



Evergreen camphor and deciduous N₂-fixer alder differently impact soil bacterial communities and N and P cycling in a *Cupressus funebris* monoculture

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Abstract

Background and Aims Transferring pure forests to more resilient and productive mixtures is commonly recommended. However, how introduced species with different identities impact the soil microbiome and nutrient cycling is not well understood.

Methods We investigated the changes of soil physicochemical properties and microbial community following admixing the evergreen deep-rooting camphor

(*Cinnamomum camphora*) and the deciduous N₂-fixer alder (*Alnus cremastogyne*) into a pure cypress (*Cupressus funebris*) forest.

Results Admixing significantly enhanced soil water, total nitrogen (N) and total phosphorus (P) contents. Soil pH, available P levels as well as alkaline phosphatase (ALP) activity were significantly increased in the vicinity of alder, while the presence of camphor improved available N and potassium (K) contents. Compared to the cypress monoculture, the two mixtures exhibited higher soil microbial diversity and a greater abundance of the dominant phyla Proteobacteria and Acidobacteria. Which contributed to improved soil nutritional status through enhanced N

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fixation, inorganic P solubilization and organic matter decomposition. In addition, genes related to methanogenesis, nitrification, denitrification and methane oxidation were markedly decreased in the two mixed stands. Our study showed that introducing alder or camphor improved N cycling without causing higher N loss. Moreover, compared to the evergreen camphor, the deciduous N₂-fixer alder favored bacterial communities stimulating organic P mineralization, nitrite and nitric oxide reduction.

Conclusion Our study reveals the significant effects of admixing on the soil bacterial community and its potential effects on nutrient cycling, highlights the importance of introduced species identities, and provides instructions for designing and managing mixed-species stands.

Keywords Bacterial community · Metagenomics · Nitrogen and phosphorus cycling · Species identity · Soil multifunctionality

Introduction

Forests covered about 31% (4.1 billion ha) of the global land surface in 2020 (FAO 2024). In addition to timber production, forests play a crucial role in maintaining biodiversity and functioning of terrestrial ecosystems. The large-scale pure cypress (*Cupressus funebris*) plantations, characterized by high initial stand density and established in the 1970s, serve as crucial shelterbelts in the hilly regions along the upper reaches of the Yangtze River due to their status as evergreen trees endemic to China and their significant contributions to soil and water conservation, climate regulation, and ecological restoration (Yang et al. 2024; Yan et al., 2022; Yang et al. 2022). However, pronounced climate change is increasing forest vulnerability to abiotic and biotic stressors (FAO 2024), particularly in monocultures, which are often less resilient to disturbances compared to multispecies plantations (Bauhus et al. 2017). Furthermore, the latter generally provide a wider range of goods and environmental services with greater resilience to abiotic and biotic disturbances (Feng et al. 2022; Liu et al. 2018; Bu et al. 2020; Ding et al. 2023b; Pan et al. 2023). Therefore, transformation from monocultures to mixed forests has been widely recognized as a promising management strategy. Among these

factors, the selection of appropriate tree species for mixed planting constitutes a critical step. *Alnus cremastogyne* is a native, fast-growing broad-leaved tree species widely distributed in southwestern China, and it possesses considerable economic and ecological value (Zheng et al. 2023). These values include its use as a source of short-cycle industrial raw materials for high-quality paper (Chen et al. 1999) and plywood (Chen et al. 2022), as well as its function as a non-leguminous nitrogen-fixing tree (Liu et al. 2021). In addition to *Alnus cremastogyne*, *Cinnamomum camphora* is a widely cultivated evergreen broad-leaved tree species in southern China (Sun et al. 2023), and it possesses considerable economic and ecological value (Liu et al. 2022; Sun et al. 2023). Moreover, its application as clonal cuttings in coppice management have gained increasing popularity in recent years. Although both alder and camphor have demonstrated considerable ecological value in previous studies, it remains unclear whether their introduction as late-successional species into pure birch forests can effectively address forest deficiencies and enhance ecological multifunctionality, as systematic research on this topic is still limited.

Soil microbes play a crucial role in regulating various biochemical processes, such as litter decomposition, nutrient transformation and cycling, as well as nutrients uptake, growth, resilience and hence productivity (Gillespie et al. 2021; Pan et al. 2023). They are also involved in buffering climate change by reduction of greenhouse gases (Delgado-Baquerizo et al. 2016; Hu et al. 2024). Soil microbes are highly dependent on abiotic and biotic factors like soil pH, carbon content and vegetation, therefore, notably vary with ecosystems and the respective inhabitants. Even a slight modification in the microbial community may influence their performances, having a substantial impact on soil multifunctionality (Delgado-Baquerizo et al. 2016). Previous studies have demonstrated that admixing with other tree species have strong effects on microbial composition not only in soil, but also in decomposing litter, forest floors, and the rhizo/mycorrhizosphere (Gillespie et al. 2021; Luo et al. 2016; Urbanová et al. 2015). Microbial diversity is generally lower within plant roots and rhizospheres than in bulk soil due to increased host selection pressure close to roots (Urbanová et al. 2015). The effects of admixing on soil biochemical and microbial properties varied with identities of introduced tree species,

e.g., the ability of N₂-fixing, litter and root properties (Gillespie et al. 2021; Liu et al. 2022; Luo et al. 2016; Qin et al. 2024), and largely declined with soil depth (Lei et al. 2024; Pan et al. 2023; Qin et al. 2024; Wu et al. 2020). For example, mixed planting significantly enhanced both the fungal and bacterial communities as well as functional genes involved in C, N cycling in Chinese fir (*Cunninghamia lanceolata*) plantations, particularly in the surface (0–20 cm) soil layer (Lei et al. 2024; Pan et al. 2023; Wang et al. 2022). N fixing trees and deciduous species had a greater effect on bacterial structure and function than non-N fixing and evergreen species, respectively (Liu et al. 2022; Luo et al. 2016). Our current understanding of the impact of different trees species, particularly species with different leaf, root, and rhizosphere traits on soil microbiome communities and associated nutrient cycling still remains insufficient. In particular less attention has been devoted to the bacterial community in comparison to the fungal community. The former also contributes to a range of essential soil processes involved in the cycling of carbon (C), nitrogen (N), and phosphorus (P) (Fenchel et al. 2012). The specificities of different forest ecosystem with various climate conditions make it even more challenging to draw common conclusions. It is therefore imperative to gain a deeper understanding of the interrelationships between tree characteristics, soil microbial communities and soil multifunctionality, the mechanisms underlying biological and abiotic changes, and therefore, providing guidance in transformation from monocultures to mixtures.

Here, we investigated the effects of different intercropping tree species on soil properties and microbial communities by comparing these characteristics in pure cypress (*Cupressus funebris*) plantation (PC) and two cypress mixtures, i.e., cypress admixed with alder (*Alnus cremastogyne*) (AC) and camphor (*Cinnamomum camphora*) (CC), respectively. The objectives were to evaluate the effects of introduced native tree species on soil nutrient dynamics, and to quantify the responses of microbial communities and key species to admixing, and to reveal the link between microbial communities and soil multifunctionality. Specifically, we tested the following hypotheses, (1) admixing can improve soil nutrient availability. (2) admixing influences microbial communities with enhanced microbial diversity and increases abundance of functional genes involved in C, N and P

recycling. (3) the admixing effects on soil property and soil microbiome are species-specific; the deciduous N₂-fixing species alder has greater impacts than the evergreen species camphor.

Materials and methods

Study area and forest information

The study area is located in Linshan Township, Yanting County, Mianyang City, Sichuan Province, China (105°45'E, 31°27'N), which belongs to the middle-upper Yangtze region. The region is characterized by a topography consisting mainly of low hills between 400 to 650 m in elevation (Yang et al. 2022). The study area falls within the subtropical humid monsoon climate zone with mean annual precipitation and mean annual temperature of 826 mm and 17.5 °C, respectively. The soils are classified as purple soils developed from weathered purple limestone without distinct pedogenic horizons (Yang et al. 2022).

The original pure cypress (*Cupressus funebris*) stands were established in the 1970s as ecological protective forests along the Yangtze River with an initial density of more than 4,000 trees per hectare. Thereafter, no management practices were undertaken (Shi et al. 1997; Wu et al. 2020; Yang et al. 2022). The prevailing shrub species are *Vitex negundo*, *Coriaria nepalensis*, *Rosa laevigata*, and *Pyracantha fortuneana*; whereas the herbaceous flora primarily comprises *Imperata cylindrica*, *Carex brunnea*, *Cyperus niveus*, and *Arthraxon hispidus*. The current stand density was about 1,600 trees ha⁻¹ with strong weaknesses, e.g., low productivity and decreased ecological functions (J Chen et al. 2019a, b; Wu et al. 2020; Yang et al. 2022). In 2010, strip-cutting and admixing were conducted in part of the pure cypress plantations. For this purpose, cypress trees were logged within 6 m wide strips from the foot to the uphill. Then 2-year-old seedlings of alder (*A. cremastogyne*) and camphor (*C. camphora*) were intercropped in the strips at a distance of 2 m × 3 m (Fig. S1). A near-natural management approach was adopted thereafter (J Chen et al. 2019a, b). Alder and camphor were originally chosen for mixing because they are both natively distributed in South China with distinct identities,

i.e., alder is a fast-growing deciduous broad-leaved tree species with the ability to fix nitrogen, while camphor is an evergreen broad-leaved tree species with a deep root system that allows it to access deep soil water (Niu et al. 2020).

Soil sampling and pre-process

Soil sampling was conducted in October 2023 in the three stands, i.e., alder-cypress mixture (AC), camphor-cypress mixture (CC) and pure cypress stand (PC), respectively. Samples of bulk soil (0–20 cm) were taken within a 50×50 cm plot predefined in the middle of a cypress tree and an alder (in AC) or a camphor (in CC) in a "S" pattern using a cylindrical soil auger. Five subsoil samples within each plot were subsequently mixed after removing stones and plant residues. Soil samples in pure stand PC were taken in the same manner but between two cypress trees. A total of five samples were collected within each stand. The samples were stored cool and transported to the laboratory on the same day. In the laboratory, soil samples were passed through a 2-mm sieve and divided into three parts: one immediately stored at −80 °C for microbial analysis, another refrigerated at 4 °C for enzyme activity determination, and the remaining portions were dried, ground, and used for assessing soil properties.

Soil biochemical property analysis

Soil properties, including pH, total nitrogen (TN), total phosphorous (TP), total potassium (TK), available nitrogen (AN), available phosphorous (AP), available potassium (AK), water content (WC), NO_3^- -N, NH_4^+ -N, and soil organic carbon (SOC), were measured as described in a previous study (Wang et al. 2023). The activities of soil alkaline phosphatase (ALP), β -1,4-n-acetylglucosaminase (NAG) and β -1,4-glucosidase (BG) were measured using the fluorescence-based protocols described by Bell et al. (Bell et al. 2013). These soil properties, reflecting various functions such as nutrient retention and utilization, soil fertility, microbial activity, and biogeochemical cycling, were selected and measured to assess the overall soil multifunctionality (Delgado-Baquerizo et al. 2016; Xu et al. 2021).

DNA extraction and metagenomic sequencing

The DNA in mixtures of AC and CC, and monoculture of PC soil was extracted from approximately 0.5 g of soil using the FastDNA™ Spin Kit for Soil (Qbiogene, Carlsbad, CA, USA). After performing quality and quantity checks on the extracted DNA, 5 µg DNA was used for 350 bp library construction through NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA), then Illumina HiSeq platform 2500 was utilized to perform metagenomic sequencing with PE150 strategy, which obtained approximately 10 Gb raw reads for each sample. The raw data were processed using MEGA-HIT software (v1.1.2) following quality filtering. Non-redundant open reading frames (ORFs) were predicted and obtained through GeneMark (v3.25) and CD-HIT software (v4.6.8) in sequence (Li et al. 2015). Subsequently, genes involved in the C, N, and P cycles were identified based on their corresponding KEGG Ontology numbers and determined key genes based on their abundance and response to the efforts of three forest stands. Sequence data associated with this project have been deposited in the NCBI Short Read Archive database (Accession Number: PRJNA1128421).

Statistical analyses

Differences in soil physicochemical properties between the three stands were tested by ANOVA and visualized by box plot. Soil multifunctionality was evaluated using methods widely employed in previous studies (Delgado-Baquerizo et al. 2016; Xu et al. 2021). This approach entails the selection of pertinent soil functions, quantification of each using specific indicators, and normalization of these values to facilitate comparison. The normalized scores are subsequently aggregated, typically by means of averaging, to derive a composite measure that reflects the overall capacity of the soil to deliver multiple functions. The variations in soil properties, multifunctionality and microbial diversity across the three forest stands (i.e., mixtures of AC and CC, and monoculture of PC) were examined using one-way analysis of variance (ANOVA).

Alpha-diversity indexes (Chao and Shannon) were computed to assess the richness and diversity of microbial communities and functional traits across

various tree stands based on QIIME2. Beta-diversity was analyzed to assess microbial community composition and functional traits based on Bray–Curtis distances and visualized using principal coordinates analysis (PCoA). In addition, permutational multivariate analysis of variance (Adonis) and similarity analysis (Anosim) utilizing the Bray–Curtis distance metric were calculated to examine the differences among the three stands, using “vegan” package in R. Linear regression analysis was performed to establish correlations between microbial diversity, dominant genes, and soil multifunctionality following tree stands.

To investigate variations in soil microbial nutrient cycling potential in response to admixing, our analysis focused exclusively on nine microbial processes related to carbon (methanogenesis, methane oxidation, and carbon sequestration), nitrogen (N fixation, nitrification, nitrite reduction, and nitrous-oxide reduction), and phosphorus (inorganic P solubilization and organic P mineralization). Through metagenomic screening, we specifically targeted 82 genes, comprising 23 related to C cycling, 49 related to N cycling and 10 involved in P cycling (Table S1). The differences in species and functional trait between the three stands were analyzed using linear discriminant analysis (LDA) effect size (LEfSe) analysis (<http://huttenhower.sph.harvard.edu/galaxy/>) and visualized using the R package DESeq2 (Love et al. 2014). To identify the microbial species responsible for the functional differences, a linear model of Spearman’s rank correlations was used to examine the relations between functional trait and microbial groups, with relations visualized using the Cytoscape package v3.2.1 (Shannon et al. 2003). Additionally, Mantel tests were conducted to identify correlations among soil properties, multifunctionality, and difference microbial species utilizing the ‘vegan’ R package. Pearson correlations were used to assess the relationship between various soil properties and were visualized with a heatmap.

Partial least squares structural equation modeling (PLS-SEM) was carried out to test the indirect or direct effects of admixing on microbial diversity, nutrient cycling and soil multifunctionality in a multivariate approach using SmartPLS 4 software with PLS algorithm (Sarstedt et al. 2024). Nonparametric bootstrap procedure (Bootstrapping) (Efron and Tibshirani 1993) was used to test the significance of

estimated path coefficients in PLS-SEM. The cross-validated predictive ability test (CVPAT) was used to calculation of average loss value, for evaluating the model’s predictive capabilities (Sharma et al. 2023). The average loss values of indicator averages (IA) and linear model (LM) should be significantly below zero to substantiate better predictive capabilities of the model compared to the prediction benchmarks. The final models were selected on the basis of the R^2 , f^2 , construct reliability and validity, discriminant validity and cross-validated predictive ability test (average $R^2=0.416$, $f^2>0.35$, $Q^2>0$, most average loss values significantly below 0).

Results

Soil physicochemical properties in pure and mixed stands

Admixing has significant effects on soil properties. Compared to monoculture (PC), soil pH, contents of water, total N, total P and available K were higher in the two mixtures (Fig. 1a, b, d, f, g). Total K contents, BG and NAG activities as well as BG/NAG ratios were significantly lower in mixed stands (Fig. 1h, n–p). Admixing with alder resulted in significantly increased soil pH, available P content and ALP activities, but significantly lowered total K ($p<0.001$, Fig. 1a, e, h). Camphor, on the other hand, significantly improved available N and available K contents, but resulted in much lower ammonium (NH_4^+) contents (Fig. 1c, g, i). No significant effects of admixing were observed for soil organic carbon and NO_3^- contents, N/P ratios (Fig. 1j–l).

Soil microbial communities in pure and mixed stands

The total number of raw sequences obtained across all samples was $1.09\text{E}+9$, with each sample ranging from $7.42\text{E}+07$ to $8.35\text{E}+07$ sequences. Compared to PC, soil microbial alpha-diversity was notably higher in mixed stands, particularly in AC as indicated by the significantly higher Chao index (Fig. 2a), although no significant differences of Simpson, Pielou_e and Shannon diversity indexes were observed between the three stands (Fig. 2b–d). Similarly, microbial beta-diversity was greatly changed by admixing

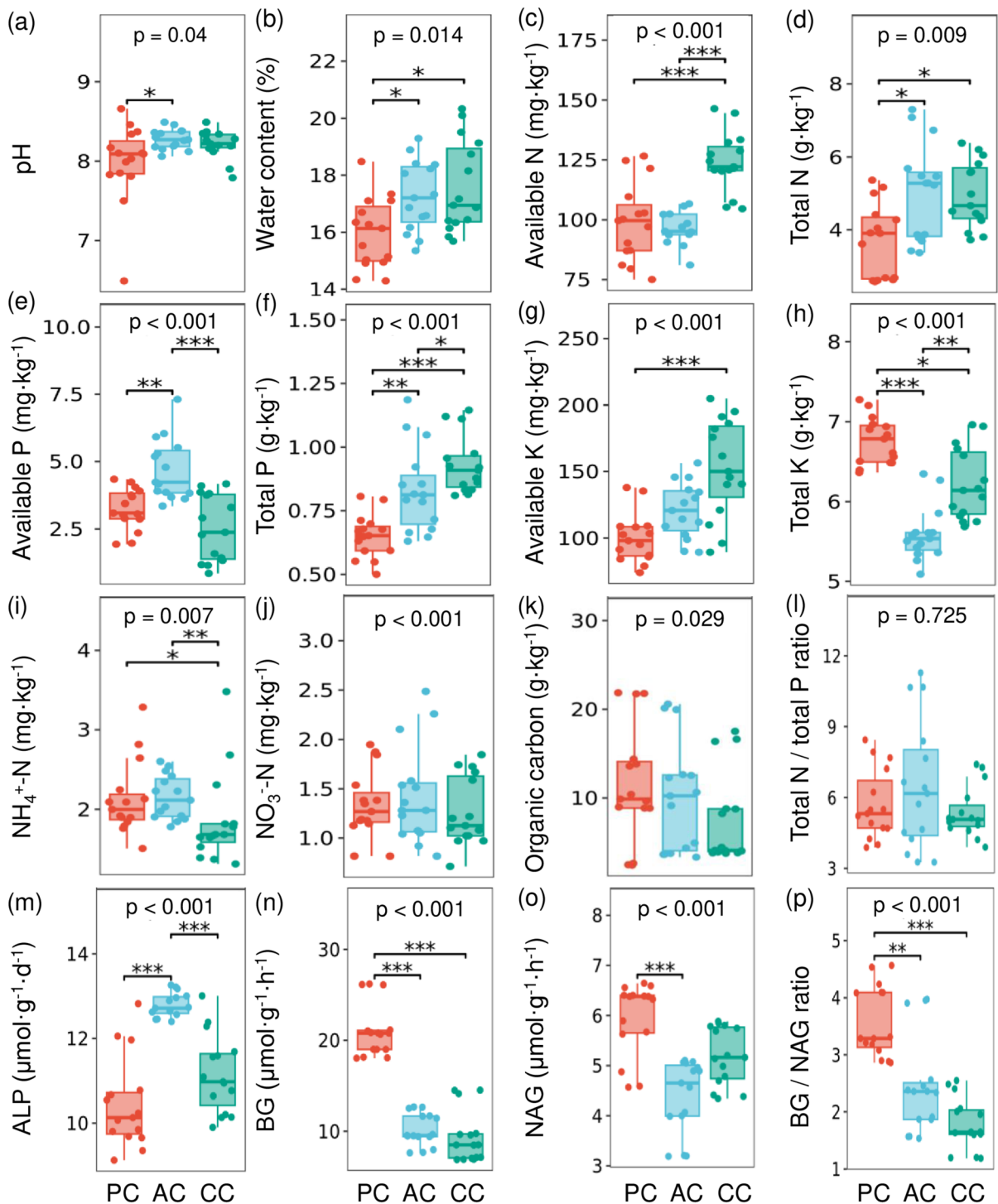


Fig. 1 Soil physicochemical properties (a-l) and enzyme activities (m-p) in pure cypress (PC), alder-cypress mixture (AC), and camphor-cypress mixture (CC). N, nitrogen; P, phosphorus; K, potassium; BG, β -1,4-glucosidase; ALP, alka-

line phosphatase; NAG, β -1,4-N-acetylglucosaminidase. Values represent mean $\pm$ SD ($n=5$). Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) between the two groups

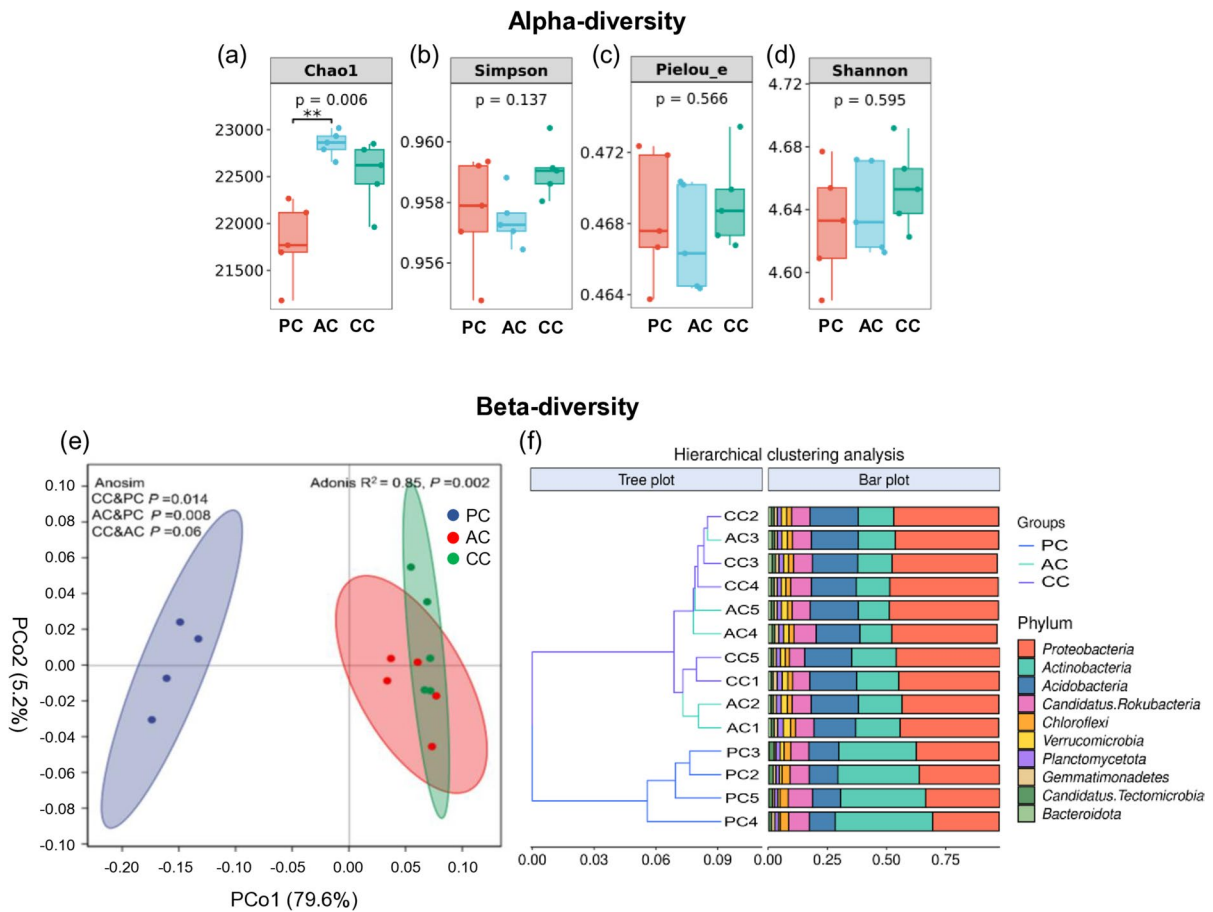


Fig. 2 Microbial alpha- (a-d) and beta- (e,f) diversity indices in pure cypress (PC), alder-cypress mixture (AC), and camphor-cypress mixture (CC). For subpanel f, the left tree plot constitutes a dendrogram of hierarchical clustering, within which samples are clustered based on their similarity to one

another. The shorter the branch length between two samples, the greater the similarity between them. The bar plot (f) represents a stacked bar chart presenting the top 10 species ranked by abundance. Asterisks indicate significant differences (** $p < 0.01$) between the two groups

as shown in the PCoA plot ($R = 0.85$, $p < 0.01$) (Fig. 2e). PCo1 alone explained 79.6% of the total variance (Fig. 2e). Acidobacteria, Actinobacteria, *Candidatus Rokubacteria* and Proteobacteria were the top five most abundant microbial phyla in the three stands. Actinobacteria represented the most abundant phyla in monoculture PC, and Proteobacteria represented the most abundant phyla in the two mixed stands (Fig. 2f).

The relative abundance of microbial species differed significantly between mixed stands and pure stand, i.e., in comparison to monoculture PC, 230 and 231 species were upregulated, while 43 and 47 species were downregulated in AC and

CC, respectively. Significant differences of microbial species were also observed between the two mixed stands, i.e., 4 were upregulated and 57 species were downregulated in CC compared to AC (Fig. 3).

Significant differences were also present at the species level. Compared to monoculture PC, relative abundances of *Flaviumicrobium solisilvae*, *Humiphysa borealis*, *Sphingomonas* sp. G124, *Sphingomonas* sp. NSE70-1, *Sphingomonas* sp. RB56-2 and *Piscinibacter defluvii* were significantly higher in CC; *Caenimonas soil*, *Povalibacter uvarum* and *Lysobacter niastensis* substantially were significantly higher in AC (Fig. 4).

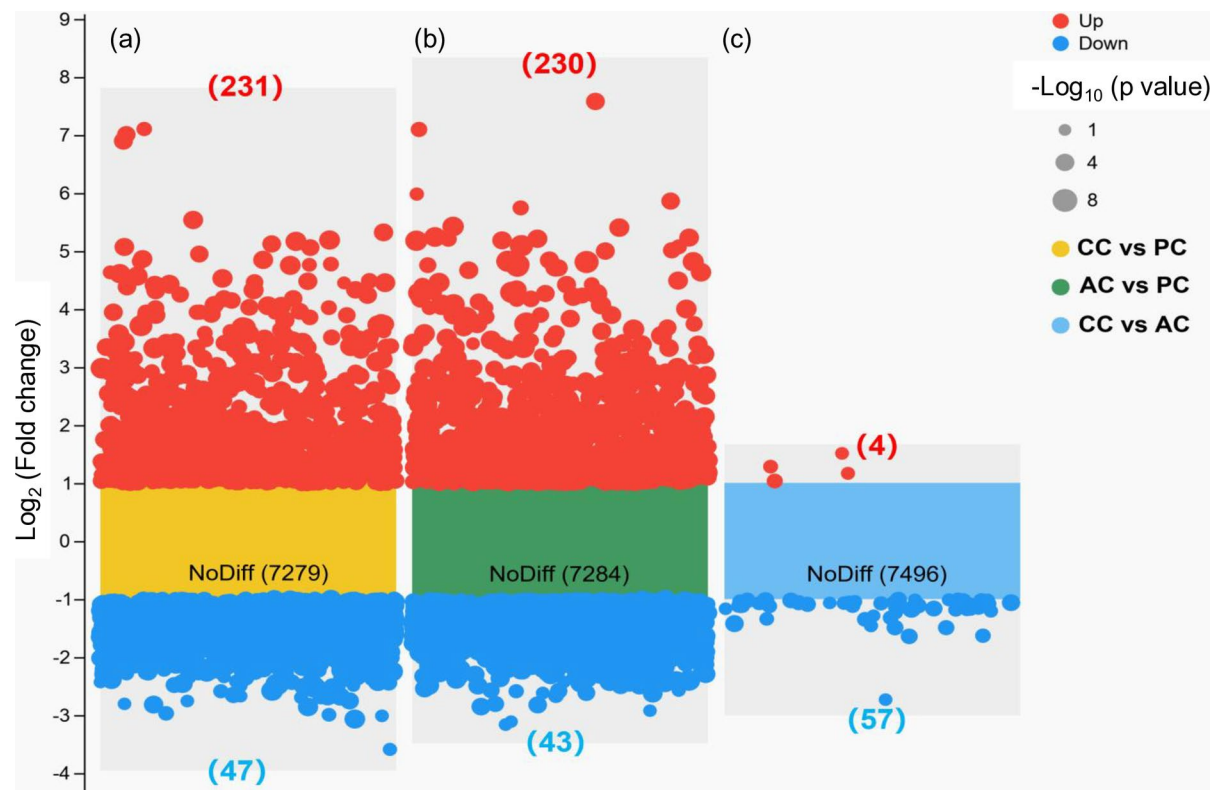


Fig. 3 Multiple volcano plot showing the differences ($\log_2$ fold change) of microbial community between pure cypress (PC), alder-cypress mixture (AC), camphor-cypress mixture (CC). The numbers within each comparing group indicate the number of microbial species which were up- (red dots) and

downregulated (blue dots), respectively. No difference was indicated by “NoDiff ($-1 < \log_2$ fold change < 1)”. The p-values were represented by the size of the dots, with larger dots indicating a lower p-value

The main function gene abundance associated with C, N and P cycling

The beta-diversity of functional traits analysis revealed a significant divergence in the relative abundance of the 79 functional genes between pure and mixed stands ($p < 0.01$, Fig. S2), which mirrored the pattern observed in microbial communities (Fig. 2). Compared to cypress monoculture, functional traits of N_2 fixation, nitrous oxide reduction and inorganic P solubilization were significantly increased in the two mixtures (Fig. 5a, c). Whereas functional traits related to methanogenesis, nitrification and methane oxidation were notably decreased in both mixed stands (Fig. 5a, c). The gene abundances of *smtA* (involved in C sequestration), *napH* and *nosL* (involved in denitrification) were significantly upregulated in the two mixtures. In addition, upregulated abundances of

norB (in CC), *napG* and *napF* (in AC) genes associated with denitrification were also observed (Fig. 5b, d). In contrast, genes related to nitrification (i.e., *pmoA-amoA*, *pmoB-amoB*, *pmoC-amoC* and *nxrA*), methane oxidation (*pmoA/B/C*), methanogenesis and N_2 fixation (*mcrA* and *nifV* in CC, respectively), as well as some genes related to denitrification (*narJ/I/X*, *norG*, *nirC/F* and *nos*) were significantly down-regulated (Fig. 5b, d). Abundances of genes related to organic P mineralization were similar between the monoculture and AC mixture, but significantly decreased in the mixture with camphor. The latter also showed upregulated abundances of genes *norB* and *nirS* related to denitrification (Fig. 5a, c, e).

Spearman's rank correlations showed that the differential functional genes can be divided into ten groups mainly involved in the processes of denitrification, nitrification, C sequestration,

methanogenesis and N_2 -fixation (Fig. 6). Bacteroidota and Gammaproteobacteria were the dominant species of groups 1, 2, 3 and 4, which were predominately associated with soil denitrification and nitrification. The *nosL* gene (groups 1 and 2), *smtA* and *norB* genes (groups 3 and 4) exhibited a positive correlation with Bacteroidota and Gammaproteobacteria. *Flaviumibacter* and *Phnomibacter* (belonging to Bacteroidota) and *Hephaestia* (belonging to Alphaproteobacteria) were significantly positively correlated with both *nosL* and *norB* genes in group 2. The abundance of these microbial groups was significantly higher in CC than in AC (Fig. 4). Negative correlation between Bacteroidota and Gammaproteobacteria and *mcrA* gene was found in group 4. Alphaproteobacteria was the most abundant phylum in groups 5, 6 and 7, which were negatively correlated with nitrification as indicated by the *pmoA-amoA*, *pmoB-amoB* and *pmoC-amoC* genes. Groups 8, 9 and 10 were largely dominated by Actinomycetota, which showed general positive correlation with genes involved in soil N turnover (e.g., *narI*, *nirC*, *narX*, *norG*, and *nirF* genes) and methanogenesis (*mcrA* in groups 9 and 10), but negative correlation with genes involved in C sequestration (groups 9 and 10). Gene *nifV* involved in N_2 -fixation showed negative correlation with groups 6 and 7, but positive correlation with group 10 (Fig. 6). The abundances of most microbial function groups showed significant correlations with soil pH, TN, TP, and TK concentrations (Fig. S3). In addition, the difference in soil function between CC and AC is mainly reflected in nitrite reduction (*nirS*) and nitrogen oxide reduction (*norB*) (Fig. 5e, f).

Correlations between microbial communities and soil multifunctionality

No significant differences in soil multifunctionality were observed between the three stands ($p = 0.34$) (Fig. 7a). Whereas, soil multifunctionality positively correlated with a number of soil properties, i.e., water content, soil organic matter, total N and P, available N and K, NO_3^- -N ($p < 0.05$) (Fig. S4), and significantly correlated with microbial beta-diversity ($r = 0.546$, $p < 0.001$), mostly via N cycling ($r = 0.513$, $p = 0.033$) (Figs 7, 8; Table S2, S3). Similarly, PLS-SEM showed positive effects of admixing on P cycling and negative effects on C cycling (Fig. 8, Table S2-S6).

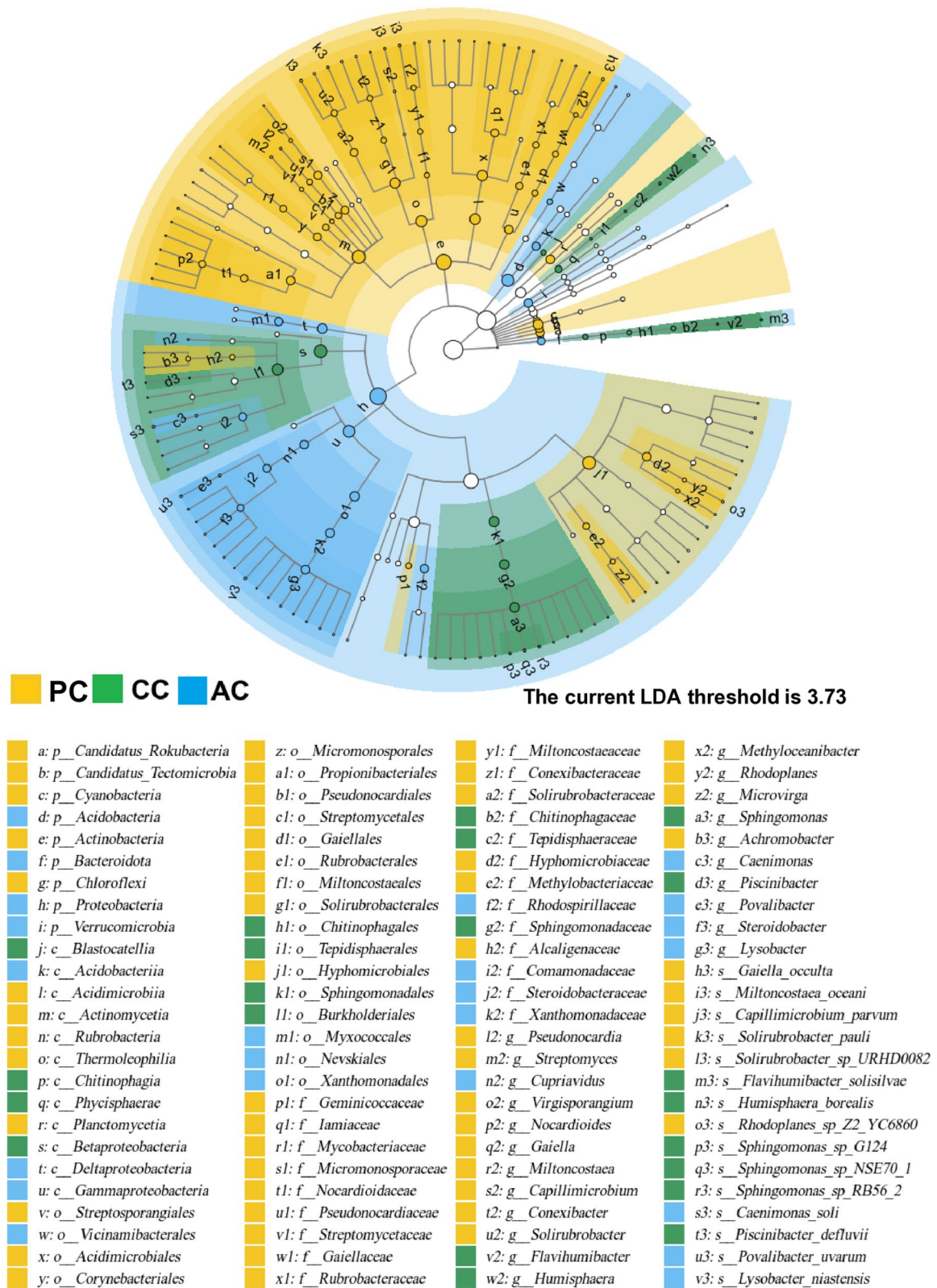
Discussion

Introduction of broad-leaved tree species into monocultures significantly affects soil physicochemical properties and microbial communities. However, our knowledge of the effects of admixing on the microbial community and its regulated soil processes, particularly the specific effects is still scarce. In the present study, we uncovered the general and species-specific effects of admixing on soil multifunctionality and bacterial communities.

Effects of admixing on soil physicochemical properties

In line with our first hypothesis, soil moisture, total N and total P contents were significantly increased by admixing regardless of introduced species (Fig. 1). Moreover, the admixing effects are highly depending on traits of the introduced species. The species-specific effects were confirmed by the differently altered soil pH, N, P and K contents, as well as the extracellular enzyme activities (Fig. 1). In addition to N nutrition, admixing with N_2 fixer alder significantly increased available P levels concurrent with increased ALP activity (Fig. 1). This, on the one hand, may indicated alleviated microbial P limitation in the vicinity of alder (Ji et al. 2023), or on the other hand increased P demand coupled with elevated N assimilation under stimulated growth of trees (Shi et al. 1997; Tian et al. 2019). Similar effects were also reported when *Cunninghamia lanceolata* plantations were mixed with N fixing trees or under N fertilization (Aleixo et al. 2020; Bu et al. 2020; Luo et al. 2016; Pereira et al. 2021). Notably, the admixing with camphor significantly increased soil available N and K contents (Fig. 1c,g), indicating improved exchangeable cations and/or limited consumption by plants and microorganisms (Ding et al. 2023b).

Alkaline phosphatase is thought to be derived primarily from microbial sources rather than higher plant material (Tabatabai 1994). Li et al. (2021) reported that the increased available P levels in the vicinity of N_2 -fixers can be attributed to the significantly increased activity of ALP under elevated soil pH, which was paralleled to our result (Figs. 1, 5e). In addition, in the present study, most likely the *Pseudomonas* and *Bacillus* (Wan et al. 2020), which favor higher organic P mineralization and exploiting



◀Fig. 4 The differences of microbial community between pure and mixed stands in lefse cladogram. The PC, CC, and AC correspond to pure cypress, camphor-cypress mixture, and alder-cypress mixture, respectively. The hierarchical classification relationships of the main taxonomic units in the sample community, ranging from phylum to species (from the inner circle to the outer circle). The size of the nodes corresponds to the abundance of the taxonomic unit; hollow nodes represent taxonomic units with non-significant differences between groups, while nodes of other colors (such as green and red) indicate that these taxonomic units show significant inter-group differences and have a higher abundance in the samples represented by the respective color. Letters identify the names of the taxonomic units with significant differences between groups

different P sources in soils (Yao et al. 2023), induced the increased ALP activities. Activities of the C-acquiring enzyme BG and the N-acquiring enzyme NAG, as well as the BG/NAG ratios were decreased in the vicinity of the two introduced species (Fig. 1n–p). Particularly, the decline BG/NAG ratios may indicate a change in litter input and microbial community after admixing (Luo et al. 2016; Mori et al. 2021; Yang et al. 2022) rather than N limitation (Mori et al. 2021). The changes of soil pH and N, P availability induced by admixing in the present study are thought to alter soil microorganisms (Lei et al. 2024; Luo et al. 2016; Pereira et al. 2018; Wang et al. 2022).

Effects of admixing on soil microbial communities

As assumed in our second hypothesis, admixing has apparent influence on microbial diversity and composition (Fig. 2a, e, f). Admixing resulted in higher abundances of the two dominant phyla, Proteobacteria and Acidobacteria (Fig. 2f), which helped to improve the soil nutritional status (Fig. 1c–f) through nitrogen fixation and organic matter decomposition (Kim et al. 2021). On the other hand, the negative effects of improved soil N contents and higher pH (Deng et al. 2018; Fierer et al. 2012) on Acidobacteria abundances were not confirmed in the present study. Similar admixing effects were observed in *Cunninghamia lanceolata* (Lei et al. 2024; Pan et al. 2023; Y. Zhang et al. 2020a, b) and *Eucalyptus* (Huang et al. 2014; Qin et al. 2024) plantations.

The saprotrophic Actinobacteria oxidatively depolymerize lignin and produce substantial soluble polyphenols, therefore, they are one of the key mediators controlling plant litter decay in forests (Chen et al.

2019a, b; Chen et al. 2024; Deng et al. 2018; Eisenlord and Zak 2010; Liu et al. 2022; Yuan et al. 2023). Prior research has also demonstrated that Actinobacteria exhibit variation across different tree species and are more prevalent in coniferous forests (Liu et al. 2022) and monocultures (Eisenlord and Zak 2010; Garau et al. 2019). Notably, increased abundance of Actinobacteria was not observed in the alder-cypress mixture containing *Frankia*-associated alder. This may be at least partly attributed to the minimal variation observed in *Frankia* between the three stands (Fig. S5), and its pervasive distribution and frequent occurrence outside the geographic range of its compatible hosts (Benson and Dawson 2007; Pöhlme et al. 2014). Another living spaces of *Frankia* are the root hair and root nodules (Yuan et al. 2023), which were not determined in the present study. The findings of the current study also indicate an association between enhanced soil nutrition levels and a decline in the prevalence of *Chloroflexi* populations following admixing. *Chloroflexi* are involved in CO₂ fixing, aerobically oxidizing carbon monoxide (CO) and nitrite (NO₂⁻) while reducing nitrate and ferric iron (Narsing Rao et al. 2022), as well as degrading cellulose, starch, long-chain sugars (Hug et al. 2013), therefore, mediate underground C and N cycling. As anaerobic phototrophic bacteria with diverse metabolism and high competitive advantage in deep soil with low nutrient level, they were found generally more abundant in nutrient poor soils (Chen et al. 2024; Fierer et al. 2012). At present, little information is known about the *Candidatus* Rokubacteria. Only recently Liu et al. (2024) found garlic–maize crop rotation system that had a positive impact on its activity compared to wheat–maize rotation system.

Furthermore, our findings revealed notable bacterial differences at the species level in a neighboring species-specific manner. Admixing with camphor resulted in an increase in the abundance of members belonging to the genera *Flavihumibacter*, *Sphingomonas* and *Humisphaera* (Figs. 3, 4). This presumably promotes N cycling, provides plants with readily available N (Dong et al. 2024; Hou et al. 2021), and may improve the abiotic resilience of trees and polysaccharide degradation in the camphor-cypress stand (Ahmad et al. 2021; Asaf et al. 2020; Dedysh 2023; Zhang et al. 2020a, b). In contrast, admixing with alder resulted in an increase in the abundance of members belonging to the genera *Poalibacter*,

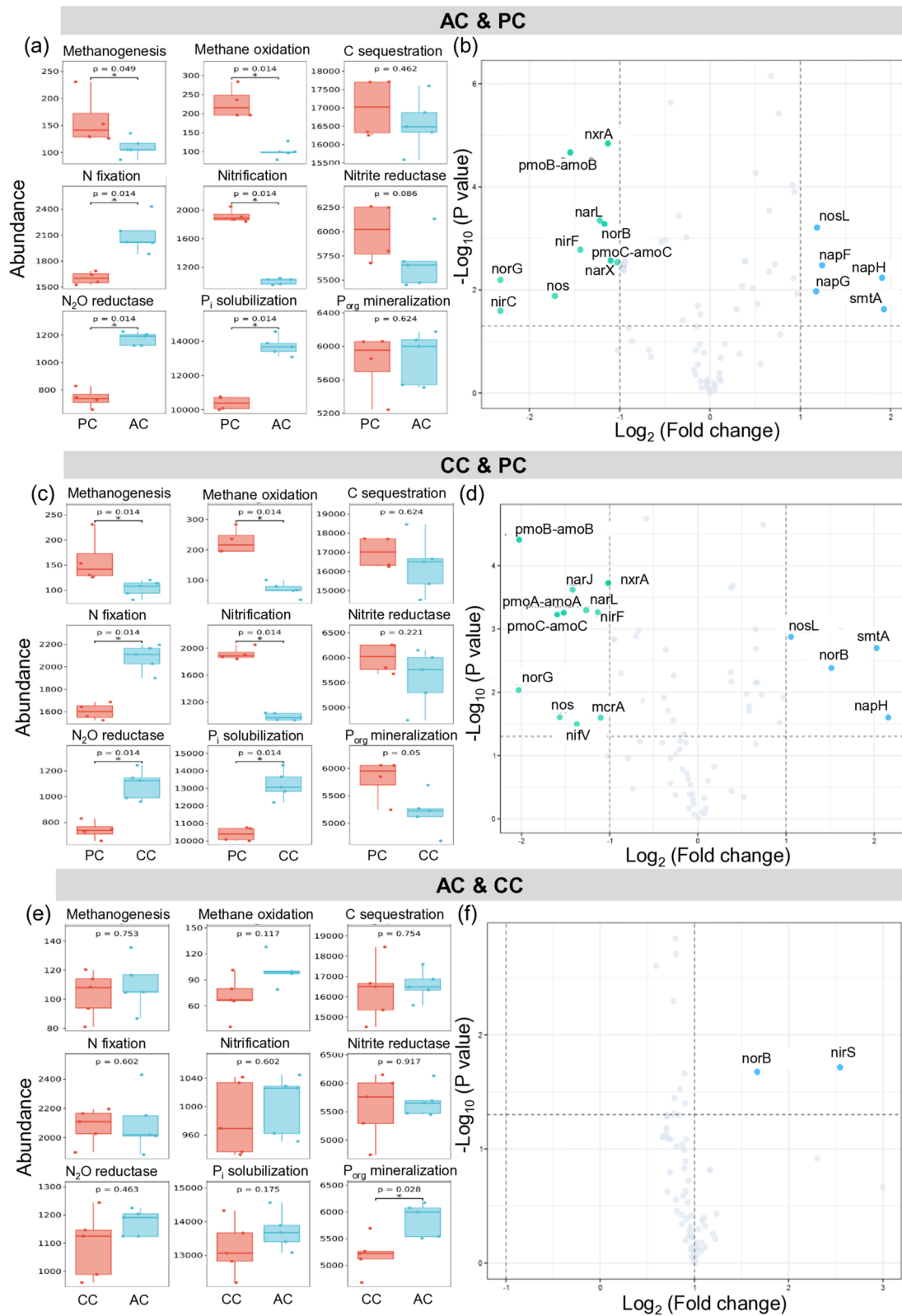


Fig. 5 The differences of functional traits (a, c, e) and gene abundance (b, d, f) among three stands. The PC, CC, and AC sites correspond to pure cypress, camphor-cypress mixture, and alder-cypress mixture, respectively. For volcano plots (b, d, f), each dot represents an individual functional gene. The blue dots represent the functional genes that exhibit a significant increase in the front stand compared to the rear stand, while the green dots represent the contrary. Asterisks indicate significant differences (* $p < 0.05$) between the two groups. P_i, inorganic phosphorus; P_{org}, organic phosphorus

Caenimonas and *Lysobacter* (Figs. 3, 4), which can promote soil fertility through N cycling and plant growth by secreting antibiotics (Panthee et al. 2016).

The present study did not reveal clear changes in soil multifunctionality across the three stands (Fig. 7a, $p = 0.34$). However, we found that soil multifunctionality was significantly positively related to beta-diversity (Fig. 6), which differs from the other study reported a strong correlation with alpha-diversity (Zhang et al. 2020a, b). The reasons for the discrepancy can be explained by our structural equation model (Fig. 8). Alpha-diversity is regulated by the C cycle and is generally associated with abundant bacteria genera, while beta-diversity is governed by the N cycle and is largely determined by the diversity of

rare sub-bacterial genera (e.g., Nitrospirae and Rokubacteria) (Chen et al. 2020).

Effects of admixing on soil bacteria regulated biogeochemical cycling

Changes in microbial composition often trigger significant variations in microbial functions. In accordance with our second hypothesis, the observed alterations in microbial communities were reflected in apparent changes to functional genes associated with biogeochemical cycling (Figs. 5, S2). In line with the more abundant communities of the phyla Proteobacteria and Acidobacteria (Figs. 2, 4) with N₂ fixation capacity (Kim et al. 2021) and the improved total N levels (Fig. 1d), the abundances of functional genes involved in N₂ fixation were significantly increased in both mixtures (Fig. 5a,c). However, significantly upregulated *nif* genes (Ding et al. 2023a) were not observed and *nifV* was rather downregulated in camphor-cypress mixture (Fig. 5a). This may be attributed to the reduced diversity of the Actinobacteria, Proteobacteria, and Chloroflexi phyla (Dos Santos et al. 2012) in the two mixed stands (Fig. 2f), the low abundance of diazotrophic microorganisms in

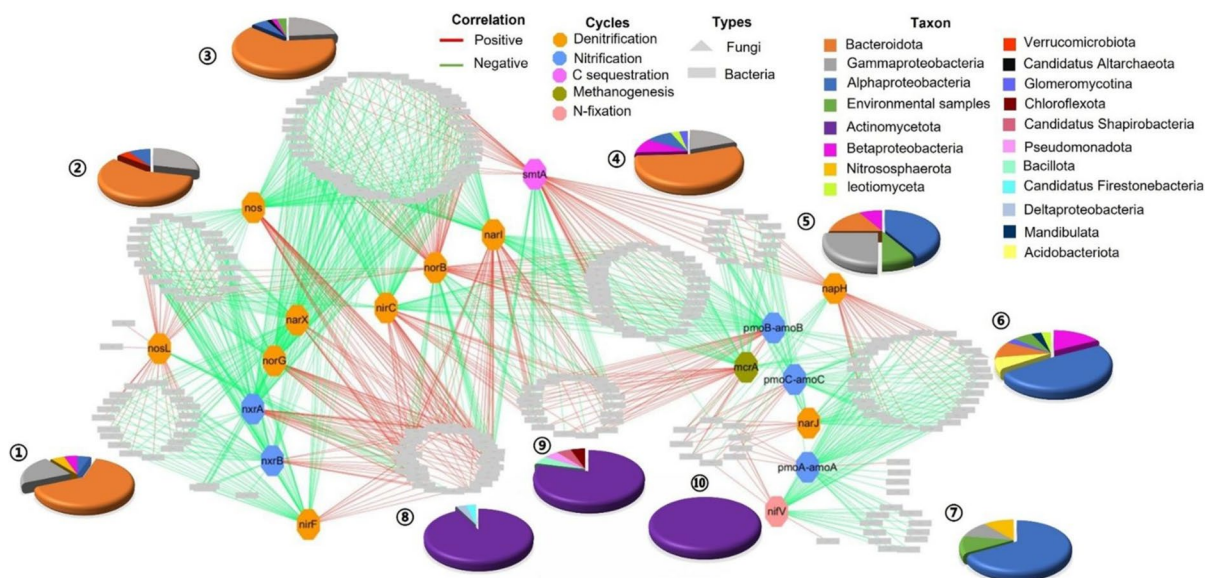


Fig. 6 The co-occurrence networks showing relationships between the different microbial species and different genes. The colors of the octagon signify functional genes with significant disparities during different cycling processes. The gray rectangles and triangles respectively denote bacteria and fungi

with differences. The components in the pie chart represent the proportions of various populations within the same functional clusters. The ten pie plots illustrate the relative proportions of various bacterial genera within each functional cluster

the sampled bulk soil (Bai et al. 2023), and/or other unknown species capable of N_2 fixation (Dos Santos et al. 2012). The increased abundance of functional genes involved in N_2 fixation observed in the camphor-cypress mixture bulk soil is probably due to the improved soil fertility and microbial community favoring N cycling (Dong et al. 2024; Sun et al. 2023).

In addition to upregulated N_2 fixation, reduced N loss was also implied by the decreased abundance of functional genes (i.e., *pmoA-amoA*, *pmoB-amoB*, *pmoC-amoC* and *nxrA*) involved in nitrification and denitrification (*narJ/I/X*, *norG*, and *nirC/F*) in the mixtures (Fig. 5b, d). The latter is the main way of N removal regulated by Bacteroidota, Proteobacteria and Actinobacteria (Hu et al. 2024; Li et al. 2024). This assumption is further supported by the largely conserved substrate contents of nitrate under decreased nitrification rates in the two mixtures (Figs. 1f, 5a, c) (Zhao et al. 2022). Overall, the present study indicated that admixing broadleaf trees into cypress monoculture improved soil N status and N cycling without leading to higher N loss (Gillespie et al. 2021; Liu et al. 2022; Wu et al. 2024).

Cycling and utilization of N by plants requires P, which is the second element limiting plant growth in most ecosystems next to N (Tian et al. 2019; Wang et al. 2020). Corroborating previous studies (Aleixo et al. 2020; Qin et al. 2024), admixing with both broadleaf trees species significantly enhanced soil total P level (Fig. 1f) and inorganic P solubilization capacity (Fig. 5a, c). The observed admixing effects on inorganic P solubilization might be ascribed to the presence of *Pseudomonas* and *Bacillus* abundant in group 9 (Wan et al. 2020).

Admixing contributed to mitigate greenhouse gas emission

In the context of global climate change, forest management strategy should also assess its impacts on C sequestration, greenhouse gas fluxes and hence, its contribution to climate change mitigation (Ontl et al. 2020). A meta-analysis of global forests showed that aboveground C stocks in mixed forests were 70% higher than the average monoculture (Warner et al. 2023). Although detailed information on C stocks in the present study is still limited (Wu et al. 2020), improved growth rates of cypress trees and coverage

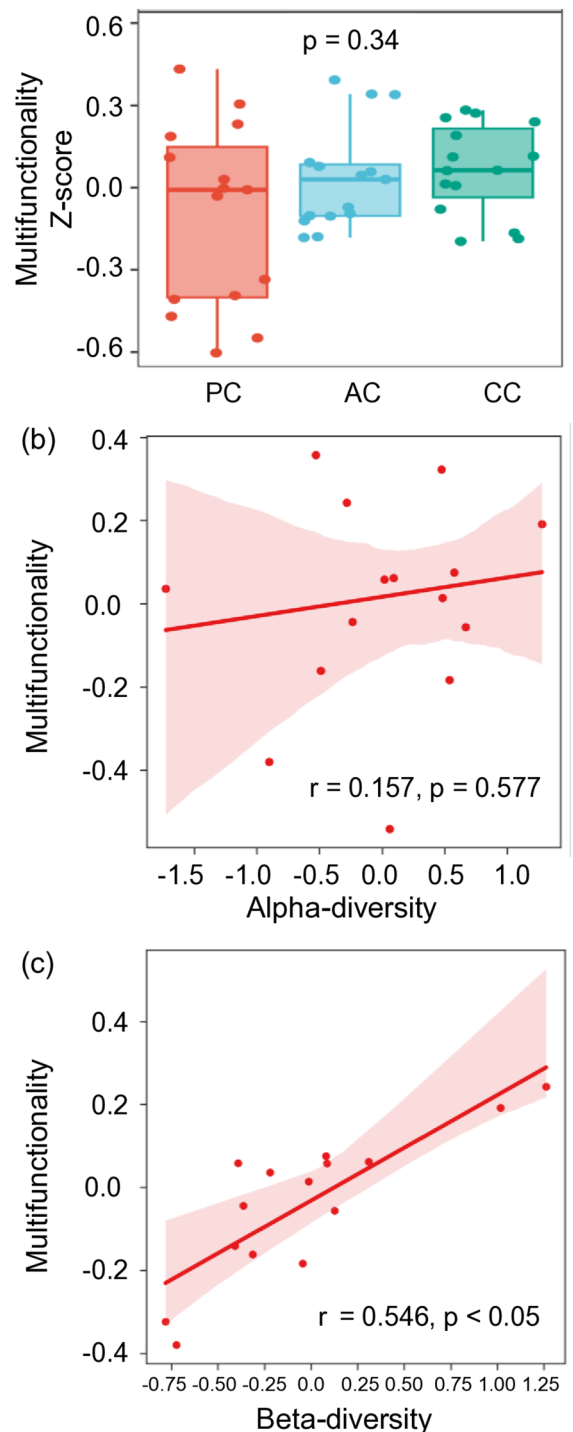


Fig. 7 The soil multifunctionality (a) and the relationships between it and microbial diversity (b, c). The shaded region represents the 95% confidence interval for the regression lines. The PC, AC and CC correspond to pure cypress, alder-cypress mixture, and camphor-cypress mixture, respectively

of shrubs and herbs in the forests were observed in the two mixtures (J Chen et al. 2019a, b). Forest soils are a net sink of methane (CH_4) and a net source for nitrous oxide (N_2O) (Gundersen et al. 2012), two greenhouse gases with a global warming potentials of 23 and 296 times that of carbon dioxide, respectively (IPCC 2001). Higher soil C stocks, particularly in the deeper mineral layer were also expected in the two mixtures (Wang et al. 2013; Wu et al. 2020). Although CH_4 fluxes were not directly measured in the present study, the addition of broadleaf tree species is likely to result in reduced production of CH_4 concurrent with decreased CH_4 uptake (Wang et al. 2013). This assumption is supported by the significantly reduced abundance of functional genes involved in methanogenesis and methane oxidation processes (Fig. 5) (Qin et al. 2023), which are sensitive to the species identities (e.g., fine root biomass, leaf litterfall mass) and the soil characteristics, e.g., pH, moisture, and N stocks (Tang et al. 2006; Wang et al. 2013).

Given the reduced abundances of the *nirS* and *norB* genes involved in the reduction of NO_2^- to NO and NO to N_2O , respectively (Dos Santos et al. 2012), concurrent with significantly increased gene abundances of nitrous oxide reductase (Fig. 5a, c), we speculated a reduction of N_2O production

and emission in the two mixed stands. This can be presumably accomplished by means of the down-regulated denitrification process concurrent with the upregulated abundances of nitrous oxide reductase catalyzing N_2O to N_2 reduction (Fig. 5). These processes are positively correlated with microbial groups 8, 9 and 10, which are dominated by the denitrifiers of Actinomycetota (Chu et al. 2020), with a decline in abundance observed in the two mixtures with moderately elevated pH values (Figs. 1, 2e 6) (Gundersen et al. 2012). The significantly upregulated *nosL* gene in the two mixtures (Fig. 5b, d), which encodes a chaperone that delivers Cu^{2+} to the NosZ apo-protein and is involved in N_2O reduction (Frostegård et al. 2022), provides further support for this hypothesis. Moreover, the reduced abundances of functional genes involved in greenhouse gases CH_4 and N_2O fluxes could be partly explained by the high C and N demand of trees in the two mixtures to support high growth rates and therefore leave less C and N for denitrification (van Haren et al. 2013). The promoted *norB* gene involved in NO reduction in CC is supposed to be regulated by *Flaviumibacter*, *Phnomibacter*, *Hephaestia* (all belonged to the Bacteroidota in group 3) (Figs. 5f, 6). Additionally, the significantly declined substrate of soil NH_4^+ concentration

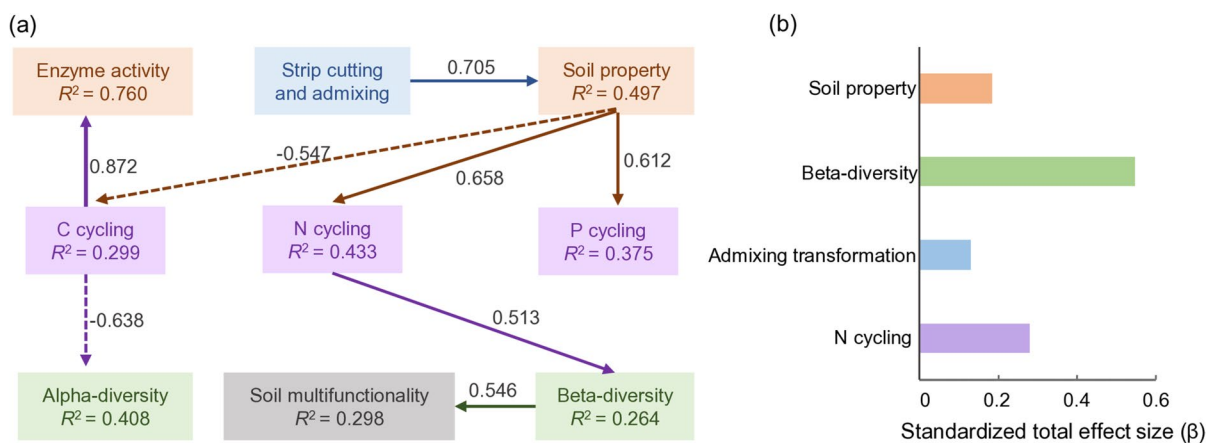


Fig. 8 Structural equation model showing the effects of the admixing on soil multifunctionality. **(a)** Path diagrams of factors influencing changes in soil multifunctionality. **(b)** Standardized total effects (direct plus indirect effects) derived from the structural equation models depicted above. Numbers adjacent to arrows are standardized path coefficients, analogous to relative regression weights. Solid and dashed arrows represent

positive and negative relationships, respectively. Only significant pathways are shown ($p < 0.05$). The proportion of variance explained (R^2) appears alongside every response variable in the model. The average loss values of most indicator averages are significantly below zero, which substantiates the model's acceptable predictive capabilities compared to the prediction benchmarks

in camphor-cypress mixture (Fig. 1i) and increased soil pH in alder-cypress mixture lend further credence to this proposition (Frostegård et al. 2022; Hu et al. 2024). Our third hypothesis assuming species-specific admixing effects on soil property and soil microbiome is fully supported. Our present study provides convincing information that the transition from monocultures to mixed forests implies a reduced climate forcing effect from plantations (van Haren et al. 2013).

Conclusion

This study showed that the incorporation of broad-leaved tree species into pure cypress forests had a significant effect on the soil bacterial community. The findings indicated that admixing led to a substantial improvement of N and P levels without higher N loss. The extent of these effects was found to be contingent upon the identity of the tree species introduced. Compared to the evergreen species camphor, the N₂-fixing species alder was found to have a significant impact on soil pH, as well as on microbial alpha-diversity and composition, favoring P mineralization. The present study also highlights the potential of mixed forest plantations as a means of mitigating greenhouse gases fluxes. Our present study examined the physicochemical and microbial changes in bulk soil. Further studies investigating changes in rhizosphere soil in response to different neighboring species are highly recommended. Furthermore, the influence of altered soil nutrients and microbial status on the physiological and biochemical processes of trees, particularly the roots, warrants further study.

Author Contributions J.W. collected the samples, performed the measurements, analyzed the data, wrote the manuscript. Z.L. and L.L. helped with sample collection. J.C. helped to initiate the project and manage the study forest. J.W., Z.L. and B.D. designed the experiment. G.J. contributed to initiate the project and revised the manuscript. J.K. and C.W. provided the instrumentation and revised the manuscript. B.D. conceived the project, supervised the research, and revised the manuscript.

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Data availability Sequence data associated with this project have been deposited in the NCBI Short Read Archive database (Accession Number: PRJNA1128421). The original physicochemical data is available upon request of the correspondence author.

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval and consent to participate Not applicable.

Consent for publication The authors gave informed consent to this publication and its content.

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