore, trait variation can be used to explain changes in the phyllosphere microbiome among different tree species [33–35], but whether intraspecific variations in plant functional traits among individuals of the same species may also significantly explain the variability in phyllosphere communities remains poorly understood.

Intraspecific variation may account for more than a quarter of the total variation in leaf functional traits [36]. In evergreen plants, needle traits change with the growth of different cohorts of needles that coexist along branches of the same individual [37–39]. The structure, chemistry, and function of leaves change with increasing "leaf age," with traits including LMA, leaf dry matter content (LDMC), and wax layer increasing significantly in "perennial" leaves compared with "newly formed" leaves, whereas the net photosynthetic rate (A) often shows an opposite trend [39–42]. Furthermore, newly formed leaves often contain a higher amount of total nitrogen (TN) and total phosphorus (TP) as they require a large supply of nutrients to synthesize proteins that promote cell growth and division, which in turn increases the leaf area available for photosynthesis [39, 43–45]. As such, the differentiation in ecological strategies between newly formed and perennial leaves suggests the existence of an "intraspecific economic spectrum" [39], similar to what has been shown among different plant species [27, 39]. Furthermore, according to the theory of microbial succession, as plants grow, modifications in ecological niches will inevitably lead to shifts in microbial community composition [46]. In line with these results, recent studies have demonstrated that phyllosphere community composition depends on both leaf morphological and physiological characteristics [18, 26, 47]. However, whether and how the diversity and structure of phyllosphere communities may also vary with leaf age within the same host plant still require further exploration.

In this study, we investigated how leaf traits influence the diversity and structure of phyllosphere communities among three representative species of evergreen coniferous trees (*Pinus koraiensis*, *Picea koraiensis*, and *Abies nephrolepis*) and how these relationships can vary with increasing leaf age. We will use the term "needles" thereafter and throughout the document, but the concepts and ideas are exactly the same as those developed for broadleaved leaves. It is noteworthy that the growth rate of *Pinus koraiensis* is greater than that of *Picea koraiensis* and *Abies nephrolepis* [48], and as a consequence, needle length and intraspecific trait variation of *Pinus koraiensis* are also higher than those of the other two coniferous plants [39]. First, we hypothesized that microbial diversity would increase with needle age because perennial needles should select for specialized microorganisms capable of surviving in a more complex environment [49], whereas new needles should stimulate the dominance of a few taxa that are able to compete for high-quality resources (H_1) [19]. Second, we tested the hypothesis that the effect of needle age on the diversity and structure of phyllosphere communities would be stronger for the relatively fast-growing *Pinus koraiensis* species, as the difference between new and perennial needles should be greater compared with slow-growing species as *Picea koraiensis* and *Abies nephrolepis* (H_2) [38]. Finally, we hypothesized that plant traits such as LMA, LDMC, TN, and TP should predict the relative proportion of copiotrophic versus oligotrophic organisms, as these traits influence nutrient use efficiency and microbial strategies (H_3) [50]. This is primarily because acquisitive plant traits, characterized by high growth rates and high resource quality, are likely to favor the presence of fast-growing organisms, such as early

decomposers and opportunists [51]. In contrast, conservative plant traits, characterized by lower growth rates and scarcer resources, tend to favor oligotrophic organisms, such as late decomposers and N-fixers [52].

Materials and Methods

Sample Site

The experimental site was located in a typical mixed broadleaved Korean pine forest in Heilongjiang Liangshui National Nature Reserve (47° 10′ 50″ N, 128° 53′ 20″ E) in northeast China. The altitude ranges from 300 to 707 m, and the terrain is relatively flat with a slope varying between 10 and 15°. The climate type of this region is classified as temperate continental monsoon climate. The annual average temperature is − 0.3 ℃, the annual average precipitation is 676 mm, and precipitation is concentrated from June to August, accounting for more than 60% of the total precipitation of the whole year. The conifer species mainly consist of *Pinus koraiensis*, *Abies nephrolepis*, and *Picea koraiensis* [53].

Experimental Design

Three representative species of evergreen coniferous trees (*Pinus koraiensis* Siebold & Zucc., *Picea koraiensis* Nakai, and *Abies nephrolepis* (Trautv. ex Maxim.) Maxim.) were selected and sampled from July 20 to August 10, 2021. Seven sample trees were selected for each tree species. The diameters at breast height (DBH) of the selected trees ranged from 30 to 50 cm, a range deemed representative of adult trees based on results from a multi-year botanical survey [39] (Table S1). The trees were spaced more than 10 m apart and distributed evenly. Samples were obtained from seven trees of each tree species. For each tree, we selected randomly three branches at the lower position of the canopy with 1- to 4-year-old branches (distinguished based on the polycyclic shoots formed on branches as the basis for distinguishing branch age) [54]. The needles were divided into newly formed needles (the 1-year-old branches that emerged in 2021 were considered newly formed needles) and perennial needles (the 2- to 4-year-old needles already existing in the branches) (Fig S1). For each needle age, three samples were collected from each individual tree. A total of 126 (2 needle ages × 3 branches × 7 trees × 3 tree species) samples were collected to measure the composition of microbial communities. Additionally, needle samples for leaf trait measurements were collected from the same locations as the phyllosphere microbial samples.

Needle Collection

Needles were collected with sterile gloves and excised with sterilized branch scissors after cutting the branch. The gloves were replaced and branch scissors disinfected. The needles were placed into sterile bags for cold storage (− 4 ℃) and quickly brought back to the laboratory. We weighed 20-g needles on a sterile table and put them into the sterile tube directly after collection. Then, 10 ml of sterile buffer (0.1 M potassium phosphate saline buffer) per gram of sample was added, and the mixture was used to extract the phyllosphere microbiome. The samples were subjected to ultrasonic washing for 1 min at 40 kHz and vortex vibration for 30 s to wash the microbial cells off the needles; this process was repeated twice. We repeated the extraction process by refilling the buffer. The two washing solutions were then mixed and filtered with a 0.22-μm sterile filter membrane. The filtered membranes were snap-frozen in liquid nitrogen and stored at − 80 ℃. All samples were shipped on dry ice to Majorbio corporation (Shanghai, China) for DNA extraction and microbial sequencing analysis.

DNA Extraction and PCR Amplification

The DNA extraction was performed using the FastDNA Spin Kit (MP Biomedicals), while DNA purity and concentration were assessed using the NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., USA). For gel electrophoresis, 1% agarose gel was used in this process. The quantitative PCR (qPCR) thermal profiling of the fungal ITS region and bacterial 16S rRNA genes was performed using primers ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC) for fungi and 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) for bacteria [55]. Bacterial PCR amplification was performed using the TransGen AP221-02: TransStart Fastpfu DNA Polymerase Reaction System. This includes 4 μl FastPfu Buffer (5 ×), 2 μl 2.5 mM dNTPs, 0.8 μl forward primer (5 μM), 0.8 μl reverse primer (5 μM), 0.4 μl FastPfu Polymerase, 0.2 μl BSA, and 10 ng template DNA. Fungal PCR was performed using the TaKaRa rTaq DNA Polymerase reaction system, which includes the following: 10 μl Taq (2 ×), 0.8 μl forward primer (5 μM), 0.8 μl reverse primer (5 μM), 10 ng/μl template DNA. The PCR reaction is divided into several phases. The initial phase involves denaturation at 95 °C for 3 min, followed by 35 cycles (27 cycles for bacteria) at 95 °C for 30 s, annealing at 55 °C for 30 s, and extending at 72 °C for 45 s. Finally, there is a last extension at 72 °C for 10 min. A negative control was included once per primer set to ensure that false positives did not occur during the PCR amplification process. All amplification procedures were performed on a PCR instrument: ABI GeneAmp® Model 9700 (Perkin-Elmer Applied

Biosystems, USA). Three replicates were performed for each sample, and we used the axyprepdna gel Recovery Kit (Axygen Corporation) to retrieve PCR products. We then eluted the PCR products with Tris–HCl elution and detected them using 2% agarose electrophoresis. Quantitation of PCR products was performed using the QuantiFluor-ST blue fluorescence quantitation system (Promega Corporation).

Illumina MiSeq Amplicon Sequencing and Sequence Analysis

After PCR amplification, sequencing was performed on the Illumina MiSeq PE300 platform (Illumina, CA, USA). Sequence processing was completed on the Majorbio Cloud Platform online platform (www.majorbio.com). The sequences were quality trimmed using FASTP version 0.19.6, and paired-end reads were assembled into full-length sequences using Flash 1.2.11 [56]. Reads with a mass value of 20 or less at the end were filtered, setting a 10-bp window,if the average mass value within the window was less than 20, the bases from the end of the window were truncated to remove reads containing N bases. Low-quality sequences (length < 200 bp) were also eliminated [47]. Sequences with overlaps longer than 10 bp were merged and mismatch densities below 0.2. The samples were then distinguished according to the barcodes and primer sequences at the beginning and end of the sequence. The number of mismatches allowed in barcodes was set to 0, and the maximum number of primer mismatches was set to 2. Original sequences underwent quality control, and unique sequences were removed. Chimeras were removed to obtain representative sequences of OTUs. The optimized sequences were then mapped to the representative OTU sequences, and the RDP classifier (version 2.13) was employed to classify the representative sequences of bacteria and fungi. We used the taxonomic databases silva 138/16s_bacteria (Release138 http://www.arb-silva.de) and unite 8.0/its_fungi Unite (Release 8.0 http://unite.ut.ee/index.php) to identify bacterial and fungal species, respectively. Subsequently, all sequences were categorized into operational taxonomic units (OTUs) and clustered based on 97% similarity using the UPARSE algorithm [57]. In this process, all chloroplast and mitochondrion sequences were removed, and all sequences that appeared only once or in only one sample were also discarded in the final analysis. Taxonomy was assigned to each OTU with a minimum confidence of 0.8 [58, 59]. To ensure the accuracy of species classification, for each classification level, we typically relied on 98%, 90.0%, 85.0%, and 80.0% similarity as a criterion for assigning an OTU to species, genus, family, or order, respectively [60].

In total, 6,157,225 optimized sequences were obtained for bacteria, with an average sequence length of 411 bp, and 8,805,101 optimized sequences were obtained for fungi, with an average sequence length of 242 bp. To ensure the consistency of subsequent analysis, all sample sequence numbers were normalized to the minimum sample size. All the raw sequencing data was deposited in the National Microbiology Data Center (NMDC). The accession number is NMDC40038831.

Needle Traits

Leaf photosynthesis (A) and stomatal conductance (GSW) were measured using a photosynthetic apparatus (Li-COR 6800, USA) in clear and cloudless weather. Leaf mass per area (LMA) was calculated by first measuring leaf volume and then leaf area according to the protocol described by Liu et al. [61]. LDMC was calculated as the ratio of the dry mass of leaves to the fresh mass of leaves. Chlorophyll (Chl) was measured using the acetone method. Total carbon (TC) was determined by combustion using a carbon–nitrogen analyzer (multiN/C2100, AnalytikJena, Germany). Total nitrogen (TN) was measured by a continuous flow elemental analyzer (AQ400, Seal, Germany) following high-temperature digestion using H_2SO_4-H_2O_2. Total phosphorus (TP) was measured by the molybdenum-antimony colorimetry method [62] after high-temperature digestion.

Statistical Analysis

To analyze the effect of tree species, needle age, and their interaction on α diversity of bacteria and fungi (Chao index and Shannon index), we used two-way ANOVA after verifying for normality and homoscedasticity. We also analyzed the effect of needle age on α diversity for each tree species separately using one-way ANOVA with Tukey HSD tests. The following sequencing data analyses and plotting were performed using the Majorbio Cloud Platform (www.majorbio.com), which is based on the R language version 3.3.1. Venn diagrams were then used to indicate the (dis)similarities in OTUs among the different tree species and needle ages. Histograms were used to represent the top ten species composition at the phylum and class level. PERMANOVA ($n = 999$ permutations) based on Bray–Curtis distances and principal coordinate analysis (PCoA) were used to assess the effects of tree species and needle ages on the structure of phyllosphere microbial community. One-way ANOVA was used to analyze the differences in the relative abundance of the top ten class-level microbial groups between newly formed needles and perennial needles for each tree species separately. Redundancy analysis (RDA) was used to detect the relationships between needle traits and phyllosphere communities. Heat maps were used to show the relationships between microbial species and needle traits in the top 20 classes of bacteria and fungi.

Results

Differences in Phyllosphere Diversity and Richness Among Needle Ages and Tree Species

The α diversity of bacterial and fungal communities varied significantly with needle age ($P < 0.05$) (Table 1). Needle age had the greatest influence on microbial Shannon index and Chao index. Tree species also significantly affected all α diversity indexes except for the Shannon index of bacteria. The interaction between tree species and leaf age affected significantly all diversity indices, except for the Shannon index of fungi (Table 1). For bacteria, the Shannon index of *Picea koraiensis* increased significantly with needle ages ($P < 0.001$) (Fig. 1a). The Chao index of *Pinus koraiensis* ($P < 0.001$) also increased significantly with needle ages (Fig. 1b). The bacterial Shannon index and Chao index of *Pinus koraiensis* were significantly lower than those of *Picea koraiensis* and *Abies nephrolepis*, and there was no significant difference between *Picea koraiensis* and *Abies nephrolepis*. Regarding the fungi, the Shannon index and Chao index of *Pinus koraiensis* were significantly affected by needle ages. Among the different species, *Pinus koraiensis* was the species most affected by needle ages ($P < 0.001$) (Fig. 1), even though it presented the lowest Chao index. Interestingly, we found that the Shannon and Chao indices were higher in *Abies nephrolepis* compared with the other species, regardless of whether we considered bacteria or fungi (Fig. 1d).

Composition of Bacterial and Fungal Communities

The composition of bacterial and fungal communities was significantly explained by the needle age, tree species, and their interaction (Table 2). Tree species alone explained 21% of the variance in bacterial communities and 26% in fungal communities. In contrast, needle age alone explained 11% of the variance in bacteria communities and 6% in fungal communities, while the interaction between tree species and needle age explained 7 and 4% for bacteria and fungi, respectively (Table 2). For each tree species individually, needle age significantly affected the intraspecific variation of phyllosphere microorganisms, with needle age explaining more of the variation in microbial community composition for *Pinus koraiensis* (Table S3). The first PCoA axis explained 20.95% of the composition in bacterial communities and 22.29% of fungal communities (Fig. 2). *Pinus koraiensis* presented a more distinct bacterial community compared with the other species, with greater differentiation for the newly formed needles (Fig. 2). For the fungal communities, we found a clear gradient among the three tree species, with the community structure of *Pinus koraiensis* being significantly different from that of the other two species (Fig. 2).

Over half of the total number of bacterial and fungal OTUs were shared by the three coniferous species (Fig S2a). For bacteria, the number of specific OTUs was about threefold higher for newly formed needles than for perennial needles, but this trend was not observed for fungi (Fig S2b). The families and classes to which the unique OTUs belong are listed in Table S5. All tree species shared about 60% or more of their OTUs between newly formed and perennial needles, except for bacteria in *Pinus koraiensis*, which shared less than 50% of its OTUs (Fig S3).

The dominant bacterial phyla were Proteobacteria (about 60%), followed by Actinobacteria, Myxococcota, Acidobacteria, and Bacteroidota, collectively accounting for about 30% of the total community across the different species (Fig S4). For fungi, the main phylum was Ascomycota (80%), followed by Basidiomycota (10%) (Fig S4). When comparing newly formed and perennial needles in *Pinus koraiensis*, we found a decrease in Ascomycota and an increase in Basidiomycota, whereas the opposite trend was observed for *Picea koraiensis* and *Abies nephrolepis* (Fig S4). At the class level, the dominant bacterial classes were Alphaproteobacteria, Actinobacteria, Gammaproteobacteria, Myxococcia, and Acidobacteria (Fig S5a). For fungi, the main classes were Dothideomycetes, Taphrinomycetes, Eurotiomycetes, and Tremellomycetes (Fig S5b).

When studying the ten most abundant classes per treatment, we found that the relative abundance of Acidobacteria

Table 1 Effects of tree species and needle age on Shannon index and Chao index of bacteria and fungi

Taxa	Index	Tree species		Needle age		Tree species × needle age	
		F	*P*	*F*	*P*	*F*	*P*
Bacteria	Shannon	2.21	0.74	21.21	$<0.001^{***}$	6.04	$<0.01^{**}$
	Chao	12.64	$<0.001^{***}$	4.24	$<0.05^{*}$	4.67	$<0.05^{*}$
Fungi	Shannon	3.60	$<0.001^{***}$	10.68	$<0.01^{**}$	2.75	0.068
	Chao	30.66	$<0.001^{***}$	12.10	$<0.001^{***}$	4.83	$<0.01^{**}$

Shannon index indicates species diversity and Chao index indicates species richness; *the significant difference, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. See Table S2 for additional details on data characteristics

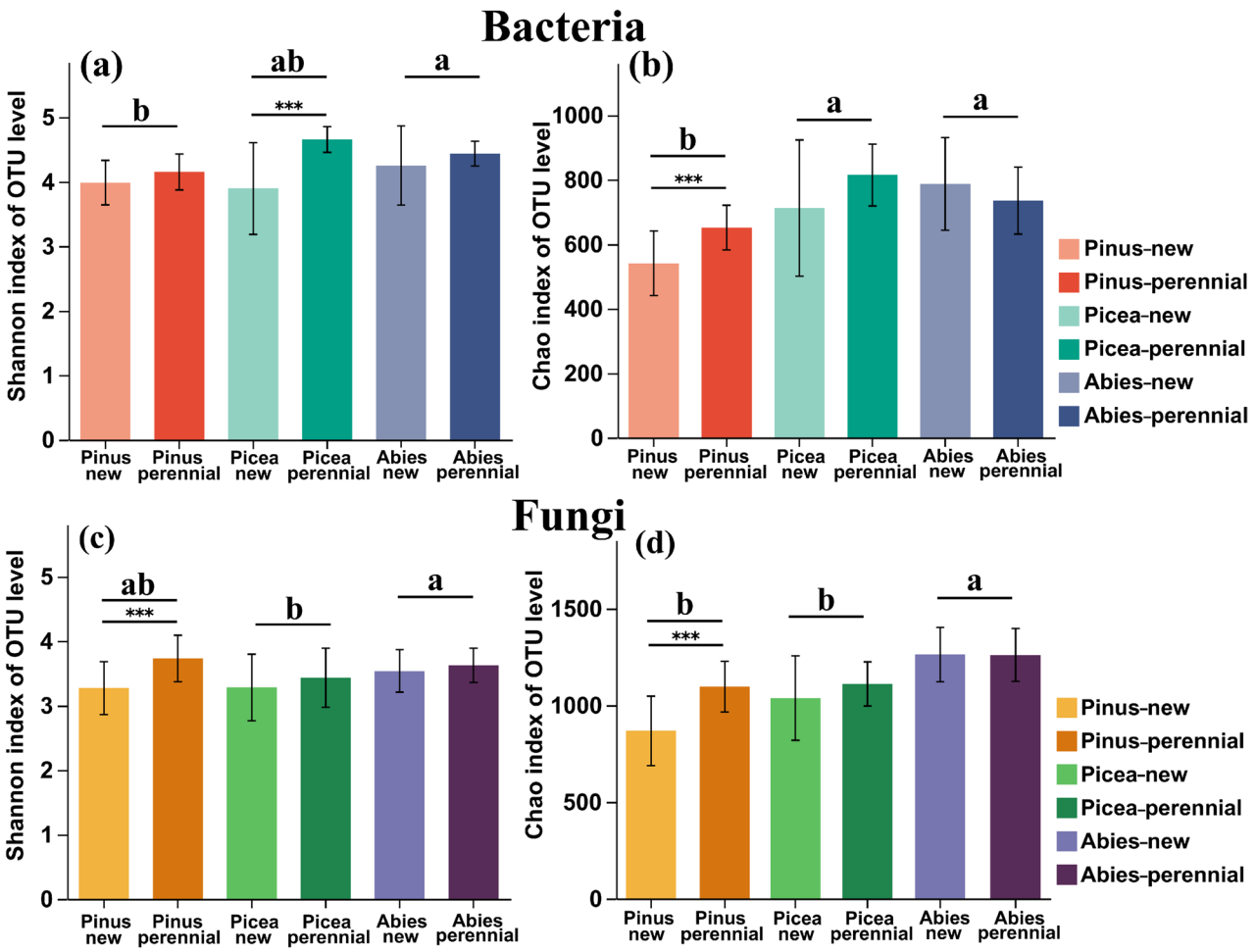

Fig. 1 Estimates of diversity indices (Shannon and Chao) for bacterial (**a**, **b**) and fungal (**c**, **d**) communities. Asterisks (*) indicate significant differences between different tree species and needle ages, $*P<0.05$; $**P<0.01$; $***P<0.001$ (only the variability between needle ages and tree species is shown). Post hoc Tukey's tests were used to determine which specific group means were significantly different from each other after finding a significant effect in the ANOVA. Abbreviations mean *Pinus koraiensis* newly formed needles (Pinus-new), *Pinus koraiensis* perennial needles (Pinus-perennial), *Picea koraiensis* newly formed needles (Picea-new), *Picea koraiensis* perennial needles (Picea-perennial), *Abies nephrolepis* newly formed needles (Abies-new), *Abies nephrolepis* perennial needles (Abies-perennial)

Table 2 PERMANOVA of the effects of tree species and needle age on microbial community structure

Taxa	Variable	*Df*	Sum of Sqs	R^2	*F*	*P*
Bacteria	Needle age	1	2.15	0.11	20.52	0.001
	Tree species	2	4.08	0.21	19.42	0.001
	Tree species × needle age	2	1.42	0.07	6.77	0.001
Fungi	Needle age	1	2.10	0.06	11.89	0.001
	Tree species	2	8.56	0.26	24.30	0.001
	Tree species × needle age	2	1.29	0.04	3.66	0.001

Df represents degrees of freedom, *Sum of Sqs* represents the total variance, and *F* represents the model test value. The R^2 value represents the proportion of variance explained by the model, with a higher R^2 indicating a greater effect. $P<0.05$ indicates that the result is statistically significant. See table S4 for post hoc test results

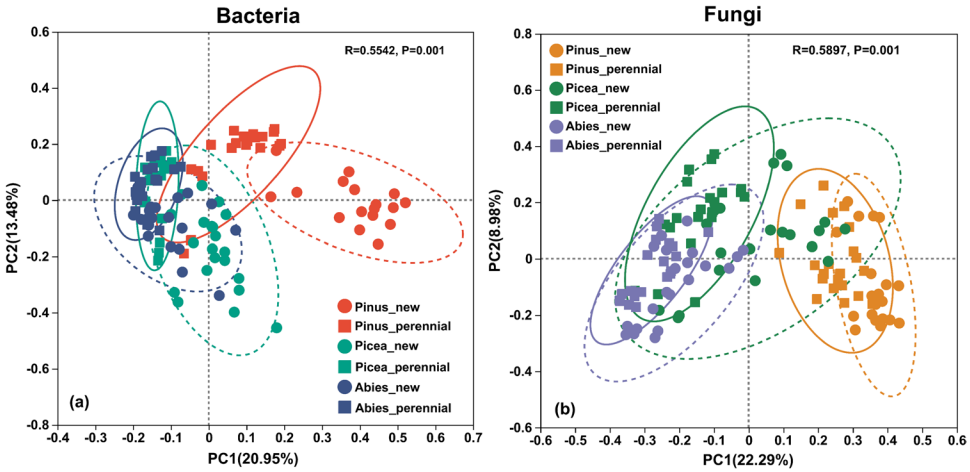

Fig. 2 Principal coordinate analysis (PCoA) of bacterial (**a**) and fungal (**b**) communities across different tree species and needle ages. Ellipses represent 95% confidence intervals. Abbreviations mean *Pinus koraiensis* newly formed needles (Pinus-new), *Pinus koraiensis* perennial needles (Pinus-perennial), *Picea koraiensis* newly formed needles (Picea-new), *Picea koraiensis* perennial needles (Picea-perennial), *Abies nephrolepis* newly formed needles (Abies-new), *Abies nephrolepis* perennial needles (Abies-perennial)

was dominant in the perennial needles across most species, while the relative abundance of Alphaproteobacteria was greater in perennial needles of *Pinus koraiensis* only. In contrast, the relative abundance of Gammaproteobacteria was greater in newly formed needles of the *Pinus koraiensis* and *Picea koraiensis* (Fig. 3a, b). Regarding the fungi, we found that the classes Dothideomycetes and Taphrinomycetes were more abundant in newly formed needles, whereas the relative abundance of Eurotiomycetes was more abundant in perennial needles of *Pinus koraiensis* and *Picea koraiensis* (Fig. 3d, e).

Relationship Between Phyllosphere Microbiome and Needle Traits

Analysis of needle traits revealed that Chl, LMA, LDMC, and TP, as well as the ratio of nitrogen and phosphorus (N/P), were significantly different among the different tree species and between two needle ages (Table S6). The variation in needle traits explained 35.39%, 36.16%, and 9.58% of the bacterial community structure for *Pinus koraiensis*, *Picea koraiensis*, and *Abies nephrolepis* along the first RDA axis (Fig. 4a). RDA Axis 1 also explained 23.94%, 17.30%, and 25.80% of the variation in fungal communities for *Pinus koraiensis*, *Picea koraiensis*, and *Abies nephrolepis* respectively (Fig. 4b), and was strongly related to plant traits such as needle nutrient concentrations (N and P contents), LDMC, and LMA. To a lesser extent, we also found that the RDA axis 2 explained a significant portion of the variation in bacterial and fungal communities (Fig. 4b), and was mainly related to photosynthetic rate (A) and total nitrogen (TN). Overall, needle traits better explained the composition of phyllosphere bacterial communities in *Pinus koraiensis* at the class level (Fig. 4).

For the three tree species, LMA, LDMC, Chl, and TP content and the C/P and N/P ratios were the main needle traits explaining the different classes of bacteria and fungi (Fig. 5). Contrary to *Pinus koraiensis* and *Picea koraiensis*, we found that LMA and Chl were related to only a minority of taxa in *Abies nephrolepis*. Interestingly, the correlations between TP and bacterial or fungal classes were opposite to those of most other plant traits (Fig. 5). Overall, most microbial taxa were more abundant on needles with high LDMC and LMA and low TP content (Fig. 5). For bacteria, Actinobacteria, Acidobacteria, Saccharymonadia, and Armatimonadia were positively correlated with LMA and LDMC, but negatively correlated with TP (Fig. 5). In contrast, Gammaproteobacteria were positively correlated with TP. For the fungi, Eurotiomycetes, Lecanoromycetes, Lichinomycetes, and Orbiliomycetes were positively correlated with LDMC, N/P, and C/P ratios, whereas Agaricomycetes, Taphrinomycetes, and Saccharomycetes were negatively related to LDMC, N/P, and C/P ratios (Fig. 5). Consistent with bacteria, these fungal species showed opposite trends in their relationship to TP (Fig. 5).

Discussion

Utilizing needles from three evergreen coniferous tree species, we investigated how needle age, tree species identity, and the variability in plant traits influenced phyllosphere communities. We found that bacterial and fungal diversity was higher in perennial compared to newly formed needles. Overall, tree species identity and needle age were the main

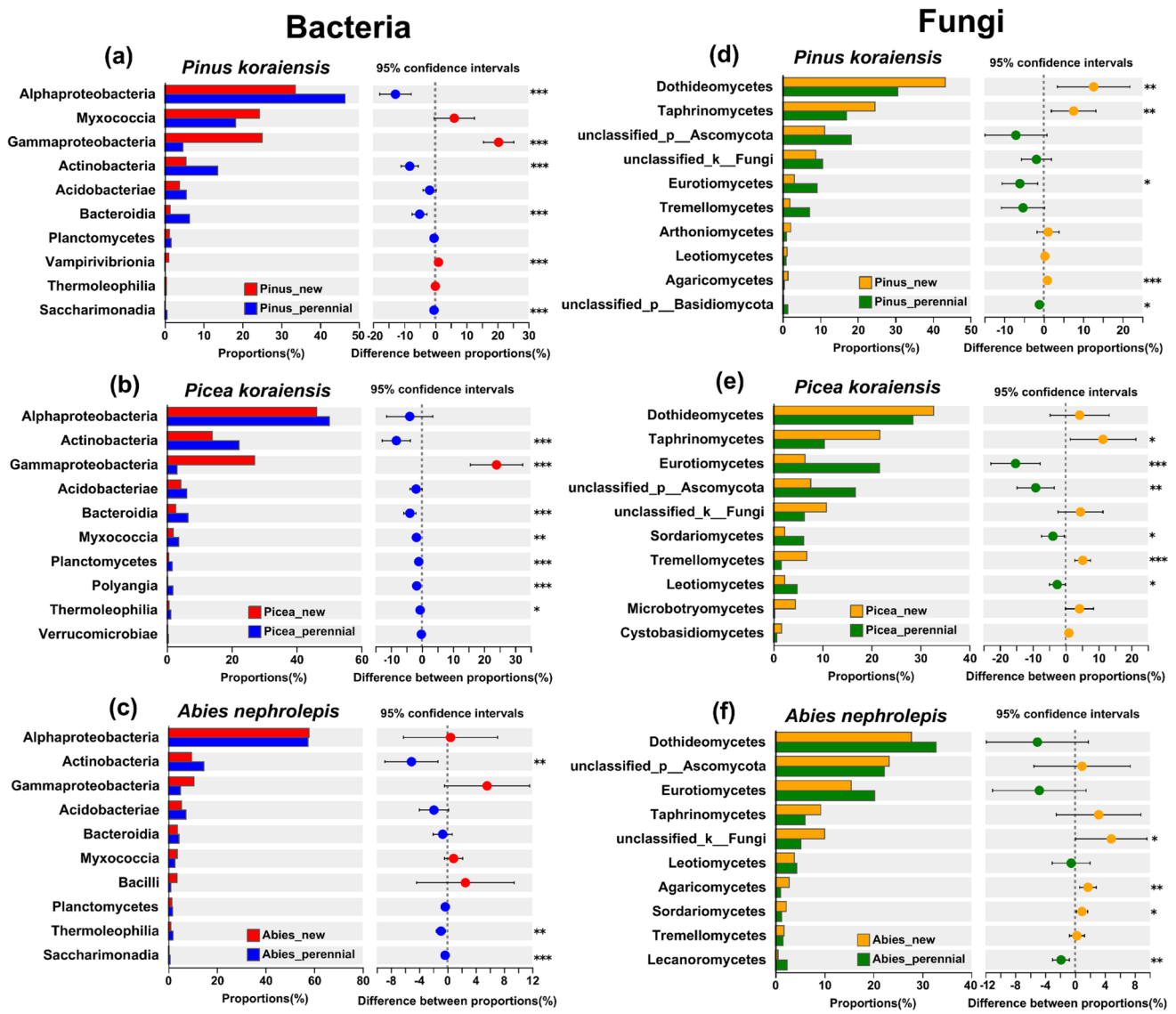

Fig. 3 The relative abundance of top bacterial and fungal classes is shown, along with the results of a one-way ANOVA analysis assessing significant differences among different tree species and needle ages. **a**, **b**, **c** Bacteria from *Pinus koraiensis*, *Picea koraiensis*, and *Abies nephrolepis*, respectively. **d**, **e**, **f** Fungi from *Pinus koraiensis*, *Picea koraiensis*, and *Abies nephrolepis*, respectively. An asterisk (*) indicates a significant difference: $*P<0.05$; $**P<0.01$; $***P<0.001$

factors explaining variations in α diversity (species richness of phyllosphere communities) and β diversity (dissimilarity among phyllosphere communities), respectively. Furthermore, the interaction between tree species identity and needle age also significantly affected the diversity and composition of phyllosphere communities, shaping microbial community structure in a more complex way than either factor alone. These changes were partly related to plant traits, such as morphological traits (LMA, LDMC) and chemical traits (N/P, TP), which are linked to the leaf economic spectrum. These findings underscore the significance of understanding the interplay between phyllosphere microorganisms and plant life strategies to advance our knowledge of phyllosphere communities and their potential role in maintaining plant growth.

Diversity of Phyllosphere Communities Across Different Needle Ages

In line with our first hypothesis, we observed an overall lower diversity of phyllosphere microorganisms in newly formed needles compared with perennial needles across different coniferous species. We attribute this difference in microbial diversity to three main, non-exclusive mechanisms: (i) environmental conditions, (ii) interspecific interactions, and (iii) microbial dispersal.

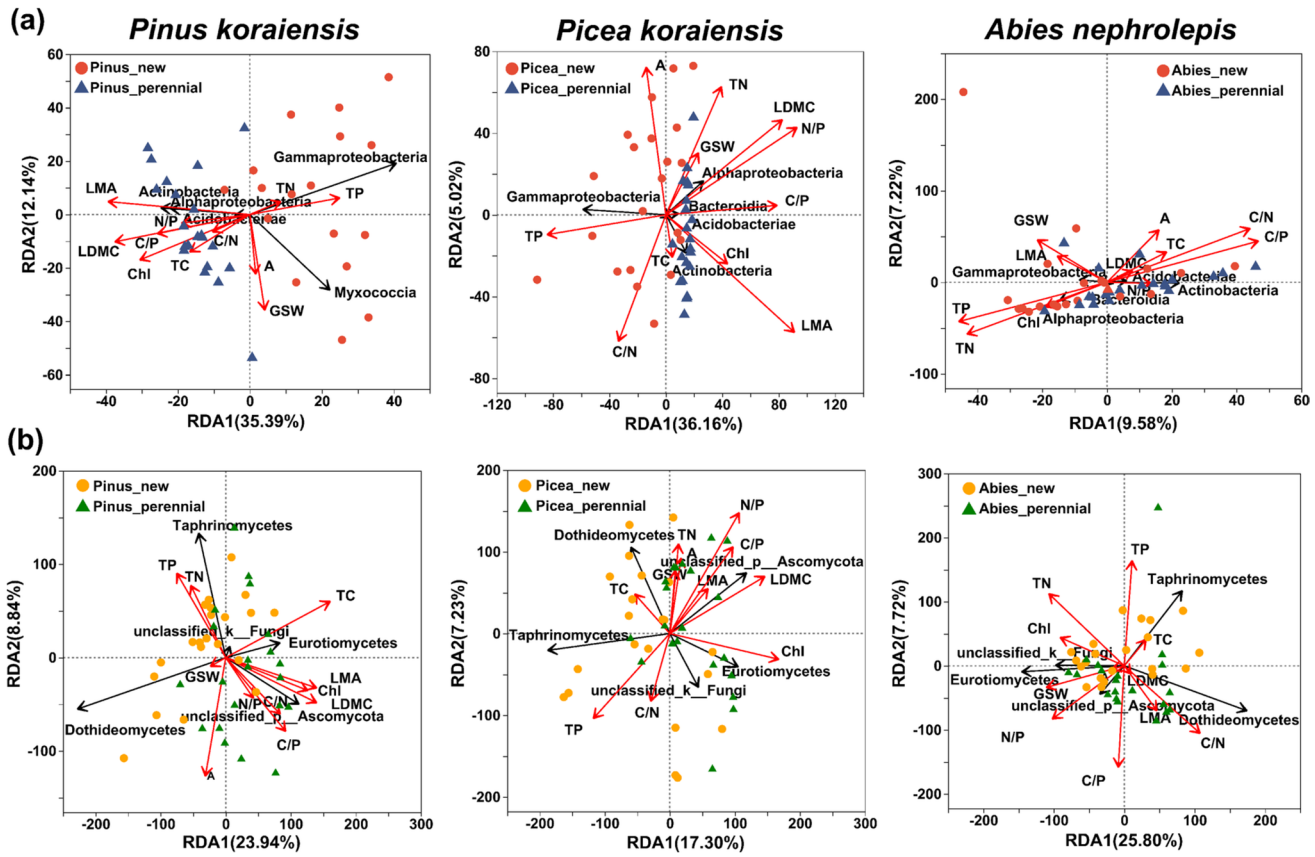

Fig. 4 Redundancy analysis (RDA) between the major bacterial (**a**) and fungal (**b**) taxa and leaf traits for each tree species separately. Black arrows indicate species, while red arrows indicate plant traits., The length of the arrows represents the degree of influence, and the distance of the projection points from the origin reflects the relative influence of plant traits on microbial communities

First, the disparity in microbial diversity may be due to varying environmental conditions, notably because newly formed needles may represent a more stressful environment for phyllosphere microbes [41, 63, 64]. While we did not directly measure light radiation in this study, our field observations suggest that environmental conditions, including UV radiation and drought, are harsher for phyllosphere microbes growing on newly formed needles. Because fluctuations in environmental conditions are stronger on sunny needles [6, 13], this can potentially exert a stronger selective pressure on phyllosphere organisms with further negative consequences on microbial diversity [13, 65]. On the other hand, because perennial needles offer more shaded conditions, the relatively more homogenous conditions may allow a higher diversity of microbes to grow and reproduce, notably because the environmental filter on species selection is lower than for sunny needles.

Secondly, newly formed needles often exhibit higher photosynthetic capacity [42, 66], which promotes the absorption of carbon and nitrogen sources and stimulates leaf growth [67, 68]. Consequently, the increased resource availability in newly formed leaves may favor the dominance of a few competitive microbial taxa, leading to a decrease in microbial diversity and evenness in the community [18, 49]. As leaf age and resources decrease at the leaf surface, complementarity among various microbial taxa may increase to improve carbon and nutrient use efficiencies when the resources become scarcer [69]. On the other hand, because perennial needles offer more diverse niches to phyllosphere microbes due to thicker layers of epidermal wax containing long-chain hydrocarbons, this may lead to an increase in the proportion of specialized microbial species in older needles [11, 28, 70, 71]. This theory aligns with the "microbial succession model" proposed by Jackson et al. [46], which suggests that while stochastic processes dominate when microorganisms first colonize a new surface, deterministic selection becomes increasingly important as the microbial community establishes itself and the ecological niche develops.

Finally, microbial dispersal caused by neighborhood effects can also be an important factor affecting the composition of phyllosphere communities [16, 17]. In particular, the strength of host filtering effects (such as the impact of tree species identity and plant traits) should gradually weaken

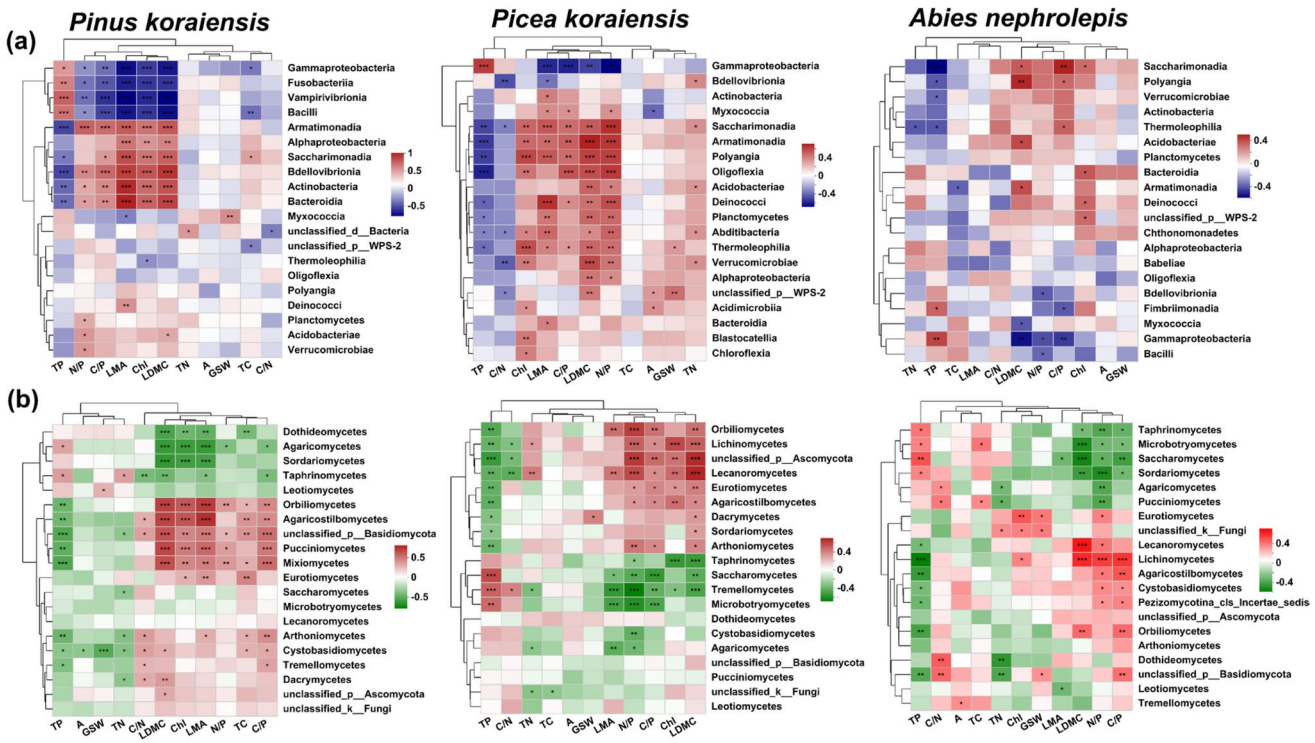

Fig. 5 Heat maps showing the correlations between the top 20 bacterial (**a**) and fungal (**b**) classes and needle traits across different tree species and needle ages. Correlations were assessed using Spearman's correlation coefficient. The X-axis represents needle traits, and the Y-axis lists the names of the classes. The color legend on the right side indicates the range of Spearman's correlation coefficients. Statistical significance is denoted by $*P<0.05$; $**P<0.01$; and $***P<0.001$

over time due to a stronger colonization by various microbes from the local environment [19, 24]. Since we found that perennial needles generally have a higher α diversity, this could be because migrating species have more opportunities to colonize older needles over longer period of time [16]. Thus, the likelihood of colonization and survival on perennial needles, which have more stable environmental conditions, is theoretically higher than on newly formed needles [24]. However, microbial community assembly relies more on stochastic processes in environments with high tree species diversity, while host plant selection is more pronounced in low-diversity environments [72]. Thus, although the influence of neighboring plants on microbial dispersion is likely of utmost importance in our forest type, which is characterized by relatively phylogenetically similar hosts, other factors such as climate and soil type can also alter the contribution of host plant species and needle age to microbial community assembly [19, 73]. As such, further research and comparative studies across diverse regions and biomes are needed to assess the generalizability of these effects. In conclusion, although we cannot disentangle the direct and indirect effects of biotic and abiotic factors affecting the diversity of phyllosphere microbes, our data highlight that microbial diversity is lower in newly formed than perennial needles, and this across various coniferous tree species.

Composition of Phyllosphere Communities Across Different Coniferous Tree Species

In partial agreement with our second hypothesis, the effects of needle age on α and β diversity of phyllosphere microorganisms were generally greater for the fast-growing *Pinus koraiensis* compared with slow-growing species *Picea koraiensis* and *Abies nephrolepis* (Figs. 1 and 2, Table S3). The greater N acquisition and photosynthetic capacity of *Pinus koraiensis* likely contributed to its higher growth rates in the newly formed leaves [41], which may further explain the superior overall plant performance of *Pinus koraiensis* compared with the other coniferous species [74]. As needles become increasingly shaded with tree growth [39, 61], and as differences in environmental conditions become more pronounced for fast-growing trees, the decrease in light conditions coupled with changes in needle physiological characteristics has likely influenced more bacterial taxa than on slow-growing trees [20, 75, 76]. In particular, we found that Gammaproteobacteria, which are often considered copiotrophic organisms [74, 77], were more abundant in newly formed needles of the relatively more productive *Pinus koraiensis*, although a similar pattern was observed to a lesser extent for *Picea koraiensis*. These results align with those of Truchado et al. [78], who showed

that Gammaproteobacteria strongly depend on high amounts of soluble carbohydrates and nutrients [78, 79], and suggest that this class is more likely to be abundant when resources are more readily available in light-exposed needles. On the other hand, we found that Actinobacteria were more abundant in the older needles of *Pinus koraiensis*. These results agree with a recent study demonstrating that Actinobacteria increase in proportion from the top to the bottom of the canopy as shading increases [13]. This supports the idea that differences between shaded and sunny needles are key factors to consider when assessing the composition of phyllosphere communities across different cohorts of needles.

Regarding the fungi, we also found significant differences with needle age, with notably a greater relative abundance of Dothideomycetes and Taphrinomycetes in newly formed needles of *Pinus koraiensis* (Fig. 3). These two groups are often considered the main taxa of phyllosphere fungi [80], probably because they are efficient at competing for nutrients and space in newly formed needles [19]. Although many of these fungi are harmless to plants, Dothideomycetes can also act as pathogens, attacking the photosynthetic tissues of plants [10, 32, 81]. Further studies will be necessary to assess if their increase in proportion was due to higher carbon and nutrient availability at the leaf surface or whether they acted as decomposers in response to the higher biomass and growth rates of *Pinus koraiensis*. Interestingly, we found that Tremellomycetes and Agaricomycetes were also relatively more abundant in newly formed needles, specifically in newly formed needles of *Picea koraiensis* and *Abies nephrolepis*. These two classes are well known as saprophytic fungi and yeasts [82, 83], which can grow relatively well on various surfaces of plants and secrete a large amount of heterologous proteins such as exopolysaccharides to enhance their high tolerance to UV [80, 83, 84]. Although the reasons why some fungal classes are more abundant in certain tree species than others are still unclear, our results underscore that the dominance of some fungal taxa is strongly host-dependent. These results are consistent with a recent study by Rodríguez-Rodríguez et al. [85], which suggests that phyllosphere fungi tend to be more dependent on tree species hosts than bacteria in coniferous forests. Furthermore, it has been recently shown that Tremellomycetes may limit the growth of other fungal classes such as that of Eurotiomycetes in *Picea glauca* [19]. These data suggest that the assemblage of phyllosphere communities is influenced not only by changes in environmental conditions but also by interspecific interactions [5, 19], and further studies will be necessary to elucidate the specific mechanisms and dynamics underlying these interactions, as well as their implications for community assembly.

Interestingly, we also found a significant interactive effect of tree species identity and leaf age on the diversity and composition of phyllosphere communities. This may be because differences in needle traits between young and perennial needles are more pronounced in the fast-growing *Pinus koraiensis* compared to *Picea koraiensis* and *Abies nephrolepis*. As a result, differences in community composition are likely greater as needles age due to more drastic changes in resource quality and quantity [19, 39], while differences are smaller in slower-growing species with more conservative needle traits across cohorts. Alternatively, this effect may be due to more pronounced changes in environmental conditions between new and perennial leaves in fast-growing *Pinus koraiensis*. Specifically, changes in leaf age reflect not only alterations in needle traits but also shifts in the microenvironment at different developmental stages [66]. These modifications in the environment, in turn, may affect microbial community composition [5, 15] and, ultimately, the diversity and function of the phyllosphere communities [19, 24]. Altogether, our results suggest that fast-growing coniferous species are more likely to exhibit differences in phyllosphere community composition between newly formed and perennial leaves likely due to changes in environmental and resource conditions, but that this effect is more consistent for bacterial communities than fungal communities, which are most host-dependent.

Importance of Plant Traits for Explaining the Composition of Phyllosphere Communities

In line with our third hypothesis, needle traits were important factors explaining the differences in the composition of phyllosphere communities. In particular, we found that the large differences in phenotypic traits such as LMA and LDMC between different cohorts of needles were significantly related to various groups of bacteria and fungi. For instance, it has been shown that LMA and LDMC are indicators of photosynthetic rate and growth rate, which predict the survival strategies of trees [41]. In line with this idea, we found that high LDMC and LMA values in perennial leaves can be used as useful predictors of some microbial taxa such as Actinobacteria and Orbiliomycetes during late stages of microbial succession (e.g., notably for the genus *Friedmaniella* in the Actinobacteria class) [13]. Alternatively, the exudation of monosaccharides such as glucose and fructose on the needle surface is often considered a key factor explaining the composition of phyllosphere communities in newly formed leaves [8, 86]. Therefore, low LDMC and LMA values can be used as useful indicators of copiotrophic taxa during earlier stages of microbial succession, particularly for the genus *Pseudomonas* in the Gammaproteobacteria class [87, 88]. In addition, it has also been proposed in the literature that nutrient supply (e.g., nitrogen and phosphorus) is essential to sustain plant growth and succession in the phyllosphere community [5, 25, 89]. In line with this idea, we found that the ratios of N/P and C/P were also significant

factors influencing the relative abundance of many bacterial and fungal taxa, with greater abundances of Saccharimonadia and Orbiliomycetes when increasing stoichiometric C:N:P ratios. However, consistent with Yadav et al. [28], we did not find that total nitrogen was a significant factor affecting the composition of phyllosphere communities. These results suggest that stoichiometric requirements are prevalent over total nutrient contents to predict the composition of phyllosphere communities, at least in the context of our study at the local scale. Finally, we also found that TP affected many microbial taxa including Saccharimonadia and Orbiliomycetes, but in an opposite direction to the effects of LMA and LDMC (Figs. 4 and 5). This may be because phosphorus-containing compounds are less likely to penetrate the cuticles of perennial needles that often present relatively high LMA and LDMC values [28], or alternatively that fast-growing organisms require more P to maintain high growth rates in newly formed needles [64, 90, 91]. Altogether, these findings suggest that needle traits can be used as useful predictors of microbial taxa during leaf aging.

Conclusion

Our study highlights that tree species and needle age are major factors affecting the diversity and composition of phyllosphere microbial communities through changes in plant functional traits and environmental conditions. These results are consistent with our hypotheses and have several implications. First, our results indicate that phyllosphere communities vary within and between tree species, even though phyllosphere communities are thought to be relatively similar within the same tree individual and/or within the same plant functional groups. This intra- and interspecific variability in microbial community composition should not be ignored, as microbes can have significant effects on tree physiology, with further repercussions on leaf development during tree growth. Second, our study highlights that examining the succession of microbial communities on leaves as they develop and age (from newly formed to perennial leaves) provides new insights into the function of phyllosphere communities in plant performance during leaf aging. These findings underscore the importance of considering the diversity and composition of phyllosphere communities during microbial succession, notably if we aim to understand their long-term effects on tree growth and productivity. Finally, our data suggest that changes in environmental conditions can also have functional consequences for tree growth through alterations in phyllosphere communities. Although the applicability of these results should be extended to other regions to confirm these findings, these data suggest that climate changes can significantly impact tree health and growth by modifying microbial community structure. Future research should focus on identifying the specific interactions between microbial communities and tree physiology to develop targeted strategies for enhancing forest resilience and productivity.

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Author Contributions LW, ZLL and GZJ designed the experiment and collected the samples, LW analyzed data and wrote the first draft of the manuscript in close consultation with ZLL and NF who contributed critically to data interpretation and ideas. CB provided many ideas of the manuscript. All authors contributed to manuscript completion and revision.

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Data Availability All the microbial data was deposited in National Microbiology Data Center (NMDC) and the number of BioProject is NMDC40038831.

Declarations

Competing Interests The authors declare no competing interests.

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