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Host species and temperature drive beech and Scots pine phyllosphere microbiota across European forests

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Tree-microbe interactions are essential for forest ecosystem functioning. Most plant–microbe research has focused on the rhizosphere, while composition of microbial communities in the phyllosphere remains underexplored. Here, we use 16S rRNA gene sequencing to explore differences between beech and Scots pine phyllospheric microbiomes at the European continental scale, map their functional profiles, and elucidate the role of host trees, forest features, and environmental factors such as climate and atmospheric deposition in phyllosphere microbiota assembly. We identified tree species and the associated foliar trait (specifically carbon:nitrogen ratio) as primary drivers of the bacterial communities. We characterized taxonomical and functional composition of epiphytic bacteria in the phyllosphere of beech and Scots pine across an environmental gradient from Fennoscandia to the Mediterranean area, with major changes in temperature and nitrogen deposition. We also showed that temperature and nitrogen deposition played a crucial role in affecting their assembly for both tree species. This study contributes to advancing our understanding on factors shaping phyllosphere microbial communities in beech and Scots pine at the European continental scale, highlighting the need of broad-scale comparative studies (covering a wide range of foliar traits and environmental conditions) to elucidate how phyllosphere microbiota mediates ecosystem responses to global change.

Forests, covering 40 million km^2 of terrestrial surface[1], play a crucial role as a carbon sink, thus contributing to mitigating climate change[2–4]. They also harbor a vast portion of terrestrial biodiversity[1], including hidden microbes in the soil and those associated with trees. This diversity has led to the recent concept of the forest microbiome, emphasizing the crucial role of microorganisms in influencing ecosystem functions and responses to global change drivers[5,6]. However, research on the forest microbiome has predominantly focused on the rhizosphere, leaving other habitats, such as tree canopies (the so-called phyllosphere), underexplored.

The phyllosphere, defined as the total above-ground surfaces of plants—including leaves, stems, flowers, and fruits— is characterized by more dynamic and stressful conditions compared to the rhizosphere[7]. Epiphytic microbes inhabiting the leaf surface face threats such as high temperatures, heavy rainfall, drought, UV radiation exposure, desiccation,

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and low nutrient availability[8]. Additionally, these microbes are often more sensitive to atmospheric chemistry, including pollutant concentrations (such as nitrogen, sulfur, and organic pollutants), which they can metabolize, thus contributing to the degradation of airborne pollutants[8–11].

Numerous studies underlined the role of plant host species as a selective filter of the phyllosphere microbiota assembly in both tropical[12] and temperate[13,14] forests. Major epiphytic taxa identified in tree canopies in undisturbed forests are dominated by Proteobacteria, Actinobacteria, and Bacteroidetes[12,13,15]. These studies suggest that there is a core microbiome for phyllospheric bacterial communities, though differences were observed between tree species in the abundance of each of the core taxa, with identified indicator taxonomic groups associated with different host species[13,15–17]. Functional profiles of phyllospheric microbes, besides allowing them to survive such harsh conditions[18], play a key role for their hosts by improving nutrient and water uptake, protecting from biotic and abiotic stresses, thus increasing plant resistance and directly acting as biological control agents[19,20].

The abiotic conditions experienced by epiphytic microbial communities living on leaf surfaces may be expected to undergo substantial shifts at the continental scale, with major changes in climate (temperature and precipitation) and anthropogenic factors (atmospheric deposition). Studies examining the latitudinal effects on forest microbial diversity and structure, mostly focusing on the rhizosphere, have shown contrasting results. Some studies found negative correlations with latitude[21], others reported positive correlations[22], and some observed a hump-shaped trend[23], with differences in the relationships between fungi and bacteria. However, the exploration of latitudinal effects has been less common in the case of the phyllosphere microbiome. To the best of our knowledge, only one study has investigated bacterial richness and composition in the phyllosphere of forests on a continental scale in China, involving over 300 tree species from 148 genera and 59 families[24]. Phyllosphere bacterial diversity and community composition followed a latitudinal gradient, primarily influenced by changes in temperature and precipitation. Our study advances understanding of factors shaping phyllosphere microbial communities in beech and Scots pine at the European continental scale, emphasizing the need of broad-scale comparative studies on foliar traits and environmental conditions to reveal how phyllosphere microbiota mediates ecosystem responses to global change.

Atmospheric deposition of pollutants, with particular reference to reactive nitrogen compounds, can interact with tree canopies and microbes living there, thus affecting not only the availability and balance of essential nutrients[25], but also changing the acidity of the phyllospheric environment[26]. Leaf associated microbes contribute to both carbon[27] and nitrogen cycling at the ecosystem scale[27–29], though evidence at continental scales is generally lacking (but see ref. 30).

Pyllospheric microbial taxonomy and functional profiles are also strongly mediated by the host species canopy and leaf traits, and therefore they vary among tree species[31]. A major axis of functional variability in tree resource economics[32] is the leaf economics spectrum[33], which is characterized by gradients in the carbon and nitrogen use, affecting leaf palatability to biotic agents, leaf lifespan and photosynthetic return on the investment related to leaf construction and reconstruction. For instance, high-nitrogen acquisitive leaves (generally in deciduous species) are expected to be low-investment and fast-return with short lifespan. Whereas conservative leaves (such as in the case of conifers) show long life span, which comes at the cost of very expensive high dry mass construction and low nutrient concentrations. How those traits potentially affect leaf microbes' assembly is still poorly investigated.

Studies assessing differences in phyllospheric microbiota across species have been mostly site-specific[12,14,15,29] and limited to taxonomical characterization[19]. Whether the 'core microbiome' and microbial structure for a given species and plant functional type is maintained along an environmental gradient has not been fully explored (but see ref. 24), particularly for European forests. In addition to the direct environmental effects, differences in climatic factors along large environmental gradients can also affect both foliar and canopy structure and functional traits[31] for a given tree species, thereby indirectly affecting the structure of the microbiota community assembly. Understanding whether it is the host species, the environment or rather the interaction between these two factors that drive the microbial assembly and functional profiles in the phyllosphere at the continental scale is pivotal to elucidate global change impacts on forest health and functioning[15]. Furthermore, this requires not only determining microbiota taxonomy but also clarifying the specific functions with which the microbes inhabiting foliar surfaces are potentially associated with.

In this study we characterized epiphytic bacterial diversity, structure, taxonomical composition and functional profiles in the phyllosphere of beech (*Fagus sylvatica* L.) and Scots pine (*Pinus sylvestris* L.) forests along a large environmental gradient from Fennoscandia to the Mediterranean area. Beech and Scots pine are two of the most widespread and economically important tree species in European forests[34], representing two plant functional types: temperate deciduous broadleaves and temperate evergreen conifers, respectively. Along the studied gradient, temperature and nitrogen deposition were the two factors that varied the most, ranging from −0.58 °C (in Finland) to 12.9 °C (in Spain), and from less than 2 kg ha^{-1} yr^{-1} (in Finland) to over 15 kg ha^{-1} yr^{-1} (in Belgium) (Table S1). Thus, they are expected to be very different in terms of leaf morphological and functional traits, which should also be reflected in the different types and amounts of nutrients available on their surfaces or those received via atmospheric deposition[30]. Our specific goals were to: (i) investigate differences between tree species foliar microbiomes and test whether these were consistent along the gradient; (ii) map the functional profiles of microbes inhabiting the phyllosphere; (iii) elucidate whether host species and characteristics (i.e., foliar carbon:nitrogen ratio, C:N, and foliar *N*%), forest features (i.e., altitude, forest age, soil C:N, soil pH) and environmental factors (including climate and atmospheric deposition) drive the assembly of the forest phyllospheric microbiota. Our central hypotheses were that the microbial diversity, structure, and functional profiles of the phyllosphere would differ between the two host tree species, regardless of the latitudinal variation. Additionally, we expected a significant effect of latitude (mostly via temperature) and nitrogen deposition on bacterial diversity, either through a direct impact on phyllosphere microbes or an indirect effect mediated by foliar traits.

Results

Structure and taxonomical composition of microbial communities in beech and Scots pine

Microbial communities of the phyllosphere were clearly segregated between tree species (R^2 0.27 and *p*-value 0.001 PERMANOVA; Fig. 1a). Alpha diversity estimated by the Shannon index was significantly higher in beech than in Scots pine (Fig. 1b).

The phyllosphere prokaryotes communities in both beech and Scots pine were largely dominated by the bacterial domain, in particular by the classes Acidobacteriae, Actinobacteria, Alphaproteobacteria, Bacteroidia, Deinococci, Gammaproteobacteria, and Myxococcia, all together accounting for at least 90% of the sequence reads in each sample (Fig. 2a). In turn, <1% belonged to the Archaea domain, which precluded from robust statistical analysis of patterns related to Archaea. The beech leaf surfaces showed higher relative abundance of Actinobacteria (on average 11.2 vs 2% in beech and Scots pine, respectively) and Bacteroidia (27.6 vs 6.1%), whereas Alphaproteobacteria (43.3 vs 54.7%) and Acidobacteria (2.5 vs 17%) showed higher relative abundance on Scots pine needle surfaces (Fig. 2a). For Scots pine at northernmost sites in Finland (Punkaharju, Kivalo and Sevettjarvi) Bacteroidia were not detected on the phyllosphere (Fig. 2a).

The core bacterial genera, defined as those taxa detected in 100% of samples for each species, reached 73.5% and 61.4% of the communities in beech and Scots pine, respectively (Fig. S1). Genera belonging to the species-specific core were more numerous in beech than in Scots pine. The number of zOTUs specific to each tree species was high (about 50% of zOTUs detected in each species), but rare (low abundance) within each community (Fig. S2). In general, beech phyllospheric communities showed more genera

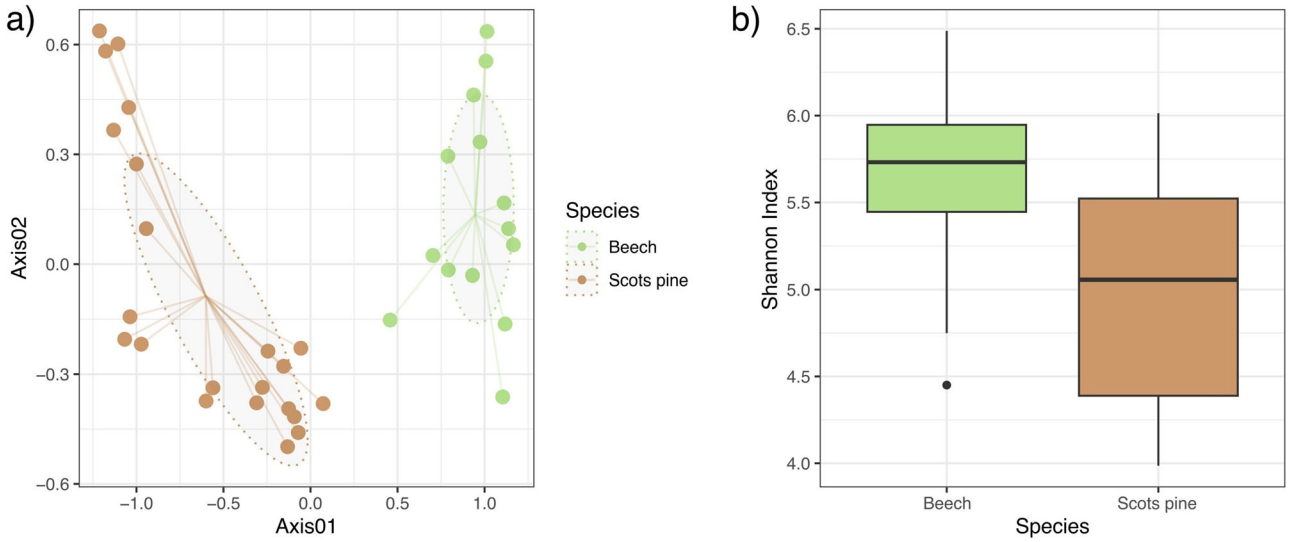

Fig. 1 | Structure and diversity of phyllospheric bacterial communities in beech and Scots pine. a NMDS ordination of phyllospheric microbial communities of beech and Scots pine along a European gradient ($n = 14$ for beech and $n = 22$ for Scots pine). **b** Boxplots representing the Shannon diversity index of the phyllospheric microbial communities of the two tree species. Boxes represent the median (horizontal line) and the interquartile ranges of binned values (Q25, Q75), and the whiskers cover Q25 − 1.5(Q75 – Q25) to Q75 + 1.5(Q75 − Q25) with $n = 14$ for beech and $n = 22$ for Scots pine.

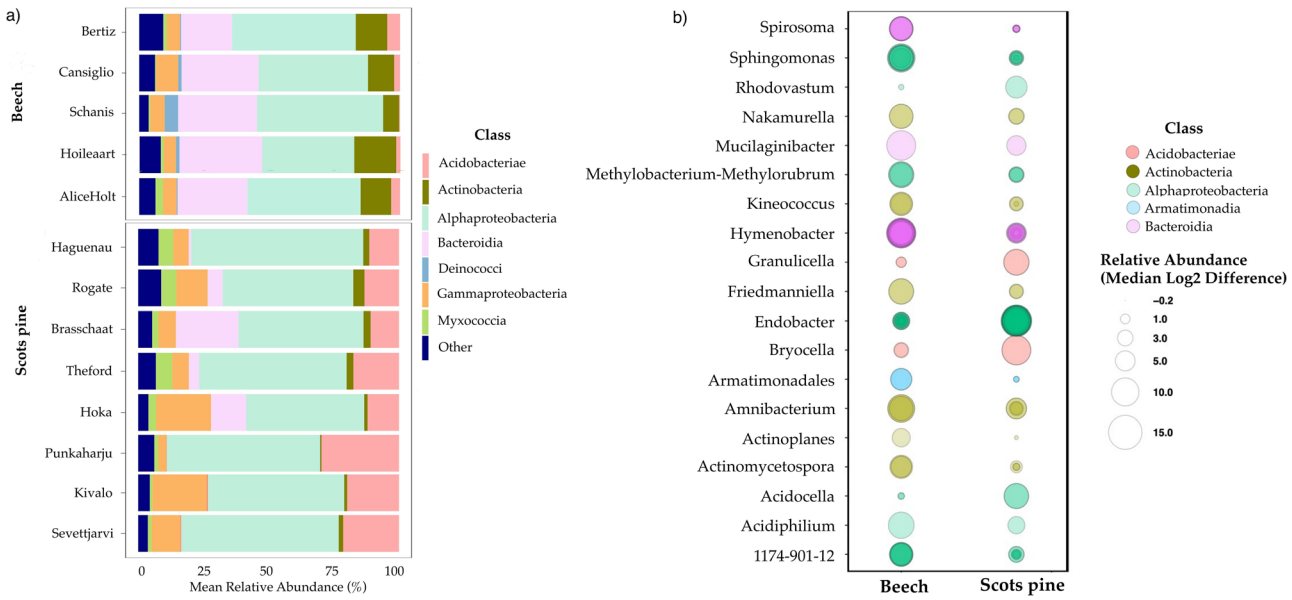

Fig. 2 | Taxonomical composition of bacterial communities in beech and Scots pine. a Taxonomic composition of the microbial communities associated to the foliar surface of beech and Scots pine at the class level, grouping samples by the tree species ($n = 14$ for beech and $n = 22$ for Scots pine) and forest site ($n = 3$, except for Punkaharju, $n = 1$ and Cansiglio, $n = 2$), and ordered by latitude from north (bottom) to south. **b** Differential abundance of zOTUs between the two tree species shown at the genus level. zOTUs with significantly different abundances (expected Benjamini–Hochberg corrected p-value of both Welch's t test <0.001 and Wilcoxon test <0.001 in Aldex2 analysis) and with a median log2 relative abundance higher than 3 in both species were shown ($n = 14$ for beech and $n = 22$ for Scots pine).

with higher differential abundances, with the exception of *Rhodovastum*, *Granulicella*, *Endobacter*, *Bryocella*, and *Acidocella* (Fig. 2b), which were more abundant in Scots pine.

Differences in functional profiles of the two tree species

In order to infer microbial functional profiles, we applied the FAPROTAX-based functional prediction, which allowed us to assign at least one functional profile to around 30% of the community, on average across all communities (see Methods for more details). Among the dominant functionalities of the phyllospheric microbial community, nitrate reduction, nitrogen fixation, methanotrophy and hydrocarbon degradation were significantly higher in Scots pine, whereas methanol oxidation and ureolysis were higher in beech (Fig. 3). *Methylobacterium* and *Methylocella*, in beech and Scots pine respectively, were the genera responsible for most of the differences observed (Fig. S3). Microorganisms with potential chemolithotrophic metabolisms (including methylotrophy) dominated on the foliar surfaces, and overall showed no difference between the two tree species.

Drivers of variation in phyllosphere microbial community structure and diversity

We investigated the influence of geographic (latitude, longitude), climatic (temperature, precipitation), host (including species and related characteristics, such as foliar *N*% and foliar C:N as functional traits), forest features (altitude, forest age, soil C:N, soil pH), and anthropogenic variables

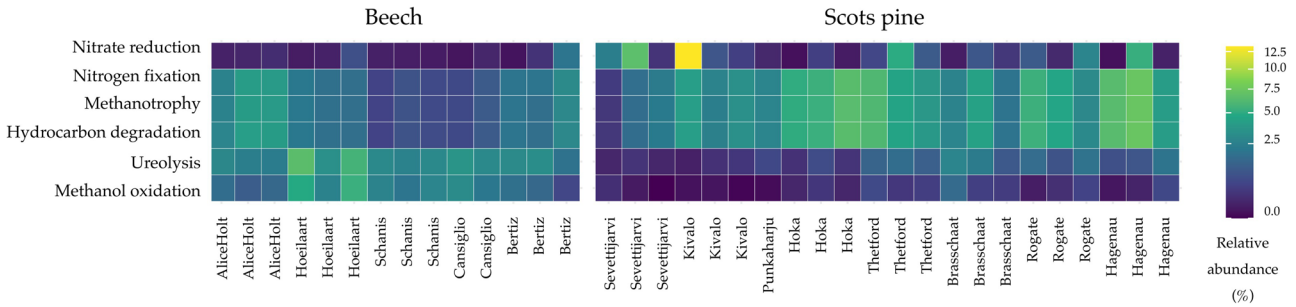

Fig. 3 | Bacterial functional profiles in the phyllosphere of beech and Scots pine. Heatmap of the functional profiles of microorganisms in the phyllosphere for beech and Scots pine grouped by tree species ($n = 14$ for beech and $n = 22$ for Scots pine) and forest site ($n = 3$, except for Punkaharju, $n = 1$ and Cansiglio, $n = 2$). Only functional profiles with significantly different relative abundances between the two tree species are shown (expected Benjamini–Hochberg corrected p-value of Welch's t test <0.05).

(bulk and throughfall depositions of nitrogen and sulfur) on the phyllospheric microbial communities. Multiple regression analysis was performed to assess the relationships between the Shannon index and tree species and characteristics, forest features, and environmental factors (both climatic and anthropogenic factors). We found that the Shannon index increased with temperature, altitude and foliar C:N, but decreased with precipitation and soil pH (Table 1). The model explained 72% of the variance, with temperature contributing the most (over 30%), followed by foliar traits and tree species (Fig. S4). Since tree species were significant predictors in the model, we further explored correlations between microbial Shannon diversity indexes and all the other variables for each species independently (Table S2). In beech, alpha diversity was negatively correlated with longitude, precipitation, and soil pH whereas positive relationships were found with temperature, and soil C:N (Table S2). In the case of Scots pine, microbial diversity was negatively correlated with latitude, longitude, and foliar C:N and positively related with temperature, foliar *N*% and with all the variables belonging to the anthropogenic group (Table S2).

Mean annual temperature and longitude were the only common variables affecting Shannon diversity in the phyllospheric microbial communities of both tree species. Most of the variables included in the analyses (geographical and climatic variables, host characteristics and species, forest features, and anthropogenic variables) influenced the microbial assemblage of the phyllosphere (Table S3), except SO_4- S and NO_3-N TF in beech, and soil C:N and pH in Scots pine. Variation partitioning analysis showed that host (including species and related characteristics), forest, climatic, and anthropogenic variables together explained 50% of the variance in phyllospheric microbial community structure (Fig. 4). Host alone explained 13% of the variance, reaching 35% when considering it simultaneously with the other three groups. Forest and climatic factors each explained 5% and 6% of the variance, respectively, while anthropogenic factors explained 3% when considered alone. Altogether the four groups explained 3% of the variance. We further explored the relationship between different variables and beta diversity for each species by correlating variables with the main axis in the NMDS ordination (Fig. 5). Bacterial communities in the phyllosphere differed across sites, with temperature and nitrogen deposition explaining these differences in both species. For Scots pine, foliar C:N was also an important factor contributing to the divergence of phyllospheric bacterial communities across sites. Additionally, we examined the correlation between temperature and zOTUs whose abundance was significantly different between beech and Scots pine according to Aldex2 analysis (Fig. 2b; Fig. S5). In both species, the correlations were negative, meaning the relative abundances of these zOTUs decreased at higher temperatures (Fig. S5). The genera affected by temperature varied between species: *Amnibacterium*, *Kineococcus*, *Methylobacterium-Methylorubrum*, *Sphingomonas*, and *Spirosoma* for beech, and *Bryocella*, *Endobacter*, and *Granulicella* for Scots pine.

Table 1 | Results from the multiple regression analysis to explore relationship between Shannon index and host (which includes both species and host characteristics) and environmental variables

Variable	Estimate	Standard error	*t*- value	*p*-value
Intercept (Beech)	3.9281	0.4228	9.290	<0.001
Species (Scots pine - Beech)	−0.9414	0.2393	0.2393	<0.001
Temperature	2.5676	0.4381	5.861	<0.01
Precipitation	−1.4497	0.5137	−2.822	<0.01
Altitude	1.7535	0.6641	2.640	<0.05
Foliar C:N	1.2869	0.5004	2.572	<0.05
Soil pH	−0.7653	0.2144	−3.569	<0.01
Adjusted R^2	0.7222			
F-statistic	1.16	$p < 0.001$		

Discussion

We have shown distinct bacterial community structures in the phyllosphere of beech and Scots pine, even across a broad environmental gradient spanning boreal and temperate/Mediterranean biomes, thus supporting our first hypothesis. The influence of host species identity emerged as a primary driver of microbial structure, consistent with earlier site-specific studies in tropical[12,35] and temperate forests[13,15]. Indeed, leaf anatomical features, such as absence or presence of trichomes or cuticular wax, and leaf thickness, have been shown to contribute to shaping the composition of the phyllospheric microbiota[36,37].

Unlike previous findings[15] where coniferous (*Abies balsamea* and *Picea glauca*) exhibited higher alpha-diversity than deciduous species (*Acer saccharum*, *Acer rubrum*, *Betula papyrifera*), our study showed a higher microbial diversity in beech compared to Scots pine. This discrepancy indicates that it may be misleading to generalize results observed at the species level to the functional type level, as microbial diversity is clearly driven by the foliar traits of the host tree species (and hence the habitat associated with it). Nevertheless, the distinctive bacterial structure and taxonomical composition of the beech and Scots pine phyllosphere may be attributed to differences in leaf and canopy structures between conifers and deciduous species. Beech has thinner and larger leaves, while Scots pine has thicker, smaller needles, which influences microbial exposure to environmental factors and nutrient limitations[38,39].

The overall taxonomic composition of the two tree species is aligned with common bacterial groups in the phyllosphere of various plant species[20]. Regardless of the site, beech and Scots pine displayed varying percentages of Actinobacteria, Bacteroidia, Alphaproteobacteria, and Acidobacteria. Notably, Actinobacteria presence accounted for 2–11% of the total microbial community, which is in line with previous studies in tropical, neotropical and temperate forests[12,15,16,40], suggesting that this is a common taxon in the phyllosphere, regardless of the biomes and climatic regions. Additionally, the higher presence of Actinobacteria in beech is in

accordance with the observation that deciduous trees host more of this taxon with respect to evergreen species[41].

Functionalities expressed by leaf-associated microorganisms have been proven to play a pivotal role not only in influencing plant growth, productivity and resistance to biotic and abiotic stresses, but also in regulating the biogeochemical cycles of carbon, nitrogen, phosphorus and sulfur[7,30,41–44].

Among the 62 functions identified by FAPROTAX, only six were significantly different between beech and Scots pine. The low functional variability observed among tree species might be due to the overall similar harsh conditions that microbes experience in the phyllosphere[14]. Nevertheless, interesting differences emerged, with functions related to ureolysis and methanol oxidation, more represented in beech than in Scots pine. The phyllosphere is a particularly privileged habitat of methanol-utilizing bacteria, such as *Methylobacterium*, which play a key role in the methanol emission mitigation thanks to their direct consumption[45]. Methanol is mostly released as a by-product of demethylesterification of homogalacturans of cell wall pectins and is associated with cell wall maturation[46]. Indeed, young growing leaves have been found to emit higher amounts of methanol relative to mature ones[47], which might be the reason for the higher abundance of microbial functions related to methanol oxidation observed in beech[17]. In addition, Scots pine phyllosphere were richer in microbes able to perform methanotrophy, nitrogen-fixation, nitrate reduction, and hydrocarbon degradation. In some methanotrophs, nifH gene encoding for N_2 fixation can be present[48,49], which partially explain the co-occurrence of the two functions (methanotrophy and N_2 fixation). Methanotrophy is a microbial function found in several environments (wetlands, marshes, rice paddies, landfills, aquatic systems) and is essential for reducing the amount of methane emitted to the atmosphere. The phyllosphere harbors a lower abundance of methanotrophs compared to the soil, probably due to the intrinsic low nutrient availability on foliar surfaces for bacterial sustenance[50]. We can hypothesize that the reduced presence of methanotrophs and N-fixing bacteria on beech vs. Scots pine could be related to the lower C:N in beech leaves. Indeed, N-fixation can be favored under C-rich but N-poor substrates, thus at high foliar C:N[51,52], whereas free-living N fixation has shown to be suppressed under high N conditions[53].

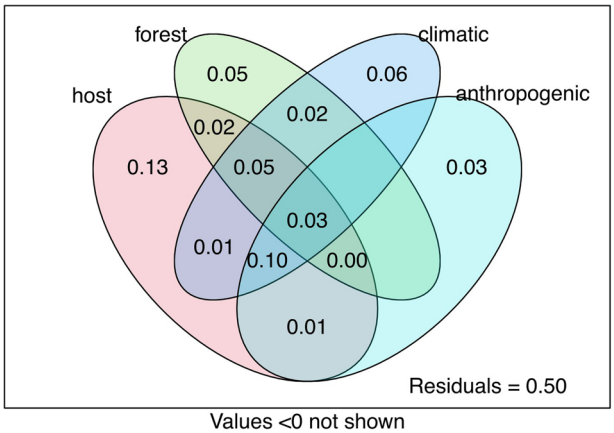

Fig. 4 | Drivers of variation in beech and Scots pine phyllosphere bacterial community structure. Variation Partitioning Analysis (VPA) of microbial community structure with explained variation by host (species and foliar C:N), forest features (altitude, forest age and soil C:N), climatic (temperature and precipitation), and anthropogenic variables (TN BD).

We also found Scots pine needle surfaces to be richer in microbial functionalities linked to hydrocarbon degradation. It has been found that the high concentration of atmospheric pollutants deposited on leaves, including PM10 and polycyclic aromatic hydrocarbons (PAHs), favor the selection of hydrocarbon degrading bacteria inhabiting the phyllosphere[54,55]. Additionally, Scots pine showing higher functionality related to hydrocarbon degradation could be linked to the foliar morphology, i.e., high foliar thickness, presence of waxes and longer lifespan compared to beech, which altogether makes needles more effective in capturing pollutants[56] in general.

It is important to emphasize that in silico functional predictions should be evaluated with caution. Tools like FAPROTAX, Picrust2, Tax4Fun, BugBase, and others[57] still have limited representation of the entire microbial community obtained by ribosomal genes analyses. Transcriptomic analysis of microbial communities remains the most powerful and reliable method for elucidating the functional profiles expressed within these ecosystems[58,59]. This approach offers unparalleled insights into the dynamic processes and gene expression profiles that drive microbial functions, providing a comprehensive understanding of the roles these communities play in situ.

Plant microbiomes are influenced by various factors, including host phenotype, species, genotype, environment, and microbe-microbe interactions[60,61]. Understanding the impact of biotic and abiotic drivers on the plant holobiont is crucial for predicting the effects of climate change on forest microbes and the ecological services they provide. In this study, we sought to determine the extent to which environmental variables influence the structure of the phyllospheric bacterial microbiota of two major European species, beech and Scots pine, at the continental scale. Along the investigated gradient, there are substantial changes in temperature (from

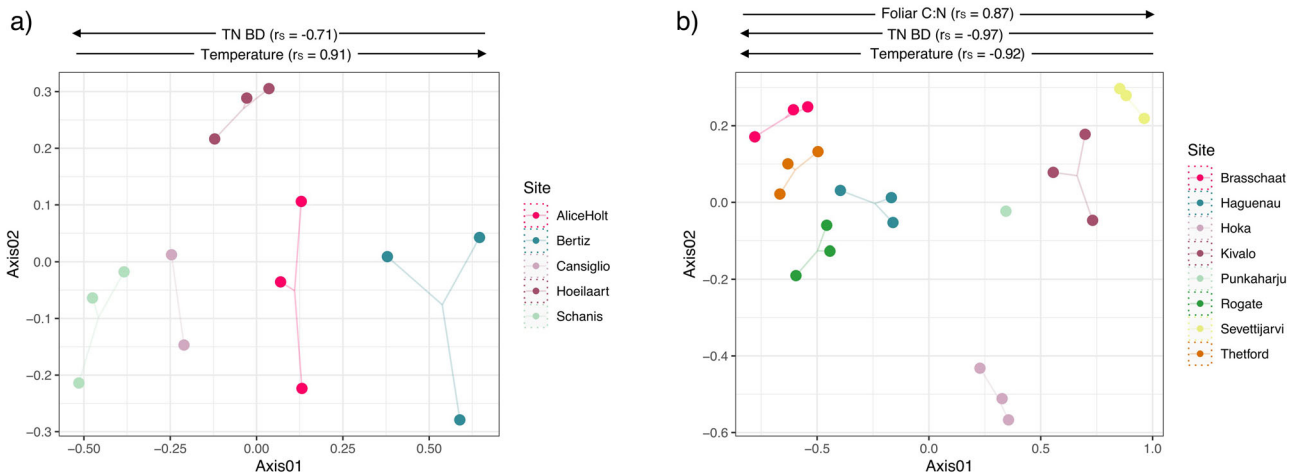

Fig. 5 | Structure of phyllospheric bacterial communities arranged per site, highlighting key variables. NMDS ordination of phyllospheric microbial communities for beech ($n = 14$) (**a**) and Scots pine ($n = 22$) (**b**) grouped by study sites ($n = 3$, except for Punkaharju, $n = 1$ and Cansiglio, $n = 2$). The arrows above indicate the Spearman's correlation coefficient (> 0.7) between the ordination scores of the first axis and variables.

−0.58 to 12.9 °C), as well as atmospheric deposition. In this latter case, we observed the highest values in Central Europe (Belgium, Switzerland), while the lowest in the Fennoscandian countries. This is also reflected in soil C:N and the foliar C:N stoichiometry, in particular for the Scots pine, i.e., lower C:N ratio at high N deposition sites.

The combination of host factors (tree species and foliar C:N as a functional trait), forest attributes (forest age, altitude, soil C:N), climatic variables (temperature and precipitation), and anthropogenic factors (deposition of total N) explained 50% of the observed variation in phyllospheric microbial assemblages. The relatively low variance explained by these variables is consistent with a study assessing diversity at a continental scale in China[24], suggesting that other unexplored biotic and abiotic factors, such as specific leaf features (e.g., nutrient concentrations, secondary metabolite emissions, leaf morphological traits, or intercepted light), could likely play a role in affecting beta diversity[62].

Although we cannot rule out a latitudinal effect related to intercontinental atmospheric bacterial deposition[63], temperature emerged as a common environmental driver influencing both alpha and beta diversity indexes. Specifically, alpha diversity increased with temperature (and decreased with latitude or altitude) along the investigated gradient in Europe. This result aligns with other studies assessing phyllospheric microbial diversity along altitudinal gradients[64], though it contrasts with a continental-scale study in China that reported a hump-shaped biodiversity pattern in relation to latitude[24]. Nevertheless, our findings support the well-established role of temperature in affecting microbial physiology and metabolism[65]. Temperature also appears to influence the relative abundance of taxa specific to beech and Scots pine along the gradient, though further exploration is needed to determine their ecological roles in the phyllosphere and their links to the host species.

The effects of nitrogen deposition on forest growth, biogeochemical processes[66–68], and vegetation diversity[69–71] have been widely explored in the literature. Recent studies have shown that increased nitrogen deposition plays a crucial role in shaping ectomycorrhizal structure and functionality in the rhizosphere[72,73], though its effect on phyllospheric microbial communities has been less explored. In our study, besides temperature, nitrogen deposition was identified as a key driver of beta diversity in both beech and Scots pine, suggesting that bacterial assemblages are sensitive not only to climatic changes but also to atmospheric chemistry conditions. For Scots pine in particular, we observed a positive correlation between microbial alpha diversity and foliar *N* %, as well as a key role of foliar C:N in explaining beta diversity. These results, together with the higher presence of potential bacterial functionality associated with nitrogen-fixation and nitrogen-reduction in Scots pine, suggests that nitrogen-limited conditions in the case of Scots pine needles vs. beech leaves may trigger the presence of microbes able to use atmospheric nitrogen for their metabolism[10,74].

In conclusion, we identified host species identity and the associated foliar trait (C and N stoichiometry) as the primary driver of the phyllospheric microbiota, both in terms of taxonomy and functional profiles. Moreover, we showed that temperature and nitrogen deposition played a pivotal role in explaining assembly of phyllosphere bacterial communities for both tree species along the large latitudinal gradient in Europe. Our results highlight the complex, yet synergic – interactions among biotic and environmental drivers in affecting forest phyllopsheric microbiota and functional profiles at the European continental scale. Extending the metagenomic approaches to other tree species, covering a wider range of foliar traits and assessing their functional profiles are important steps forward to elucidate causes of variations in phyllospheric microbial communities and their role in nutrient cycling, stress resistance, and other processes underpinning ecosystem functioning under global change.

Materials and methods

Sites description and foliar samples collection

Thirteen forested sites within the Level II ICP Forests network (http://icp-forests.net/), including as dominant species (Table S4) two of the most common European tree species (*Fagus sylvatica* L. and *Pinus sylvestris* L.), were selected to span a wide range of environmental conditions, including forest features, climate and atmospheric nitrogen and sulfur depositions (Table S1).

At each site, professional tree climbers collected foliar samples from five trees chosen amongst those already considered for nutrient analysis in the ICP Forests network. Three shoots from each tree were sampled in the upper, middle, and lower third of the canopy in August 2016, except for Sweden and Finland where the samples were collected in October 2016 and August 2017, respectively. To avoid the contact between foliage and the ground (and possible contamination with soil microbes), shoots were sampled from the canopy and were immediately placed in labeled sterilized bags, which were sealed when the tree climber was still in the canopy. The sealed bags were then dropped to the forest floor and immediately placed in a box containing dry ice. The foliar samples were stored in the laboratory at −20 °C until the microbial DNA extraction.

Forest and environmental data

For each site, we considered the following variables: Forest features (altitude, forest age, soil carbon:nitrogen ratio (soil C:N) and soil pH) and host characteristics (foliar nitrogen concentration in percent of dry mass (foliarN %) and foliar carbon:nitrogen ratio (foliar C:N)). Soil C:N was obtained from the top 10 cm, while foliar samples collected in 2016 at most of the sites, except for the Finnish sites where sampling was carried out in 2017. Protocols for soil sampling and laboratory analyses at ICP Forests sites are described in ref. 75 and ref. 76, respectively. Site information included several variables, ranging from longitude and latitude (named as geographic) and environmental variables, including climate and anthropogenic factors. Within the climate factors, we included mean annual temperature and total annual precipitation for the year sampling was carried out, whereas anthropogenic factors included bulk (BD) and throughfall (TF) depositions of inorganic nitrogen (NH_4-N, NO_3-N, total N (TN)) and sulfur (SO_4- S). TN was obtained as the sum of inorganic N and dissolved organic nitrogen. For more details on atmospheric deposition fluxes quantification, please refer to ref. 77 and ref. 30.

DNA extraction and sample preparation for metabarcoding analysis

Of the five trees originally considered for sampling shoot, only three were used for the analysis of phyllospheric microbiota, and the remaining two were used for repeating DNA extraction when not enough microbial DNA (e.g., the forest sites in Sweden and Finland) was available. Microbial DNA extraction and sample preparation were performed as described in ref. 30. Briefly, epiphytic microbial DNA was obtained from 5–6 g (for beech leaves) and 8–10 g (for Scots pine needles) of foliage randomly collected from each of the three shoots sampled per tree and placed (as a composite sample for each tree) in sterile 50-mL Falcon tubes[20]. This allowed to have a canopy-level information on epiphytic bacteria. Thirty-five milliliters of 1:50 diluted Redford buffer wash solution (1 M Tris·HCl, 0.5 M Na EDTA, and 1.2% CTAB[12]) was added to each tube, which was then stirred for 5 min. Then, the washing solution containing the leaf epiphytes was centrifuged for 30 min at 3000 g. The obtained pellet was transferred to 2-ml MO BIO PowerSoil bead beating tubes for DNA extraction, which was conducted following the manufacturer's instructions (DNeasy PowerSoil Kit, Qiagen, Benelux BV; previously the PowerSoil DNA isolation kit from Mo Bio laboratories). The 16S rRNA V5-V6 region was targeted by 799 F and 1115 R, cyanobacteria and chloroplast excluding primers[12]. Overhang adapters (forward 5′TC GTCGGCAGCGTCAGATGTGTATAAGAGACAGAACMGGATTA-GATACCCKG and reverse 5′GTCTCGTGGGCTCGGAGATGTGTA-TAAGAGACAGAGGGTTGCGCTCGTTG) were attached to the primers, as recommended in the Illumina manual for 16S Metagenomic Sequencing Library Preparation. Reaction for the first amplification had a total volume of 25 µL consisting of 1–2 µL of Amplicon PCR Forward Primer (5 µM), 1–2 µL of Amplicon PCR Reverse Primer (5 µM), 2.5 µL of microbial DNA, 12.5 µL of 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems), and MilliQ water. Reactions were carried out following the Illumina procedure:

95 °C for 3 min and then 34 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 30 min. LabChip electrophoresis indicated that the amplicons were 400 bp long. The reactions were purified using CleanPCR beads (CleanNa) and ethanol to remove free primers and/or primer dimers. A second PCR was performed with the cleaned PCR product as template to attach dual indices and Illumina sequencing adapters using the Nextera XT Index primers and following Illumina protocols. Multiplexed 16S libraries were prepared by mixing 5 μL of each reaction at a concentration of 8 pM with 30% PhiX (the internal DNA control from Illumina). The microbial DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Aliquots of microbial DNA obtained as described above were used to prepare amplicon libraries for Illumina 16S rRNA gene sequencing using an Illumina MiSeq instrument with a 500-cycle cartridge. DNA extraction, amplicon preparation, and sequence analysis were carried out at Servei de Genòmica i Bioinformàtica (Universitat Autonoma de Barcelona, Spain).

DNA sequencing processing

Raw 16S rRNA gene sequences were processed by following the UPARSE pipeline[78] (Edgar, 2013) as follows (i) forward and reverse reads were paired to obtain consensus sequences, (ii) low-quality sequences were discarded based on the expected error filtering, set to 0.5 and (iii) sequences were denoised (error-correction) and defined into operational taxonomic units at 100% identity, that is zero-radius OTUs (zOTUs)[79]. SINA[80] and SILVA 138 database[81] were used for the taxonomic assignment. Chloroplast, mitochondrial and unclassified sequences were excluded from further analyses, and a total of 2'827'137 high quality reads were used. Functional Annotation of Prokaryotic Taxa (FAPROTAX) tool was used to determine the potential functional profile of each zOTU[82]. The functional profiles of microorganisms identified in the phyllosphere of beech and Scots pine with high relative abundances were all related to common metabolic functions in both species. For diversity and community structure analyses, the original zOTU table was rarefied and set to a minimum depth of 4500 reads per sample in order to minimize biases for differences in sampling effort. One replicate of the forest site Cansiglio was removed due to the low sequencing depth. All the three samples of the site located in Collelongo were removed from the dataset because late frost in 2016 affected the beech forest in Collelongo, causing complete defoliation, which was followed by a second green-up of the canopy in June[83]. Analysis of the microbial community structure clearly showed a separation of microbial communities from the rest of the sites (Fig. S6).

Statistical analysis

Shannon diversity was calculated using the 'diversity' function from the vegan package[84]. To identify factors affecting Shannon diversity, we performed multiple linear regression analysis, using forest features (altitude, forest age, soil C:N, and soil pH), environmental (including climatic and anthropogenic factors), and host variables (including tree species and related characteristics) as predictors. We applied a two-directional stepwise model selection based on the Akaike information criterion (AIC) and ensured that the variance inflation factor (VIF) for all variables remained below 10. The assumptions of the model were checked using the sjPlot package[85].

The relative importance of predictors in the final model was assessed using the Lindeman, Merenda, and Gold (lmg) method, which partitions R^2 by averaging over all orders, through the 'calc.relimp' function in the relaimpo package[86]. Spearman correlations between the Shannon diversity index and environmental variables were computed separately for the beech and Scots pine datasets.

Continuous variables considered for the forest and environmental data were first rescaled using the formula (x - xmin) / (xmax - xmin), where x represents the given variable. To reduce collinearity in multivariate analyses, we calculated pairwise Pearson correlation coefficients between variables and removed those with a correlation coefficient greater than 0.9. As a result, foliar N%, all anthropogenic variables in TF, as well as NH_4-N and NO_3-N in BD, were excluded from the analysis. Geographic coordinates were also not considered due to their relationship with climatic factors.

A hypothesis contrast test (Student's *t*-test) was used to assess the effect of tree species on Shannon diversity.

For community composition, we calculated a Bray–Curtis distance matrix based on Hellinger transformed community data[87]. Nonmetric multidimensional scaling (NMDS) analysis was performed to show the variation in composition between the two tree species, and across the study sites for each species, where NMDS scores of the first axis were correlated with continuous variables of forest and environmental data by calculating the Spearman's correlation coefficient. The relation of forest, environmental and host variables on the microbial structure was analyzed separately by permutational analysis of variance (PERMANOVA). Each variable was analyzed separately, with community data transformed by Hellinger and 999 permutations, as implemented in the 'adonis' function in vegan package. We also tested to which extent the variation in the community structure was related to four groups of variables: (i) host (species, foliar *N*%, foliar C:N), (ii) forest, (altitude, forest age, soil C:N, soil pH) (iii) climatic (temperature and precipitation), and (iv) anthropogenic variables (bulk (BD) and throughfall (TF) depositions of inorganic nitrogen (NH_4-N, NO_3-N,total N (TN)) and sulfur (SO_4- S)). For this purpose, we carried out a variation partitioning analysis (VPA) of the Bray–Curtis dissimilarities matrix as a function of these four groups by the 'varpart' function in vegan. For each group, variables were selected by two directional permutation tests using the 'ordistep' function to avoid multicollinearity in the VPA model.

The identification of zOTUs and functional profiles with significantly different abundances between beech and Scots pine was carried out using 'aldex.clr' function for compositional data, in Aldex2 package[88]. We calculated the Spearman's correlation between temperature and relative abundance of differentially abundant zOTUs for beech and Scots pine, respectively. The false discovery rate (fdr) was applied to adjust probability (*p*) values for multiple comparisons.

Plots and statistical analyses were performed in R studio[89] by using ggplot2[90], ggpubr[91], tidyverse[92], and ggfortify[93] packages.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Genetic data from 16S sequence analyses are available in the Sequence Reading Archive at the National Center for Biotechnology Information under accession no. PRJNA859654. All variables included in the statistical analyses are reported in Table S1.

Code availability

The coding involved in this study is for statistical analyses, using the specific packages described in the 'Statistical analyses'.

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Author contributions

R.G., M.M., and J.P. conceived the study. R.G. and M.M. led the experimental design. A.V., E.V., P.W., A.T., D.E., B.D.C., S.H., P.M., M.N., F.M., D.R., and G.M. led foliar sampling. R.G. was responsible for extracting microbial DNA, with the supervision of A.B. and S.M.; A.B. sequenced the DNA using an Illumina platform. J.C. processed the raw DNA sequences obtained from the Illumina platform. D.S. conducted bioinformatic analyses and finalized figures with the support of J.C. and E.O.C.; D.S. and R.G. prepared the original draft of the manuscript, with input from J.C., M.M., E.O.C., and J.P.; All authors discussed the results and commented on the manuscript.

Competing interests

Rossella Guerrieri is an Editorial Board Member for Communications Earth & Environment, but was not involved in the editorial review of, nor the decision to publish this article. The remaining authors declare no competing interests.

Additional information

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