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**Plant-plant interactions and N fertilization shape soil
bacterial and fungal communities**

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Abstract The impact of conspecific and heterospecific neighboring plants on soil bacterial and fungal communities has never been explored in a forest ecosystem. In the present study, we first investigated soil microbial communities in three plantations: *Larix kaempferi* monoculture, *L. olgensis* monoculture and their mixture. Then, a two-year growth experiment was conducted to investigate the effects of intra- and inter-specific plant interactions of *L. kaempferi* and *L. olgensis* on rhizosphere microbial communities in different nitrogen conditions. The results demonstrated clear differences in the beta-diversity and composition of bacteria and fungi among the three plantations, which implied different effects of plant-plant interactions on soil microbial communities. The results of the pot experiment showed that *L. kaempferi* suffered from greater negative effects from its conspecific neighbor regardless of the N fertilization, whereas the negative effect declined when *L. kaempferi* was grown with *L. olgensis* under N fertilization. Changes in intra- and inter-specific plant interactions significantly impacted the chemical and biological properties of soil under N fertilization, with lower concentrations of NH_4^+ , and lower soil microbial biomass (C_{Mic}) and soil carbon nitrogen biomass (N_{Mic}) under intra-specific plant interactions of *L. kaempferi* (KK) compared to inter-specific interactions of *L. kaempferi* and *L. olgensis* (KO). N fertilization increased bacterial and fungal alpha diversities in the rhizosphere soil of KO. For the beta diversity, the PERMANOVA results demonstrated that there was a significant impact of intra- and inter-specific plant interactions on soil microbial communities, with KK significantly differing from intra-specific plant interactions of *L. olgensis* (OO) and KO. The two plant species

and N fertilization showed specific effects on the soil microbial composition, particularly on the fungal community. Both *L. olgensis* and N fertilization increased the abundance of *Ascomycota* but reduced that of *Basidiomycota*, and even shifted the dominance of *Basidiomycota* to *Ascomycota* in KO under N fertilization. Based on our results, we suggest that *L. kaempferi* planted with *L. olgensis* under N fertilization may be an efficient way to promote the productivity of plantations.

Keywords Forest plantations; Plant neighbors; Plant-soil feedback; Fungal communities; Carbon investment

1. Introduction

Many types of forest plantations (mostly monocultures) have been established around the world to provide wood products or restore degraded lands (Paul et al., 2010; Richards et al., 2010). However, the productivity of monocultures has commonly declined due to reasons, such as decreasing nitrogen (N) availability or autotoxicity (O’Hehir and Nambiar, 2010; Chen et al., 2014; Deng et al., 2014). For example, the declined productivity of Chinese fir (*Cunninghamia lanceolata*) monocultures is mainly caused by a novel allelochemical, cyclic dipeptide (Chen et al., 2014), which alters fungal communities in soil by inhibiting the spore germination of *Glomus cunnighamia* and *Gigaspora alboaurantiaca* (Xia et al., 2016). Mixed-forest plantations always have higher productivity than monocultures (Lovelock and Ewel, 2005; Richards et al., 2010). Lovelock and Ewel (2005) have found differences in arbuscular mycorrhizal compositions between different tropical tree mixtures and respective monocultures, and the fungal diversity was correlated with the net primary productivity of plantations. Similarly, Mortimer et al. (2015) have observed that the biomass of soil fungi and bacteria as well as the productivity of tea were higher in a mixture of *Alnus nepalensis* and tea species (*Camellia sinensis* var., assamica) compared with a tea monoculture.

Plants interact intensively and reciprocally with soil microbial communities (Kuzakov and Xu, 2013). Previous studies have confirmed that plants show specific effects on the structure of their own microbial communities, for example, by secreting organic compounds and through input on litter (reviewed in Bakker et al., 2013a;

89 Cline and Zak, 2015; Kumar et al., 2016; Cheeke et al., 2017). However, the majority
90 of these studies have focused on isolated plants or have overlooked the impacts of
91 plant-plant interactions. Conspecific or heterospecific plant neighbors always cause
92 different effects on processes, such as nitrogen uptake, the secretion of root exudates
93 or growth (McKane et al., 2002; Kozovits et al., 2005; Broz et al., 2010; Kunstler et
94 al., 2015; Guo et al., 2017). If plants suffer from their conspecific or heterospecific
95 neighbors, for example as a result of competition, there may be consequent effects on
96 the microbial communities of soil. As research increasingly demonstrates the
97 connection between plants and microbes, there remains a need to understand how
98 intra- and inter-specific plant-plant interactions impact belowground microbial
99 communities.

100
101 Plant-plant interactions are affected by nitrogen availability (Wilson and Tilman, 1991;
102 Guo et al., 2017; Broadbent et al., 2018) and plant-soil feedback processes (Harrison
103 and Bardgett, 2010; Hendriks et al., 2015). A negative effect means that a particular
104 species changes its biological community or abiotic soil properties in a way that its
105 growth is reduced, while the other species shows less harmful or even beneficial
106 effects (Harrison and Bardgett, 2010). Reduced plant growth can result from changes
107 in the composition of beneficial arbuscular mycorrhizal fungi (Castelli and Casper,
108 2003). It has been discovered that nitrogen fertilization can alleviate plant-soil
109 negative effects and plant-plant interactions (Guo et al., 2017), probably not only by
110 increasing the N level of soil but also by improving microbial communities (Xia et al.,

2016). Many studies have reported that nitrogen availability largely impacts bacterial and fungal communities (e.g. Fierer et al., 2012; Dietrich et al., 2017; Carrara et al., 2018; Treseder et al., 2018). Based on a nitrogen fertilization experiment, Yuan et al. (2016) found that *Chloroflexi* and *Bacteroidetes* show positive responses to N fertilization, while *Acidobacteria* and *Verrucomicrobia* have negative responses. Treseder and Allen (2002) discovered that the fungal community of soil tends to shift from *Gigasporaceae* under low N availability to *Glomeraceae* under high N. A few studies have investigated the effects of intra- and inter-specific plant-plant interactions on soil bacterial communities at different N levels in grass lands or agroecosystems (Bakker et al., 2013b, 2014; Pivato et al., 2017). For instance, Pivato et al. (2017) showed that the total bacterial abundance in the rhizosphere of some inter-specific plant-plant mixtures was significantly higher than that of either plant species in a monoculture at a low nitrogen level in an agroecosystem, whereas the difference disappeared at a high nitrogen level. Thus, their results implied that the impacts of plant-plant interactions on soil bacterial communities largely depend on N levels. However, there is less knowledge of the effects of N availability and plant-plant interactions on soil bacterial and fungal communities in forest ecosystems.

Larch plantations are widely distributed and have a high economic and ecological value across the Northern Hemisphere in areas such as Siberia, north-eastern China and Japan (Agathokleous et al., 2017). The larches *Larix kaempferi* and *L. olgensis* are two important plantation species. *L. kaempferi* grown in isolation has a higher

growth rate, biomass production and N accumulation than does *L. olgensis* (Li et al., 2016; Guo et al., 2017). However, our previous results have suggested that the growth of *L. kaempferi* is suppressed when grown in a mixed culture with *L. olgensis* in soil from a *L. kaempferi* plantation (Guo et al., 2017), which may result from the presence of different soil microbial communities. The present study consists of two parts. Firstly, bulk soil samples were collected from three types of plantations (*L. kaempferi* monoculture, *L. olgensis* monoculture and a mixed plantation of these two species). The plantations are characterized by even-aged individuals and there are some herbaceous species in low abundance. This setting enables studies on intra- and inter-specific interactions in natural conditions. Secondly, a two-year pot experiment was established, including isolated plants and combinations of two plants under intra- and inter-specific interactions with and without N fertilization. In the pot experiment, we used soil collected from a mature *L. kaempferi* monoculture to investigate the effects of plant-plant interactions and N fertilization on soil microbial communities. We aimed to answer the following questions: 1) do different plantations contain specific types of soil bacterial and fungal communities? Based on the pot experiment, 2) if the intra- or inter-specific plant interactions of the two studied species were different, would the rhizosphere soil bacterial and fungal communities also differ from each other? 3) If N fertilization differently affects intra- and inter-specific plant interactions, how does the interplay of N fertilization and plant interactions affect rhizosphere soil communities?

2. Materials and methods

155

156 In the first part of this study, bulk soil samples were collected from three types of
157 plantations: *L. kaempferi* monoculture plantation, *L. olgensis* monoculture plantation
158 and a mixed-plantation of these two species. In the second part of the study,
159 rhizosphere soil samples were collected from the pot experiment. The pot experiment
160 was conducted outside on an open space at the Qingyuan Experimental Station of
161 Forest Ecology, Institute of Applied Ecology, Chinese Academy of Sciences, Liaoning
162 Province, Northeast China (41°51'N, 124°54'E, 560 m above sea level). The mean
163 annual rainfall is 811 mm and the mean annual air temperature varies between 3.9 °C
164 and 5.4 °C. *L. kaempferi* and *L. olgensis* plantations are widely distributed in the study
165 area.

166

167 *2.1. Plantation selection*

168

169 The following principles were followed when selecting the three types of plantations:
170 1) similar age, 2) close location, and 3) similar exposure and slope. Eventually, we
171 found only one pure *L. kaempferi* plantation (about 20 years old, 3.6 ha and 2050
172 stems per ha), one pure *L. olgensis* plantation (about 20 years old, 4.0 ha and 1750
173 stems per ha) and one mixed plantation (about 24 years old, 6.0 ha and 600 *L.*
174 *kaempferi* stems per ha, and 640 *L. olgensis* stems per ha), about 30 km away from the
175 research station. The two monoculture plantations were adjacent, and the *L. olgensis*
176 plantation experienced thinning forest management in 2010 according to the

description of a local farmer. The mixed plantation was about 400 m away from the two monoculture plantations but on another hillside.

In the center of each plantation, ten plots (10 m × 10 m) were established. From each plot, ten bulk soil samples (0-10 cm in depth) were collected from ten subplots (1 m × 1 m) and then mixed to form one composite sample. Finally, ten samples from each plantation were collected. Each bulk soil sample was divided into two equal subsamples: one subsample was used for the analysis of soil properties, while the other subsample was stored at -20 °C for the subsequent DNA extraction. Five subsamples from each plantation were randomly selected for later DNA extraction.

2.2. Pot experiment and sampling

For the pot experiment, soil was collected from the *L. kaempferi* plantation near the research station and then homogenized (pH 5.65, C 18.61 g kg⁻¹ and total N 1.82 g kg⁻¹) early October, 2013. One-year old seedlings of *L. kaempferi* and *L. olgensis* with approximately the same crown size and height were chosen from a local nursery garden to establish intra- and inter-specific plant interactions. Before planting, all roots were carefully washed with sterile water to remove adhering soil. Two seedlings were planted 10 cm apart from each other into each plastic pot (external diameter and height 56 cm and 33 cm, respectively) late October, 2013. KK refers to two *L. kaempferi* seedlings in a pot, KO refers to one *L. kaempferi* and one *L. olgensis* in a

pot, and OO refers to two *L. olgensis* seedlings in a pot. Each seedling combination included thirty-two pots. In May 2014, sixteen pots were randomly selected from each treatment to carry out N fertilization by applying 5.1 g urea (46.3% N). N application was repeated three more times (each time 5.1 g urea), in June 2014, and in May and June 2015. In addition, single *L. kaempferi* seedlings (K) and single *L. olgensis* seedlings (O) were planted into pots in late October, 2013, six pots for each species. Similarly, three pots from both K and O were randomly selected to apply urea as above. The combination of results from the plantations and pot experiment will provide a better understanding of the effects of intra- and inter-specific plant interactions on soil bacterial and fungal communities.

Double-plant pots were harvested twice, late August, 2014 and early September, 2015. The harvested plants were dried at 70°C for 72h to determine their dry masses. Neighbor effect index (NEI), estimated to measure plant-plant interactions, used the following formulas (Manea and Leishman, 2011):

$$NEI_{k/kk} = (Y_{k/kk} - Y_k) / (Y_{k/kk} + Y_k), NEI_{o/oo} = (Y_{o/oo} - Y_o) / (Y_{o/oo} + Y_o),$$

$$NEI_{k/ko} = (Y_{k/ko} - Y_k) / (Y_{k/ko} + Y_k), NEI_{o/ko} = (Y_{o/ko} - Y_o) / (Y_{o/ko} + Y_o),$$

where $NEI_{k/kk}$ and $NEI_{o/oo}$ indicate intra-specific plant interactions for *L. kaempferi* and *L. olgensis*, respectively; $NEI_{k/ko}$ and $NEI_{o/ko}$ indicate inter-specific plant interactions for *L. kaempferi* and *L. olgensis*, respectively. Y_k and Y_o are the biomass of *L. kaempferi* and *L. olgensis* grown in isolation, respectively. $Y_{k/kk}$ and $Y_{o/oo}$ are the biomass of *L. kaempferi* and *L. olgensis* with intra-specific plant interactions,

respectively. $Y_{k/ko}$ and $Y_{o/ko}$ are the biomass of *L. kaempferi* and *L. olgensis* with inter-specific plant interactions, respectively. Because the biomass of isolated plants is higher than that of plants exposed to plant-plant interactions (Kozovits et al., 2005; Guo et al., 2017), the NEI values are negative. A more negative NEI value indicates that a plant suffers a more negative impact from its neighbor.

Soils were sampled following the last harvest. Using a soil corer (4 cm in diameter), soil samples were collected from four pots of KK, KO and OO, as well as from corresponding pots with N fertilization. The sampling position was at the center between the two plants in a pot. Then loose soil was removed by careful shaking and the tightly-adhering soil was sampled from root surfaces as rhizosphere soil. Rhizosphere soil samples were collected also from the pots of K and O as well as from corresponding pots with N fertilization. The sampling center was 5 cm away from the basal stem. Each soil sample was divided into two equal subsamples: one subsample was used for the analysis of soil properties, while the other subsample was stored at -20 °C for the subsequent DNA extraction. Three subsamples from each treatment were chosen for later DNA extraction.

2.3. Determination of soil properties

Soil pH was determined from soil-water suspensions (1:2.5 v/v). Available phosphorus (AP) was extracted with sodium bicarbonate and then determined using

the molybdenum blue method (Xiong et al., 2015) Soil organic matter (SOM) was determined using the potassium dichromate external heating method (Ciavatta et al., 1991). Total N (TN) was determined by the Kjeldahl method (Buchi K370, Switzerland). Soil NH_4^+ and NO_3^- were extracted with 1M KCl and the indophenol-blue colorimetric and double wavelength (220 nm and 275 nm) methods (Nie et al., 2018), respectively. Soil microbial biomass carbon (C_{Mic}), nitrogen (N_{Mic}) and phosphorus (P_{Mic}) were determined by the chloroform fumigation extraction method (Yao et al., 2017). For the pot experiment, two-way ANOVAs were applied to check the impact of N application and plant-plant interactions on the soil properties.

2.4. DNA extraction and Illumina sequencing

Genomic DNA was extracted using the Power Soil kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The primers used for the amplification of the V4-V5 hypervariable regions of the bacterial 16S rRNA gene were 515F (5'-GTGYCAGCMGCCGCGGTA-3') and 909R (5'-CCCCGYCAATTCMTTTRAGT-3') (Yao et al., 2017). The primers used for the amplification of the partial ITS region of fungi were ITS4: 5'TCCTCCGCTTATTGATATGC-3' and ITS3_KYO2: 5'GATGAAGAACGYAGYRAA-3' (Bokulich and Mills, 2013). The PCR reactions, quality control and purification processes followed the instructions of Yao et al. (2014). A library was constructed and all sequences were generated with the

Illumina's MiSeq platform using paired-end reads. All above-mentioned steps were completed at the Environmental Genome Platform of Chengdu Institute of Biology, Chinese Academy of Sciences, China.

2.5. Bioinformatics

The paired-end reads of the bacterial 16S rRNA gene and fungal ITS region amplicons were processed using the mothur pipeline (V.1.35.1) (Schloss et al., 2009), based on the MiSeq standard operating procedure (Kozich et al., 2013). For quality control, the 16S and ITS sequences that contained ambiguous (N) bases and homopolymers longer than 8 nucleotides were screened out. The remaining sequences were pre-clustered to allow for up to 1 bp difference per 100 bp bases to remove potential sequencing errors before the identification of the chimeric sequences using the UCHIME algorithm. The SILVA full length reference sequences (V.128) were used for the alignments of the bacterial 16S rRNA sequences. For the fungal ITS sequence analysis, due to a lack of reference templates for sequence alignment, we trimmed the raw sequences to the same size (300 bp) after the removal of the forward primer sequence. After removing the chimeric sequences, the unique bacterial 16S rRNA gene sequences were classified using the SILVA reference database (V.128), and fungal ITS sequences were classified using the mothur-formatted UNITE ITS reference database (UNITE v6_sh_99) with the default bootstrapping algorithm (cutoff value 80%). All sequences were assigned to operational taxonomic units

(OTUs) using the OptiClust clustering algorithm at 97% similarity. Bacterial 16S sequences were assigned to OTUs using classify as the split method, whereas fungal ITS sequences were assigned into OTUs using fasta as the split method based on nearest neighbor clustering. Singletons were removed from both bacterial and fungal ITS datasets. For the bacterial OTU dataset, OTUs that were classified as non-bacterial or chloroplast were removed. For the fungal OTU dataset, all non-fungal OTUs were removed. The raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA) database under accession no. SRP125300.

2.6. Alpha diversity estimation

The alpha diversity of bacteria and fungi in each sample was rarefied and estimated at the sampling depths of 2873 and 5558, respectively, using the R package phyloseq (McMurdie and Holmes, 2013). The normalization of the alpha diversity data was visualized using Q-Q plots. The homogeneity of variances was examined using the Bartlett test. The differences of the means between treatments and their interactions were tested using ANOVA, assuming that the alpha diversity data were normally distributed and the variances were equal among treatment groups. The non-parametric Kruskal-Wallis rank sum test of each single treatment factor was used when the data were not normally distributed or the group variances were heterogeneous. Tukey's multiple comparisons of means were used when the differences between groups were significant. The significance of the differences was concluded at the 95% confidence

level ($P < 0.05$).

2.7. Beta diversity estimation

The relative abundance of each OTU was calculated by dividing its read count by the total read count of the corresponding sample, prior to the beta diversity analysis. Principal coordinate analyses (PCoA) were applied to the relative abundance data to visualize the broad pattern of bacterial and fungal communities between treatment groups based on the Bray-Curtis distance, using the R package phyloseq (McMurdie and Holmes, 2013). PERMANOVA was used to assess, whether the treatment groups of Species and N fertilization and their interaction resulted in a different bacterial community composition with the default 999 permutations. Betadisper was used to test, whether the dispersions of observations between the treatment groups were equal with 999 permutations. PERMANOVA and Betadisper were performed based on the Bray-Curtis distance using the R package Vegan (Oksanen et al., 2007). Pairwise PERMANOVA tests were used when the differences were significant between a priori groups following PERMANOVA tests. Constrained analysis of principle coordinates (CAP) was performed to visualize the significant differences in the community composition between the treatment groups based on the Bray-Curtis distance using the R package phyloseq (McMurdie and Holmes, 2013) with 999 permutations. The significance of the differences was concluded at the 95% confidence level ($P < 0.05$).

2.8. Biomarker discovery

The linear discriminant analysis effect size LEfSe (Segata et al., 2011) was used to determine the differentially abundant OTUs 1) among treatments without interaction (isolated *L. kaempferi* and *L. olgensis*) and with intra-specific and inter-specific interactions, irrespective of the N treatment (Class: Interaction; Subclass: N treatment), and 2) between the N-treated and control pots (Class: N treatment; Subclass: Interaction) in the pot experiment. For the soil sampled from the three plantations, LEfSe was used to explore the OTUs with differential abundance between the different interaction patterns (Class: Interaction).

3. Results

3.1. Neighbor effect index

L. kaempferi suffered stronger negative effects from its conspecific neighbor compared to *L. olgensis*, regardless of N fertilization. However, the negative effect declined when *L. kaempferi* was grown with *L. olgensis* under N fertilization. *L. olgensis* suffered less negative effect from *L. kaempferi* when they were grown together (Fig. 1).

3.2. Changes in soil properties

The mixed plantation soil had higher NH_4^+ , C_{Mic} and N_{Mic} contents compared to soil from the two monoculture plantations (Table S1). In the pot experiment, soil pH, SOM and the NO_3^- concentration were significantly impacted by N fertilization and plant-plant interactions (Table 1). N fertilization decreased rhizosphere soil pH in each treatment (Table S2). The intra-specific plant interactions of KK showed lower NH_4^+ , C_{Mic} and N_{Mic} compared to KO under N fertilization (Table S2).

3.3. Taxonomic composition and alpha diversity

The bacterial communities of the bulk soil samples from the three plantations and from the pot experiment were dominated by *Proteobacteria*, *Acidobacteria* and *Actinobacteria*, whereas the fungal communities were dominated by *Basidiomycota*

and *Ascomycota* (Figs. 2 and 3, Supplementary Fig. S1). The abundance of *Basidiomycota* was lower, whereas the abundance of *Ascomycota* became higher in KO and OO relative to KK, especially under N fertilization in the pot experiment (Fig. 3).

In the bulk soil samples from the three plantations, the average Shannon values of the bacterial and fungal communities were 5.98 and 2.52, respectively, and neither intra- nor inter-specific plant interactions had any effects on the bacterial and fungal diversity ($P > 0.05$, Supplementary Fig. S2). In the rhizosphere soil of single-plant pots, N treatment showed no significant effect on bacterial and fungal diversity ($P > 0.05$, Figs. 3a and 4a). However, N treatment increased bacterial diversity ($P < 0.001$, Fig. 4b) and also fungal diversity in the rhizosphere soil of KO ($P < 0.05$, Fig. 5d). In addition, plant-plant interactions had a significant impact on bacterial diversity ($P < 0.001$, Fig. 4b). For instance, the rhizosphere soil of KK exhibited a lower bacterial diversity compared to KO ($P < 0.001$) and OO ($P < 0.001$).

3.4. Beta diversity

The PCoA showed that the plantation soils had distinct compositions of both bacterial and fungal communities (Supplementary Fig. S3), unlike the rhizosphere soils from the pot experiment (Supplementary Fig. S4). Intra- and inter-specific interactions had a significant effect on bacterial and fungal community structures in soil from the three

plantations (PERMANOVA, $P < 0.01$, Supplementary Fig. S5). In the rhizosphere soil of double-plant pots, plant-plant interactions and N treatment significantly affected both the bacterial and fungal community composition (Supplementary Figs. S6e-h, PERMANOVA, $P < 0.01$). Pairwise PERMANOVA demonstrated that the structure of both bacterial and fungal communities in the rhizosphere soil of KK was different from those of KO and OO (Supplementary Figs. S6e and f, PERMANOVA, $P < 0.05$).

3.5. Biomarker discovery

We used the LEfSe analysis to discover biomarkers (different abundances of bacterial and fungal taxa) in the plantation and pot experiment soil. From phyla to genera, the three plantations showed their specific influence on bacterial and fungal compositions (Supplementary Fig. S7).

In the rhizosphere soil of single plants, the abundance of the bacterial orders like *Ktedonobacterales* and *Sphingomonadales* and the fungal order *Chaetothyriales* and the genus *Ciliophora* decreased, whereas the fungal family *Corynesporascaceae* became more prevalent under N fertilization (Fig. 6a and b). In the rhizosphere soil of double-plant pots, N fertilization significantly increased the abundance of the bacterial orders *Gemmatimonadales*, *Xanthomonadales*, *Propionibacteriales*, *Methylophilales* and *JG37-AG-4*, whereas the abundance of the phylum *Acidobacteria*, including the orders *Solibacterales*, *Blastocatellales* and *Subgroup 6* and the orders *Rhodocyclales*

and *Rhodospirillales*, reduced under N fertilization (Fig. 7a). The abundance of *Basidiomycota*, such as class *Tritirachiomycetes* and order *Boletales*, reduced but that of *Ascomycota*, such as the order *Hypocreales*, increased under N fertilization in the double-plant pots (Fig. 7b).

In the pot experiment, we also identified the bacterial and fungal taxa responding to species and plant interactions, irrespective of N fertilization (Figs. 6c and d, Fig. 8). The orders *Rhodospirillales*, *Rhizobiales*, *Acidimicrobiales* and *Acidobacteriales* were more abundant in the rhizosphere soils of K than O (Fig. 6c). Among fungal taxa, *Ascomycota* preferred the soil of O, whereas *Basidiomycota* and the order *Rhizophydiales* preferred the soil of K (Fig. 6d).

In the double-plant pots, the bacterial orders *Acidobacteriales* and *Micrococcales* were generally more abundant in the soil of KK, whereas *Actinobacteria* became more prevalent in the soil of KO (Fig. 8a). The taxa from the phyla *Firmicutes*, *Chloroflexi* and *Planctomycetes* were more frequent in the soil of OO (Fig. 8a). The fungal taxa *Basidiomycota* were generally more abundant in the rhizosphere soil of KK, whereas fungi from the other dominant phylum *Ascomycota* were more abundant in the rhizosphere soil of OO (Fig. 8b). In addition, the orders *Microascales* and *Hymenochaetales* became more abundant in the rhizosphere soil of KO (Fig. 8b).

4. Discussion

Many plant attributes, such as physiological processes, root exudates and leaf N content largely depend on the identity of a plant's neighbors and on the soil nitrogen content (Broz et al., 2010; Pierik et al., 2013; Chen et al., 2017). It follows that soil microbial communities can be differently impacted depending on, whether plants grow in the absence of neighbors or in the presence of conspecific or heterospecific neighbors in different environments.

4.1. Different plantations have specific bacterial and fungal communities

Different plantations have specific effects on soil chemical properties (Nazaries et al., 2015; Suz et al., 2017). For example, a pine plantation was found to decrease soil pH and increase the soil nitrate (NO_3^-) content, whereas a birch plantation declined the total carbon and NO_3^- contents (Nazaries et al., 2015). Our results also showed that the chemical traits of soil were different among the three studied plantations, particularly in the KK plantation. Differences in the chemical properties of soil largely depend on plantation characteristics, such as litter production and decomposition (Hättenschwiler et al., 2005; Helfrich et al., 2015), nutrient mineralization (Richards et al., 2010) and root exudates (Chen et al., 2014). Guo et al. (2016) have revealed that inter-specific interactions of *L. kaempferi* and *L. olgensis* decrease leaf C/N compared to intra-specific plant interactions without N fertilization, which may lead to biochemically heterogeneous plant litter in different types of plantations.

It has been shown that functions, compositions or diversities of soil microbe communities closely correlate with soil properties, such as pH, and soil carbon and nitrogen contents (Bakker et al., 2013b, 2014; Nazaries et al., 2015; Gunina et al., 2017; Suz et al., 2017). Suz et al. (2017) found that there were no significant differences in root colonization by generalist ectomycorrhizal fungi between mixed and pure plantations but, on average, there were more generalist ectomycorrhizal fungi in mixed plantations, where plants connect with their neighbors through common mycorrhizal networks. In this study, the phylum level abundance and the Shannon index of bacterial and fungal communities showed little difference; however, the beta diversity and biomarkers of bacterial and fungal taxa displayed greater dissimilarities, possibly different nutrient translocation or litter decomposition. For example, the abundance of *Basidiomycota* was higher but the abundance of *Ascomycota* was lower in KO and OO plantations compared with KK. The ability to degrade lignin is mainly conserved in *Basidiomycota* (Baldrian, 2006). The accumulation of soil organic matter exerts a positive and significant effect on the fungal beta-diversity (Cline and Zak, 2015), thus implying an important role of soil organic matter in shaping fungal communities and in regulating ecosystem carbon dynamics (Cheeke et al., 2017).

4.2. Direct and indirect effects of N fertilization on microbial communities

Nitrogen addition directly drives changes in bacterial and fungal communities in

different terrestrial ecosystems by increasing N availability (Cox et al., 2010; Fierer et al., 2012; Yuan et al., 2016; Treseder et al., 2018; Zhu et al., 2018). Our results found a decrease in the abundance of *Acidobacteria* after N fertilization in the pot experiment. An enhanced soil N availability increased the abundance of copiotrophic bacterial taxa, including *Proteobacteria* and *Bacteroidetes*, but lowered the proportion of oligotrophic *Acidobacteria* (Fierer et al., 2012). Similarly, Yuan et al., (2016) have found that *Acidobacteria* show a negative response to N fertilization. For fungal communities, shifts in the fungal composition have been considered to be the main driver of the decomposition response to enhanced N addition (Baldrian, 2006; Cox et al., 2010; Morrison et al., 2016). Cox et al. (2010) have reported that *Russula ochroleuca* and *Thelephora terrestris* respond positively to increasing N, whereas *Pseudotomentella tristis* and *Piloderma* respond negatively to increasing N. In our pot experiment, we discovered a shift from *Basidiomycota* to *Ascomycota* under the combined effects of *L. oligensis* and N fertilization. Changes in bacterial and fungal communities reflect corresponding alterations in functional consequences (Cox et al., 2010; Dietrich et al., 2017).

Nitrogen fertilization affects soil microbial communities also indirectly through changing chemical properties of soil. We found that N fertilization significantly impacted soil pH and soil organic matter, which indirectly control microbial communities (Fierer and Jackson, 2006; Cline and Zak, 2015; Yuan et al., 2016; Carrara et al., 2018). Soil pH is a major driver in shaping soil microbial communities

(Constancias et al., 2015; Ochoa-Hueso et al., 2018). For instance, the relative abundances of *Bacteroidetes*, *Planctomycetes* and *Thaumarchaeota* show a positive correlation with soil pH across an agricultural landscape (Constancias et al., 2015). Fierer and Jackson (2006) have suggested that any significant deviation from extracellular pH should impose stress on single-celled organisms and restrict the survival of taxa exposed to pH beyond their optimum.

4.3. *Effects of plant-plant interactions*

Mortimer et al. (2015) have reported fewer significant differences in the chemical properties of soil, whereas the ectomycorrhizal biomass, as well as the biomass of Gram-positive, Gram-negative, and actinomycetes bacteria were significantly higher in a mixed plantation than in a monoculture. Our results also demonstrated greater differences in the composition and beta-diversity of soil microbes between monocultures and a mixed-plantation. These results indicated that intra- and inter-specific plant interactions possibly have great impacts on soil bacterial and fungal communities. Our PERMANOVA and LEfse results indicated significant differences between KK and KO as well as KK and OO, with a higher bacterial and fungal diversity in KO compared to KK. At first, we observed a stronger neighbor effect from conspecific species in both species. Available P, total N (under fertilization) and NH_4^+ in the rhizosphere soil of a double-plant *L. kaempferi* (KK) pot were the lowest among all double-plant pots, implying a higher demand for nutrients and a

stronger intra-specific competition than previously suggested by Kunstler et al. (2015). Changes in soil nutrients induced by plant-plant competition contribute to alterations in soil microbes (Fierer et al., 2012; Yuan et al., 2016; Treseder et al., 2018; Zhu et al., 2018). Secondly, we observed a weakened neighbor effect caused by *L. kaempferi* on *L. olgensis* in the inter-specific plant interactions. There is a general acceptance that soil microbes are important in determining plant-plant interactions in different environments (Hodge and Fitter, 2013; Keymer and Lankau, 2017). *L. olgensis* has been found to enhance its ability to absorb NO_3^- under inter-specific plant interactions (Guo et al., 2018), which may result from the increase in the fungal phylum *Ascomycota* (Leroy et al., 2017). Finally, we found that after N fertilization, the neighbor effects on both species were less negative in KO, which implied declined inter-specific plant competition compared to KK and OO. In the present study, strong combined effects between plant-plant interactions and N fertilization on the rhizosphere soil were observed, particularly on the fungal communities. Both *L. olgensis* and N fertilization increased the abundance of fungal species belonging to *Ascomycota* but reduced abundance of *Basidiomycota* species, and their combined effect even led to a dominance shift from *Basidiomycota* to *Ascomycota* and to a higher fungal diversity. The changes in rhizosphere soil microbes were possibly an important reason to drive changes in plant-plant interactions.

4.4. Negative feedback from *L. kaempferi* plantation conditioned soil

In the pot experiment, the soil was selected from a *L. kaempferi* plantation. Lower bacterial and fungal diversities of KK were probably caused by *L. kaempferi* continuing to prefer its own soil microbial communities, whereas *L. olgensis* selected different microbes and increased bacterial and fungal diversity particularly under N fertilization. Guo et al. (2017) have reported that the *L. kaempferi* conditioned soil showed negative effects on the growth of *L. kaempferi*. The more negative neighbor effect of *L. kaempferi* on its conspecific neighbors also confirmed that. A given plant species alters its biological soil communities and abiotic soil properties that may decrease its own growth rate, resulting in a negative feedback (Harrison and Bardegett, 2010; Hendriks et al., 2015). The introduction of *L. olgensis* to *L. kaempferi* conditioned soil greatly changed the composition, abundance and diversity of bacterial and fungal communities, particularly under N fertilization. Previously, Van der Putten et al. (2016) have emphasized the role of bacterial and fungal communities in the development of plant-soil feedback under environmental changes through plant species loss or nitrogen enrichment. For example, the negative effects of Chinese fir conditioned soil on Chinese fir is alleviated by an introduced foreign plant species through increasing arbuscular mycorrhizal fungi and improving chemical properties of soil (Xia et al., 2016). The above results suggested that an introduced foreign plant species could improve soil conditions by changing microbial communities to alleviate the negative soil feedback.

5. Conclusions

573

574 Our work demonstrated that intra- and inter-specific plant interactions were
575 differently affected by plant neighbors and N fertilization, and there were distinct
576 changes in soil microbial communities. In turn, the changing soil bacterial and fungal
577 communities probably influence plant-plant interactions. Based on the present study,
578 we suggest that *L. olgensis* and *L. kaempferi* growing together with N fertilization
579 may be an efficient way to promote the productivity of plantations. The introduced
580 foreign plant species and N fertilization could improve the chemical and biological
581 conditions of soil and, consequently, plantation productivity. However, further studies
582 are needed to explore, how soil microbes mediate plant-plant interactions in different
583 ecosystems all around the world. Such knowledge would be crucial for revealing and
584 understanding plant-soil-microbe relationships.

585

586

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823

824 **Table 1** Effects of plant-plant interactions and N fertilization on soil properties (two-way ANOVA).

		pH	AP	SOM	TN	NH ₄ ⁺	NO ₃ ⁻	C _{Mic}	N _{Mic}	P _{Mic}
N	F	56.674	1.087	10.246	0.173	0.120	0.024	3.358	0.455	1.507
	P	<0.000	0.305	0.003	0.680	0.732	0.012	0.076	0.505	0.229
Interactions	F	8.297	0.140	26.386	48.904	11.358	7.143	12.470	1.686	0.528
	P	0.007	0.711	<0.000	<0.000	0.002	0.012	0.001	0.203	0.473
N* Interactions	F	0.002	0.301	0.119	1.460	0.003	0.936	1.103	0.153	0.153
	P	0.967	0.587	0.732	0.236	0.960	0.341	0.301	0.699	0.698

825 AP: available phosphorus, SOM: soil organic matter, TN: total nitrogen, C_{Mic}: soil microbial biomass, N_{Mic}: soil carbon nitrogen biomass and

826 P_{Mic}: soil carbon phosphorus biomass.

Table 2 Summaries of PERMANOVA tests with 999 permutations based on Bray-Curtis distances.

Taxonomy	Tree species or interactions			N treatment	
	Experiment	PERMANOVA	Betadisper	PERMANOVA	Betadisper
Bacteria	Single-plant	ns	ns	ns	ns
	Double-plant	*	***	**	ns
	KO vs KK	**	ns	ns	ns
	KK vs OO	**	ns	ns	ns
	KO vs OO	ns	ns	ns	ns
	Plantations	***	ns	—	—
	KO vs KK	*	ns	—	—
	KK vs OO	**	ns	—	—
	KO vs OO	ns	ns	—	—
Fungi	Single-plant	*	ns	ns	ns
	Double-plant	**	ns	***	ns
	KO vs KK	*	ns	ns	ns
	KK vs OO	**	ns	ns	ns
	KO vs OO	ns	ns	ns	ns
	Plantations	***	ns	—	—
	KO vs KK	**	ns	—	—
	KK vs OO	**	ns	—	—
	KO vs OO	**	ns	—	—

When a plant was grown in isolation, we analyzed the effect of species, and when grown under intra- or inter-specific interactions, we analyzed the effect of plant-plant interactions. KK and OO refer to intra-specific interactions of *Larix kaempferi* and *L. olgensis*, respectively. KO refers to inter-specific interactions of the two species. In the plantations, KK and OO refer to monoculture plantations of *Larix kaempferi* and *L. olgensis*, respectively. KO refers to a mixed plantation of the two species.

Figure legends

Figure 1 Neighbor effect index (NEI) of *L. kaempferi* and *L. olgensis* in different plant-plant interactions. KK and OO refer to *L. kaempferi* and *L. olgensis* in intra-specific plant-plant interactions, respectively. K/KO and O/KO refer to *L. kaempferi* and *L. olgensis* in inter-specific plant-plant interactions, respectively. N- and N+ refer to soil without and with N fertilization, respectively. Negative NEI values indicate that a plant suffers negative impacts from its neighbor. Tukey's HSD tests are conducted for multiple comparisons.

Figure 2 Taxonomic composition of bacterial communities in the rhizosphere soil at the phylum level under N fertilization. (a) Rhizosphere soil sampled from single-plant pots (without plant-plant interactions); (b) rhizosphere soil sampled from pots with two plants, where KK, KO and OO refer to *L. kaempferi* + *L. kaempferi*, *L. kaempferi* + *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively. N- and N+ refer to pots without and with N fertilization, respectively. For bacteria, only phyla with relative abundance over 1% are shown.

Figure 3 Taxonomic composition of fungal communities in the rhizosphere soil at the phylum level under N fertilization. (a) Rhizosphere soil sampled from single-plant pots (without plant-plant interactions); (b) rhizosphere soil sampled from pots with two plants, where KK, KO and OO refer to *L. kaempferi* + *L. kaempferi*, *L. kaempferi*

+ *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively. N- and N+ refer to pots without and with N fertilization, respectively. For fungi, only phyla with relative abundance over 1% are shown.

Figure 4 Effects of different plant-plant interactions on the alpha diversity (Shannon index) of rhizosphere soil bacteria under N fertilization. (a) Rhizosphere soil sampled from single-plant pots (without plant-plant interactions); (b) rhizosphere soil sampled from pots with two plants (intra- or inter-specific interactions). KK, KO and OO refer to *L. kaempferi* + *L. kaempferi*, *L. kaempferi* + *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively. N- and N+ refer to pots without and with N fertilization in the pot experiment, respectively. Tukey's HSD tests were conducted for multiple comparisons.

Figure 5 Effects of different plant-plant interactions on the alpha diversity (Shannon index) of rhizosphere soil fungi under N fertilization. (a) Rhizosphere soil sampled from single-plant pots (without plant-plant competition); (b) rhizosphere soil sampled from pots with two plants (intra- or inter-specific plant interactions). KK, KO and OO refer to *L. kaempferi* + *L. kaempferi*, *L. kaempferi* + *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively. N- and N+ refer to pot experiment without and with N fertilization in the pot experiment, respectively. Tukey's HSD tests were conducted for multiple comparisons.

Figure 6 Bacterial (a and c) and fungal (b and d) taxa with different abundance changes in single-plant pots between N-treated (N+) and control (N-) soil, irrespective of plant species (a and b, Class: N treatment; Subclass: Species) and ii) between plant species, irrespective of N treatment (a and b, Class: Species; Subclass: N treatment), as detected by LEfSe analysis. The taxa with the absolute LDA scores over 3 and *P* values less than 0.05 are shown.

Figure 7 Bacterial (a) and fungal (b) taxa with different abundance changes in two-plant pots between N-treated (N+) and control (N-) soil, irrespective of plant-plant interactions (Class: N treatment; Subclass: Plant-plant interactions), as detected by LEfSe analysis. The taxa with the absolute LDA scores over 3 and *P* values less than 0.05 are shown.

Figure 8 Bacterial (a) and fungal (b) taxa with different abundance changes in two-plant pots between intra- and interspecific plant interactions, irrespective of N treatment (Class: Plant-plant interactions, Subclass: N treatment), as detected by LEfSe analysis. The taxa with the absolute LDA scores over 3 and *P* values less than 0.05 are shown. KK, KO and OO refer to plant-plant interactions *L. kaempferi* + *L. kaempferi*, *L. kaempferi* + *L. olgensis* and *L. olgensis* + *L. olgensis*, respectively.

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Figure 2

Figure 3

Figure 4

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