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Factors Affecting the Natural Regeneration of the *Larix principis-rupprechtii* Mayr Plantations: Evidence from the Composition and Co-Occurrence Network Structure of Soil Bacterial Communities

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Abstract: Bacterial communities living in the soil can affect forests natural regeneration, but the effects of their composition and network inference on regeneration of *Larix principis-rupprechtii* Mayr plantations remain largely elusive. Therefore, the redundancy analysis and structure equations modeling of affecting elements for the regeneration of *L. principis-rupprechtii* plots including the diversity, composition and network structure of soil bacteria, topographic factors, light factors, and soil physicochemical properties have been conducted. It was found that the increased modularity of the soil bacterial community co-occurrence network and the enrichment of metabolic pathway bacteria had a significant positive effect on the successful regeneration (total effect of 0.84). The complexity of the soil bacterial community gradually decreased with the increase of stand regeneration, and the composition and structure of the flora became simpler (with standard path coefficients: −0.70). In addition, altitude also had a positive effect on regeneration with a total effect of 0.39. Soil nutrients had significantly negative effects on regeneration with total effects of −0.87. Soil bacterial communities may mediate the effects of soil nutrients, altitude, litter thickness, and herbaceous diversity on regeneration in *L. principis-rupprechtii* plantations. The results provide a great contribution to our understanding of regeneration-soil bacterial community interactions and the basis and important data for sustainable management of *L. principis-rupprechtii* plantations in the Lvliang Mountains located in northern China.

Keywords: plantation natural regeneration; soil bacterial community; composition and co-occurrence network; soil characteristics

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1. Introduction

Forest regeneration provides the next generation of overstory trees for forest ecosystems and is considered to be an important link in achieving forest ecosystem succession and restoration [1]. The natural regeneration of forests is the process of using the reproduction itself to complete the self-renewal of trees, which can drive the natural formation of multi-species communities. It can reflect the spatial pattern and function of the forest ecosystem and is one of the essential pathways to achieve high quality ecosystem structure of forest; moreover, it is the basis to maintain the dynamic stability and sustainable development of forests [2–4]. Forest regeneration will ultimately determine the composition of future forests and thus the ecosystem services they provide [5].

Based on the concept of near-natural forest management, ensuring regeneration to obtain adequate density and seedling growth is one of the most challenging problems in forest management [6,7]. Natural regeneration is based on recruitment from seeds (seedlings) without artificial help [8], which have the significant advantages of low-cost, adaptability to microenvironment and higher seedling density, enabling the advantages

of compound heterogeneous forests [9,10]. Due to the complexity of forests, the different stages of the stand regeneration process are also influenced by competing biotic and abiotic factors above and below ground [11–15]. A comprehensive understanding of the factors driving regeneration is critical to determining the best forest management strategy based on forest sustainable development goals.

Larix principis-rupprechtii Mayr, as a strong masculine species, is the endemic and dominant tree species of coniferous forests in northern China and is commonly found in pure forests. However, the poor survival of understory seedlings and saplings is a critical problem for tree regeneration for *L. principis-rupprechtii* plantations. Therefore, maintaining and re-establishing the sustainability of the plantation forest ecosystem of *L. principis-rupprechtii* has become an important issue that needs to be addressed in forest management at present. Previous studies have shown that the litter thickness, soil nutrients such as soil organic matter and ammonia nitrogen have an influence on the regeneration of *L. principis-rupprechtii* compared to altitude, slope, stand density, biomass of accumulated litter, and soil total nitrogen and total phosphorus [16]. This shows that the natural regeneration of *L. principis-rupprechtii* plantations is closely related to the changes in environmental factors. The complex relationship formed by a forest environment plays a crucial role in regulating forest ecosystem functions and also strongly influences microbial diversity in soil.

As the most abundant and functionally diverse microbial taxa in forest soils, bacterial communities drive the cycling of mineral nutrients such as soil nitrogen and phosphorus, the transport of material and energy between above- and below-ground communities, etc., and are closely associated with soil nutrient and vegetation production [17–19]. Previous studies have shown that soils with higher bacterial diversity exhibit more ecological functions, higher resistance to environmental stresses and vegetation productivity [20,21]. The interactions between plant and soil bacterial communities promote the metabolic activities of bacteria, thus releasing nutrients and other compounds that can be used by the plant body and directly or indirectly contribute to the formation of plant communities [22]. It has been shown that monoculture plants reduce soil bacterial community diversity [23,24]. In addition to bacterial community diversity, stand light intensity, soil moisture, soil temperature, soil alkaline nitrogen, available phosphorus, available potassium, soil enzymes such as phosphatase, urease, and translaminar enzymes, and composition of the understory layer may affect the regeneration [24–27]. There is a close relationship between the environmental factors mentioned above and soil bacterial communities [28–35]. The effects of soil bacterial communities on the regeneration of *L. principis-rupprechtii* plantations have rarely been addressed in previous studies.

With the rapid development of high-throughput 16SrRNA sequencing technology, we can study the unculturable soil bacterial communities at the genetic level. Currently, we lack studies on the composition, network inference and potential ecological functions of soil bacterial communities in a *L. principis-rupprechtii* plantation. We selected a study area with naturally regenerated *L. principis-rupprechtii* in the middle part of the Lvliang Mountains in northern China, but the degree of regeneration is varying. In this study, we analyzed soil bacterial community richness, diversity and composition characteristics at different levels of stand regeneration and revealed potential functional information of the community at the amplicon sequencing level using functional annotation tools newly developed, and visualized information about species interactions within bacterial communities using co-occurrence network diagrams. Combining the other environmental indicators related to stand regeneration, including topographic factors, light factors, soil physicochemical characteristics, litters thickness and water holding capacity, and herbaceous diversity, we further quantified the extent to which soil bacterial community composition and symbiotic network structure influenced the natural regeneration of *L. principis-rupprechtii* plantations. This study provided a theoretical basis for understanding the plantation natural regeneration, soil bacterial communities interaction and provided important basic data for sustainable management of *L. principis-rupprechtii* plantations in the Lvliang Mountains of northern China.

2. Materials and Methods

2.1. Study Area

The study sites were located at 1983–2168 m in the Guandi forest region in the middle of the Lvliang Mountains, located at 37°45′–37°55′ N, 111°22′–111°33′ E in northern China. This forest region has a temperate continental monsoon climate. The mean annual temperature and mean annual precipitation are 4.3 °C and 822.6 mm, respectively. The relative humidity is 60%, and the annual sunshine duration ranges from 1900 to 2100 h. The growing and cultivation season is generally from June to September. The soil is vertically zoned from low to high altitude, with granitic gneiss-textured montane brown loam soil (Chinese classification) found at our study sites, and the soil thickness ranges from 70 to 80 cm and contains a 10 cm thick humus layer. The study area included *L. principis-rupprechtii* Mayr plantation forests dominated by *L. principis-rupprechtii* (80–90%), accompanied by a small proportion of *Picea asperata* Mast and *Betula platyphylla* Suk plantations. Understory vegetation primarily consists of *Caragana jubata*, *Potentilla glabra*, *Potentilla fruticosa*, *Rosa xanthina*, and *Daphne giraldii Nitsche*. The herb community includes *Ranunculus japonicus* Thunb., *Carex rigescens*, *Fragaria orientalis* Losinsk., and *Plantago asiatica* L.

2.2. Experimental Design

The study was conducted in July 2021. We established 20 plots (30 × 30 m), including five plots which were considered high regeneration stands (Group H), five plots considered as moderate regeneration stands (Group M), five plots considered as low regeneration stands (Group L) and five plots considered as no regeneration stands (Group N). If the number of seedlings > saplings > adult trees, it was categorized as high regeneration stands; if the number of saplings > seedlings, it was categorized as moderate regeneration stands; if the number of saplings > adult trees > seedlings, it was categorized as low regeneration stands; if there was little to no regeneration of seedlings, it was categorized as no regeneration stands. Tree stems were classified according to height (Height) and diameter at breast height (DBH): seedlings (0.1 m $\leq$ Height < 1 m), saplings (Height $\geq$ 1 m, DBH $\leq$ 5 cm), and adult trees (DBH > 5 cm). The number of seedlings in each plot was counted as the amount of stand regeneration. The average height of stand regeneration is referred to as ATH.

2.3. Data Collection

2.3.1. Soil Bacterial Community

After removing the litter layer in each plot, five soil subsamples were collected from points C, E, S, W, and N at a depth of 0–20 cm using an "X"-shaped collection scheme and mixed into a composite soil sample, and another composite soil sample was collected from points C, NW, SE, NE, and SW (Figure 1), each with three replicates (n = 20 plots × 2 composite soil samples × 3 replicates). Soil samples were removed from stones and roots and were air dried, homogenized and sieved (<2 mm). All soil samples were stored in coolers filled with dry ice with sterilized plastic bags and shipped back to the laboratory within 3 days and stored in a low temperature refrigerator at −80 °C awaiting genomic DNA extraction for 16SrRNA amplicon sequencing analysis.

2.3.2. Environmental Factors

The main variables measured in each plot were as follows: (1) topographic factors (altitude, slope); (2) stand structure factors (species, the number of trees (DBH > 10 cm) per hectare (Density), diameter at breast height, height(H)); (3) light factors (canopy opening (CO), relative light intensity (RLI), and leaf area index (LAI) in the sample plot); (4) soil characteristics: soil moisture (SW), soil temperature (ST), available nitrogen (AN), available phosphorus (AP), available potassium (AK), phosphatase (PHO), invertase (INV), urease (URE); (5) litter characteristics: litter thickness (Tit T), and their maximum water holding capacity (Tit Max.); (6) herbaceous diversity: Shannon index (H. Shannon), Simpson index (H. Simpson), evenness index (H. Pielou).

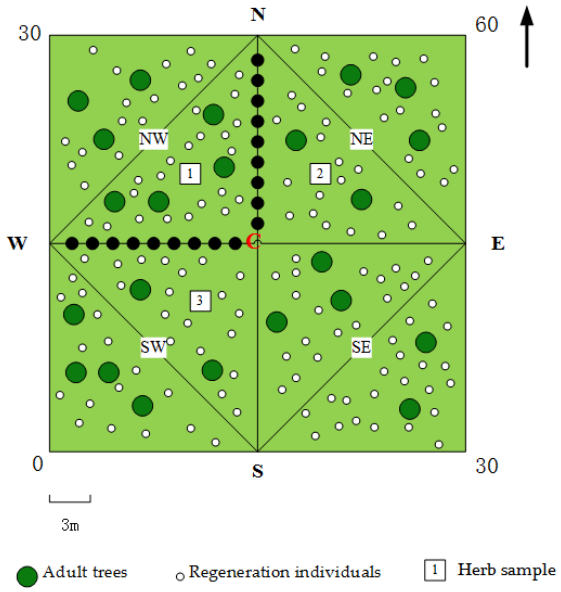


Figure 1. The distribution of the regeneration individuals of each sampling plot. Abbreviations: C = center; N = northern transition; E = eastern transition; S = southern transition; W = western transition; NE = northeast; SE = southeast; NW = northwest; SW = southwest.

We characterized CO and understory light availability based on hemispherical photographs taken 1.2 m above the ground at 1 m intervals positioned along the WE and NS directions, respectively, using a digital camera (Canon EOS-1D Mark IV, Canon Inc., Tokoyo, Japan) fitted with an EF 8–15 mm f/4 L USM fisheye lens on cloudy days during the growing season (Figure 1). Imaging software Gap Light Analyzer v.2.0 (GLA V2.0, Frazer, Canham, & Lertzman, Copyright © 2022: Simon Fraser University, Burnaby, British Columbia, and the Institute of Ecosystem Studies, Millbrook, New York) was used to calculate direct, diffuse, and relative light intensities per gap [27]. ST and EC were measured at each sub-sampling site under each forest gap respectively. ST and EC were determined using a multi-parameter WET sensor (WET-2, Delta-T Devices Ltd., Cambridge, UK) [36]. All composite soil samples were used to determine soil moisture and soil nutrients. SW was measured gravimetrically by drying the soil at 105 °C for 24 h [37]. AP was extracted using 0.5 M $NaHCO_3$ and determined using the molybdenum blue colorimetric method [38]. AN was measured using the alkaline permanganate method [31]. URE activity was assessed using the sodium phenol hypochlorite colorimetric method [39]. PHO activity was measured using the phenyl phosphate sodium colorimetric method. INV activity was directly measured using the 3,5-dinitro-salicylic acid colorimetric method [40]. The cover of herbs was estimated as the ratio of the vertical projection of leaf area to the area of each square. The mean vegetation cover of each square effectively described the basic profile of understory vegetation in each square. Three small sample squares with an area of 30 cm × 30 cm were set up near the three herbaceous sampling points to record the thickness of the above-ground litter layer.

2.4. DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Total DNA was extracted from soil samples using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA) and quantified using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The recovered DNA was purified using agarose gel electrophoresis, and PCR was performed using the forward primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V3–V4 regions of bacterial 16S rRNA genes. Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR conditions involved initial denaturation at 98 °C for 5 min, followed by 25 cycles of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s, with a final extension for

5 min at 72 °C. The PCR products were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA).

2.5. High-Throughput Analysis

Soil bacterial communities were analyzed with high-throughput sequencing on the Illumina NovaSeq platform using the NovaSeq 6000 SP Reagent Kit (500 cycles) (Biotechnology Co., Ltd., Shanghai, China). QIIME2 (version 2019.4, https://qiime2.org/, 1 May 2022) was used to perform sequence data analyses [41]. Raw sequence data were demultiplexed using the demux plugin. The primers were cut using the QIIME cutadapt trim-paired plugin to remove the unmatched primer sequence [42]. For quality control, the sequences were filtered, denoised and merged, and chimeras were removed using the DADA2 method [43]. After denoising all libraries, the non-singleton amplicon sequence variant (ASV) feature sequences and ASV tables were aligned using MAFFT and used to construct a phylogenetic tree using FastTree2 [44].

2.6. Statistical Analysis

Sequence data analyses were mainly performed using QIIME 2 and R packages (version 4.2.0, http://www.R-project.org/, 1 May 2022). To estimate alpha diversity, we drew the un-drawn ASV abundance table flat and selected the average of the scores at the maximum draw depth as the alpha diversity indices. The soil bacterial alpha diversity indices in all plots were analyzed by nonparametric tests and the Wilcoxon rank sum test. Differences in alpha diversity between groups were analyzed using the Kruskal–Wallis test, and the Dunn test was used for post hoc analysis. The number of observed species and Chao1 indices of each sample were estimated by counting the number of ASVs. The Shannon index was calculated based on the richness and evenness of each sample. The Pielou index of the community was calculated by dividing the Shannon index by the total number of species (total ASV number). The Good's coverage was calculated as the proportion of non-singleton ASVs in all species. The higher the Good's coverage index, the lower the proportion of undetected species in the sample. The calculation of Faith pd index needs to be based on the evolutionary tree file. Beta diversity was analyzed to examine structural variations in bacterial communities across soil samples using Bray Curtis distance metrics, and the results were visualized via principal coordinate analysis (PCoA). The intergroup variability of soil bacteria was assessed via analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA), with the number of permutation tests set to 999.

Co-occurrence network analysis was performed using Spar CC. The pseudocount value in Spar CC was set to 10^{-6}. The R package "RM Threshold" was used to determine the cutoff of correlation coefficients as 70 based on random matrix theory. Based on the correlation coefficients, a co-occurrence network was constructed, with nodes representing ASVs and edges representing correlations between these ASVs. The average degree (avg K) reflected the network complexity of bacterial communities [19]. The negative and positive connection proportions (N/P cohesion) reflected the potential competition among bacterial communities [45]. The network was visualized using Gephi (version 0.9.2 for Mac OS X).

For microbial ecology studies, we are equally interested in the functional potential possessed by the flora. The PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) and R software were used to predict the functional unit composition of samples with only sequencing data of colony marker genes (16S rRNA genes). The 16S rRNA signature sequences are then aligned with the reference sequences to construct a new evolutionary tree. The Nearest Sequenced Taxon Index (NTSI) of a signature sequence is inferred from gene copy number corresponding to the reference sequence in the evolutionary tree, using the Castor hidden state prediction algorithm. Note that it should be excluded from any subsequent analysis if the sequence has an NTSI > 2. Combining the abundance of each sample's characteristic sequence, the gene family copy number of each sample is calculated. Here, we obtained the functional-species

correspondence gene families and output the results in a hierarchical processing (i.e., the functional units of different signature sequences are not combined). Finally, the gene families are "mapped" to the KEGG (https://www.kegg.jp/, 3 June 2022) database, and the presence of metabolic pathways is inferred using Min Path to obtain the abundance data of metabolic pathways in each sample.

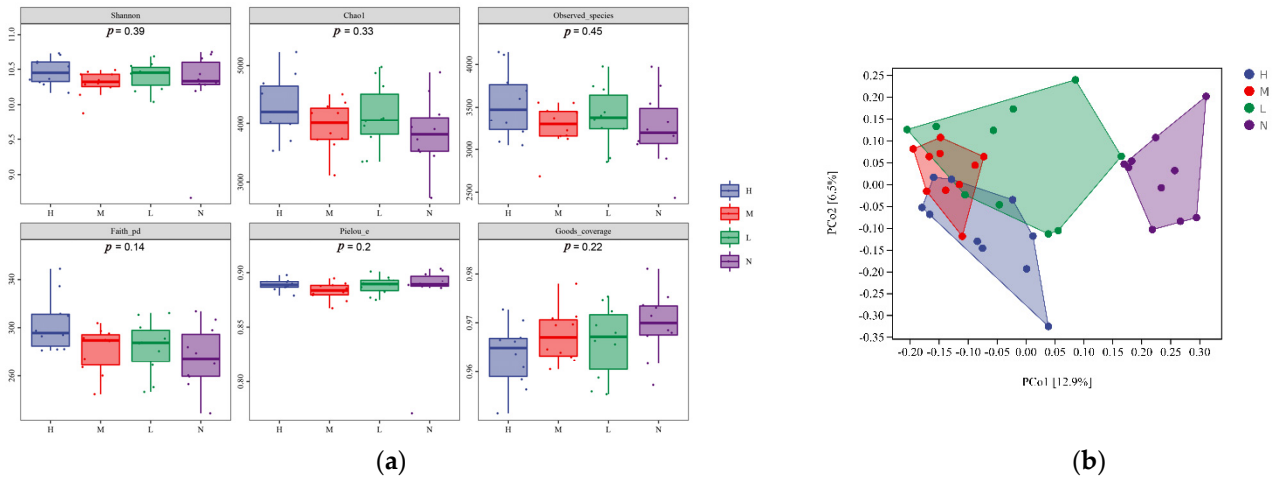
The core of the KEGG database is the KEGG Pathway Database (http://www.genome.jp/kegg/pathway.html, 7 June 2022), which categorizes metabolic pathways into six major groups, including Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems and Human Diseases. Currently, the second class includes a total of 45 metabolic pathway sub-functions. Using a normalized pathway/group abundance table, the average abundance or full number of pathways in the second level is calculated based on the selected samples.

Redundancy analysis (RDA) was used to identify the dominant environmental factors (i.e., soil bacterial communities, topographic factors, stand structure, light properties, and soil physicochemical characteristics etc.) impacting on stand regeneration, implemented in the R (version 4.2.0) software and CANOCO 5.0 software package. The ranking scale focused on interspecies correlations, and the significance of data was evaluated via the Monte Carlo test. Standardizing all the data allows for the elimination of magnitude relationships between variables, thus making the data comparable. The Amos Graphics 24 software (IBM/International Business Machines Corporation, Armonk, NY, USA) was used to perform structural equation modeling (SEM) to examine the relationship between multiple dependent and independent variables.

3. Results

3.1. Soil Bacterial Community Diversity, Composition and Structure: Regeneration Categories

High-throughput 16SrRNA sequencing technology was used to analyze the composition and distribution of soil bacterial communities in *L. principis-rupprechtii* plantations with different regeneration degrees. The results showed that 1, 196, 766 sequences were classified into 90,312 operable ASVs ranging from 4156 to 8373, which indicated that the number of ASVs in the soil bacterial community varied significantly among samples. The dilution curve of Shannon index, the number of observed species, Chao1 and Faith pd indices were saturated (Figure S1). This indicated that the sequencing depth was sufficient to represent most soil bacterial microorganisms. The value of Good's coverage index remained above 0.95, indicating that there were few species which were undetected in all samples. (Figures 2 and S1).



(**a**) (**b**)

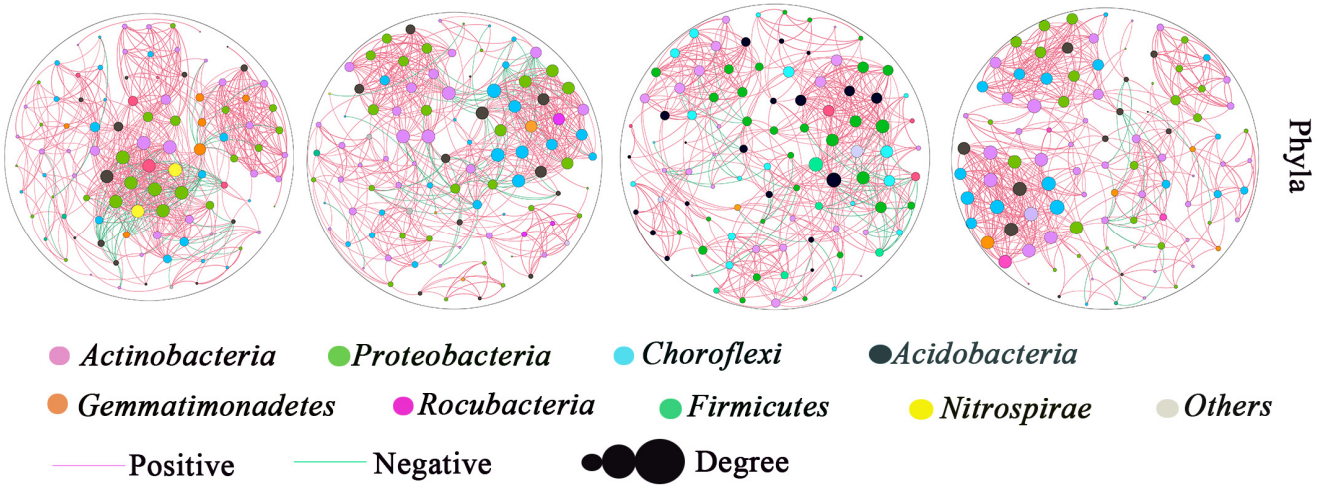
Figure 2. (**a**) Shannon index, observed species, Chao1, Pielou index of soil bacterial communities in plots with different regeneration degree; (**b**) PCoA analysis based on Bray Curtis distances of soil bacterial communities with different degrees of regeneration.

First, we calculated the Alpha diversity of soil bacterial communities, and the results are shown in Figure 2a. There was no significant difference in Shannon, the number of observed species and Chao1, Faith pd indices of soil bacterial community between groups ($p > 0.05$). These indicated that the richness and diversity of soil bacterial communities between groups was not significantly different for various regeneration degrees. The diversity and richness of soil bacterial communities showed an increasing trend with the increase of regeneration degree.

PCoA based on Bray Curtis distances explained 12.9% of the variation along the first axis and 6.5% along the second axis, the results of which are presented in Figure 2b and Table S1. All samples with different regeneration degrees were clustered individually (ANOSIM R = 0.404, $p < 0.001$; ADONIS $R^2 = 0.172$, $p < 0.001$). The results of the PERMANOVA and ANOISM analyses showed that the median line within Group N was lower than inter-group comparisons. The above-mentioned results indicated our grouping is valid (Figure S2, Table S1). The results of Beta diversity analysis showed that the showed the tightest clustering within Group N and M. Among all groups, the lowest intra-group clustering in Group L indicated that the greatest variation in soil bacterial communities between samples occurred at low regeneration levels (Figure 2b). In addition, the greatest significant difference between groups H and N indicated that the largest changes in soil bacterial communities between groups occurred in highly regenerated stands (Figure S2).

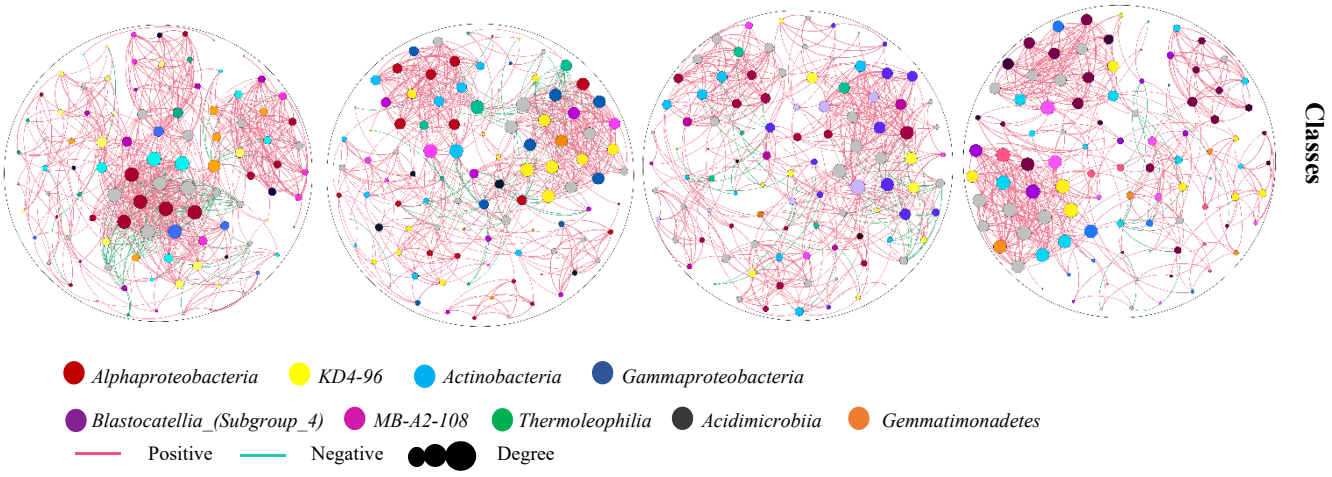
3.2. Soil Bacterial Community Diversity, Composition and Structure: Co-Occurrence Networks Analysis

Based on META tools and RMT theory, we constructed a taxonomic and modular co-occurrence network of soil bacterial communities with different degrees of regeneration. The operational taxonomic units ASV nodes in the network were mainly from Actinobacteria, Proteobacteria, Chloroflexi, and Acidobacteria, while being divided into six main modules. The co-occurrence network analysis results showed a significant succession in soil bacterial communities with increasing stand regeneration. The relative abundance of Actinobacteria (35%) was significantly increased in Group H, while the relative abundance of Proteobacteria showed a neutral pattern. The relative abundance of Acidobacteria (19.19%) in Group M increased significantly. The relative abundance of Chloroflexi (23%) in Group L increased significantly (Figure 3a).



(**a**)

Figure 3. *Cont.*



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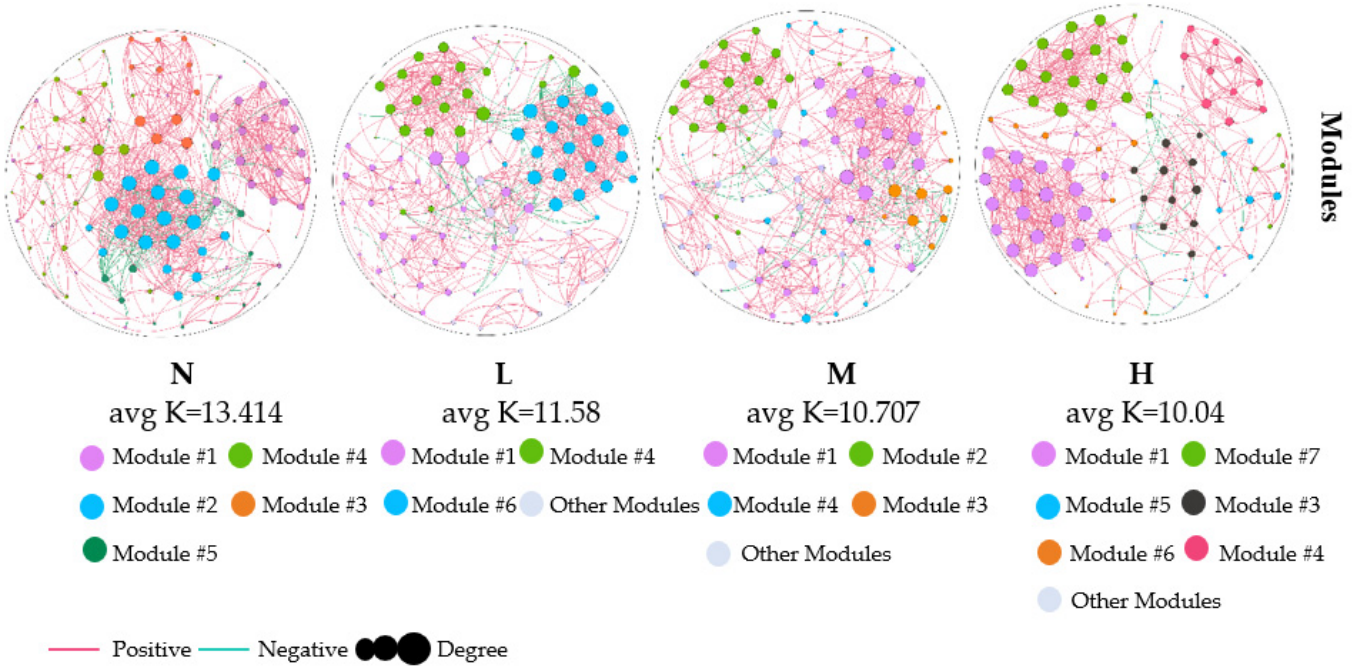

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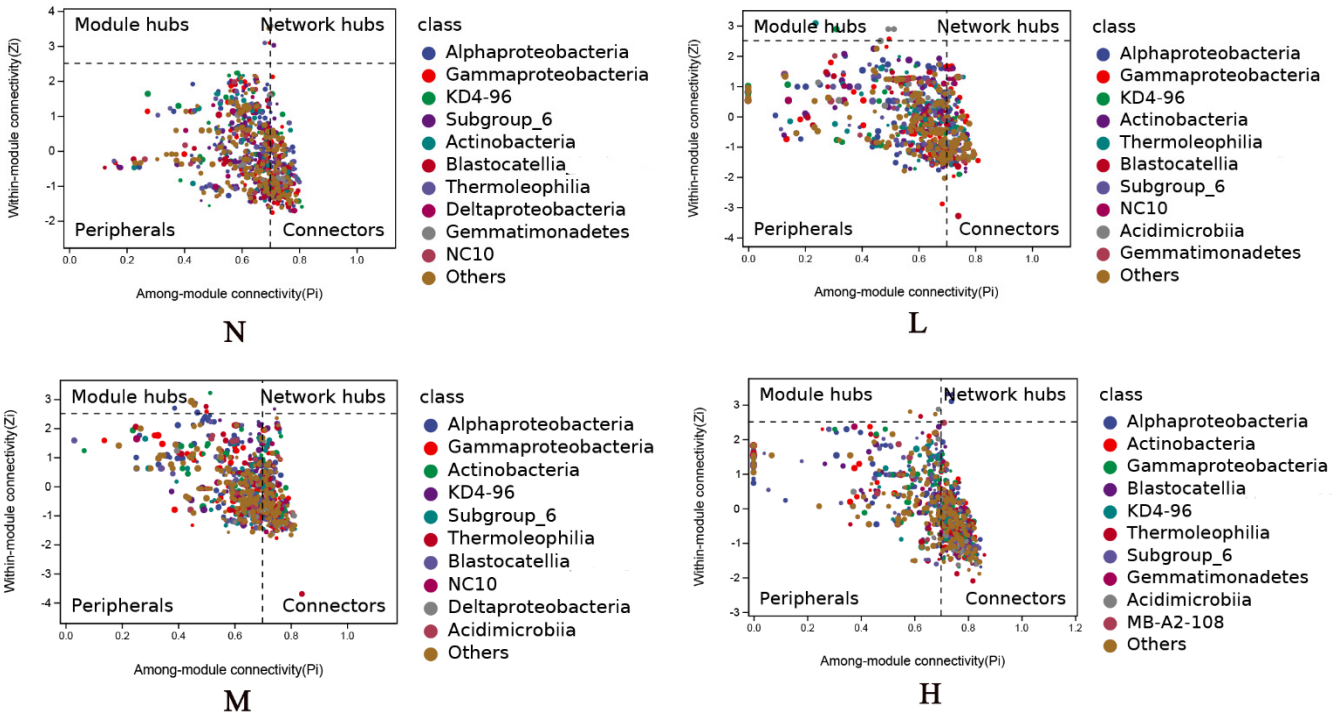
Figure 3. *Cont.*



(**d**)

Figure 3. The effects of *L. principis-rupprechtii* regeneration degree on soil bacterial structures and diversities. (**a**–**c**) Network of soil bacterial populations among different *L. principis-rupprechtii* regeneration degree. Red lines indicate positive correlations and green lines indicate negative correlations. The area of the node is proportional to node degree, calculated from correlations of abundances for each ASV. Only correlations with an r of >0.6 or <0.6 and a *p* value of <0.05 were included in the network. (**d**) Topological roles of ASVs in the soil bacterial co-occurrence networks in four regeneration degrees as indicated by the *Zi-Pi* plot. The nodes with $Zi > 2.5$ are identified as module hubs, and those with $Pi > 0.62$ are connectors. The network hubs are determined by $Zi > 2.5$ and $Pi > 0.62$, and the peripherals are characterized by $Zi < 2.5$ and $Pi < 0.62$.

The relative abundance of the soil bacterial community at classes level in Group N ranged from 1% to 14.14%. Alphaproteobacteria (14.14%), KD4-96 (14.14%), Actinobacteria (12.12%), Blastocacaliia_(Subgroup_4) (8.08%) became predominant bacteria in Group N. Alphaproteobacteria (18%), KD4-96 (17%), Actinobacteria (11%), Gammaproteobacteria (9%) became dominant bacteria in Group L. KD4-96 (11.11%) was further reduced in groups M and H (9%). Alphaproteobacteria (18%), Actinobacteria (14%) became the dominant bacteria in Group H (Figure 3b).

The average degree of soil bacterial symbiotic network (avg K) decreased from Group N (avg K = 13.414), Group L (avg K = 11.58), Group M (avg K = 10.707) to Group H (avg K = 10.04). Group H had the lowest network complexity and the highest modularity degree, average clustering coefficient (ACC), and average path distance (GD). The trend of modularity degree was N < L < M < H group (Table S2). In Group L, 3 modules accounted for more than 10% of the overall network including Module 1 (34%), Module 4 (24%), and Module 6 (21%). In Group M, the module composition changed, with Module 1 (33.33%), Module 2 (23.23%), Module 4 (14.14%), and Module 3 (11.11%) accounting for more than 10% of the overall network. Among Group H, 6 modules accounted for more than 10% of the overall network, including Module 1 (26%), Module 7 (22%), Module 5 (13%), Module 3 (12%), Module 4 (10%), Module 6 (11%) (Figure 3c). The trend of negatively correlated connections proportions (N/P cohesion) in the network was H < M < N < L

groups (Table S2). To assess the potential topological role of operable taxonomic units in the network, we further analyzed the intra-module connectivity Zi and inter-module connectivity Pi of co-occurring network nodes. The composition of key species maintaining network stability at different levels of regeneration is shown in Figure 3d. The topological properties of the inter-collaborative network of soil bacteria in Group H were found to have the lowest complexity, but the largest proportion of network key species. 356, 232, 373, and 476 nodes were classified as module centers and network connectors, in groups N, L, M, and H, respectively. They have particularly strong dependencies with many nodes in their own modules from Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Gemmatimonadetes, Rokubacteria and Firmicute (Figure 3d).

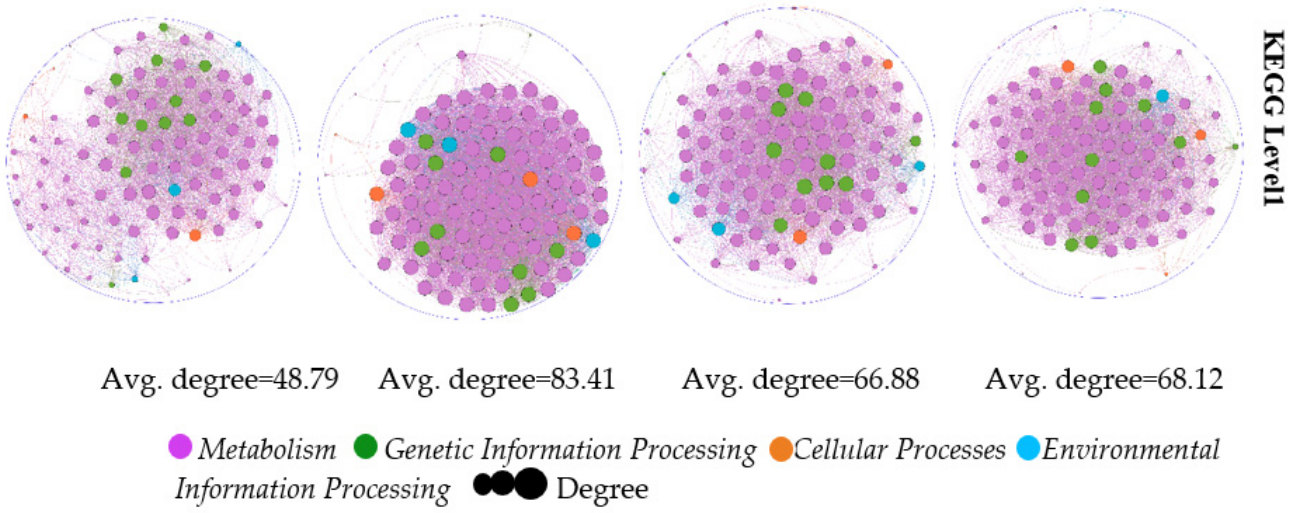
3.3. Soil Bacterial Community Diversity, Composition and Structure: Prediction of the Functional Potential of the Flora

We likewise focused on the potential function possessed by the soil bacterial community at different regeneration degrees. A co-occurrence network analysis based on KEGG pathway annotations showed that the N group exhibited the lowest average degree of soil functional composition (Avg. degree = 48.79) and the highest degree of modularity of community functional components in soil (Mod. Degree = 0.258), as shown in Figure 4a,b. Group L exhibited the highest average degree of soil functional composition (Avg. degree = 83.41), but the lowest degree of modularity of community functional components in the soil (Mod. Degree = 0.015). The modularity of the functional components of the soil community increased with regeneration degree, reaching 0.129 in Group M and 0.093 in Group H (Table S3). Results of the KEGG level 1 annotation analysis showed that metabolism was the core functional category of the soil bacterial community in the forest under the four regeneration levels, and the proportion of metabolism increased progressively with higher regeneration degree. The metabolic functional pathway of soil bacteria in Group H reached 83.84%, higher than those in groups M (83%), L (82.83%) and N (82.83%) (Figure 4a).

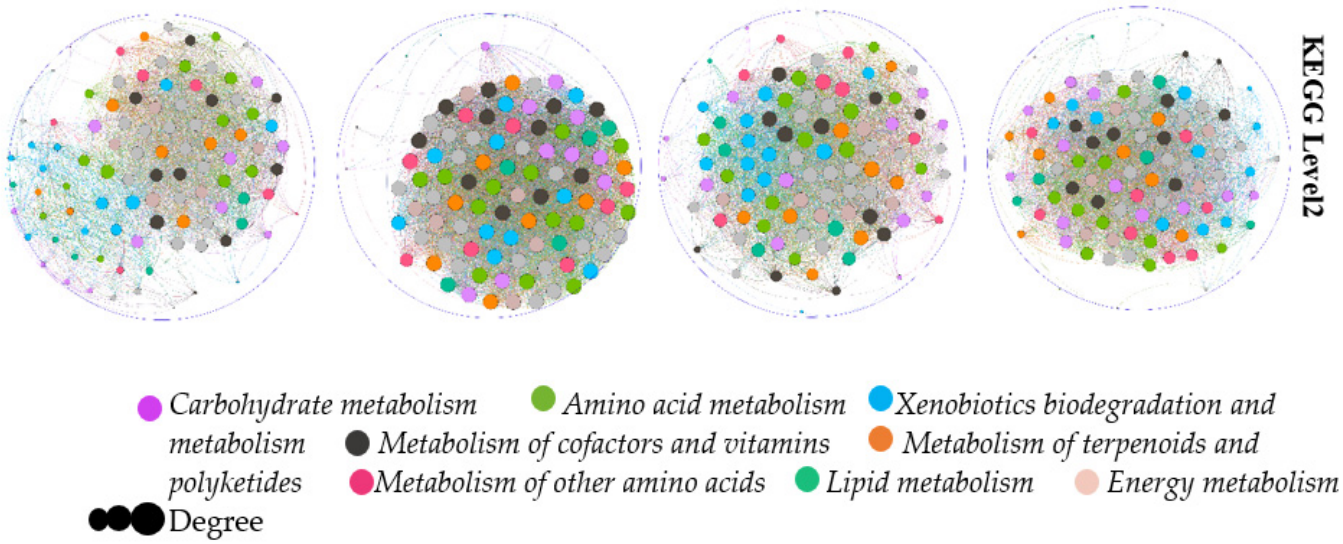
The analysis of KEGG level 2 annotations showed that the core functions of soil bacteria in different regeneration stands differed. The functions of soil bacterial communities in group N mainly include Carbohydrate metabolism (12.12%), Amino acid metabolism (13.13%), Xenobiotics biodegradation and metabolism (12.12%), Metabolism of cofactors and vitamins (11.11%), Metabolism of terpenoids and polyketides terpenoids and polyketides (9.09%), Lipid metabolism (6.06%), Metabolism of other amino acids (7.07%). Carbohydrate metabolism (13%) became more prominent in Group L and M stand soils. In Group H stand soils, Metabolism of other amino acids (8.08%) became more prominent, and Amino acid metabolism (13.13%) replaced Carbohydrate metabolism (12.12%) as the most prominent core functional pathway in highly regenerated stand soils (Figure 4b).

3.4. Soil Bacterial Community Diversity, Composition and Structure: RDA Analysis

The results of RDA showed that 52.47% of the stand regeneration could be explained by the first axis and 44.7% of the stand regeneration could be explained by the second axis (Figure 5a). The metabolic core functions and the avg K of co-occurrence network of soil bacterial communities had the largest contribution to (>20%) to stand regeneration in *L. principis-rupprechtii* plantations (Table S4). Metabolism was highly significantly and positively correlated with modularity degree of the co-occurrence network (0.922). The avg K of co-occurrence network of soil bacterial communities were highly significantly and positively correlated with the N/P cohesion (0.726). Metabolism functions, modularity, Shannon index, Chao1 of soil bacterial communities, CO, ST, stand density, average height of stand(H), and altitude were positively correlated with stand regeneration. Metabolism function and the modularity of the soil bacterial community co-occurrence network had the greatest effects on stand regeneration (0.964 and 0.891, respectively), while CO had the least effect on stand regeneration (0.023).



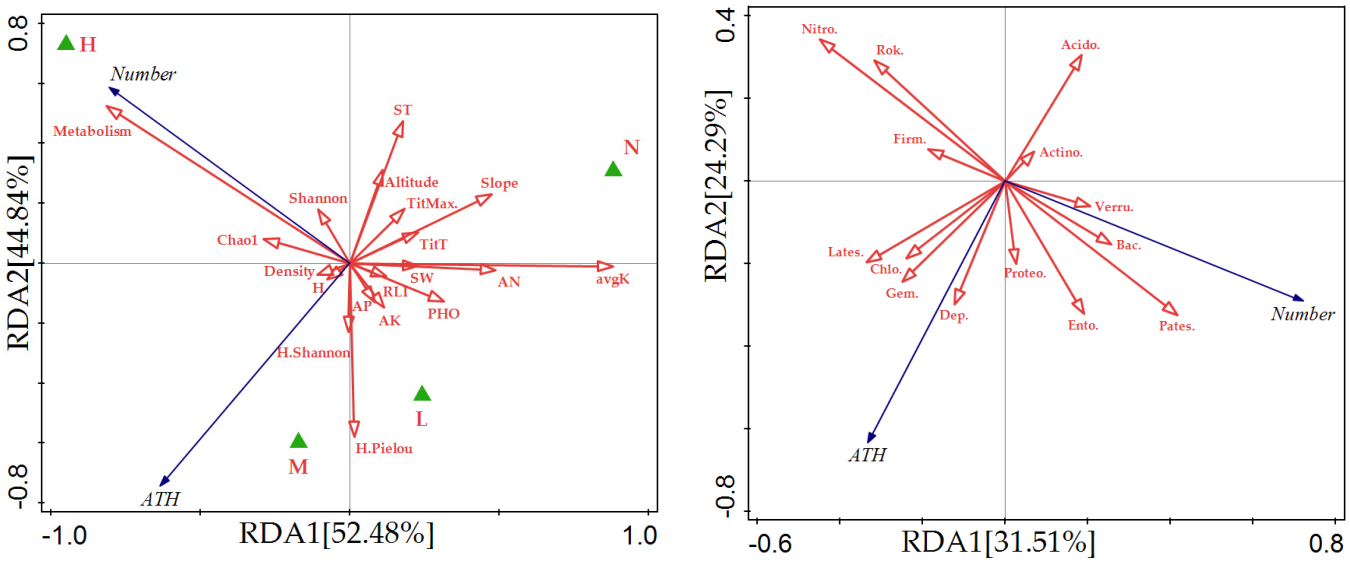
(**a**)



(**b**)

Figure 4. The effects of *L. principis*−*rupprechtii* regeneration degree on soil bacterial potential functions. (**a**,**b**) Variation in core soil microbial community functions associated with *L. principis*−*rupprechtii* regeneration degrees. Functions are based on KEGG annotations. The area of the node is proportional to the node degree, which is calculated from correlations of abundances for each KEGG pathway. Only correlations with an r of >0.6 or, <−0.6 and a *p* value of <0.05 were included in the network.

SW, AN, AP, AK, PHO, URE, H. Simpson, Tit T and Tit Max., and avg K, N/P cohesion of soil bacterial community co-occurrence network were negatively correlated with stand regeneration. N/P cohesion and avg K of the soil bacterial community co-occurrence network had the most significant negative effects on stand regeneration (−0.947 and −0.715, respectively), while H. Simpson had the least negative effect on stand regeneration (−0.044). Each of the four regeneration degree samples showed a consistent pattern. Overall, the metabolic core functions of the soil bacterial community, soil bacterial community richness and diversity, and soil bacterial community complexity had the most significant effects on regeneration (Figure 5a and Table S5).



(**a**) (**b**)

Figure 5. An ordination diagram of the results of the RDA analysis between diversity, composition and network structure of soil bacterial communities, stand structure, soil nutrients, litter, topographic variables and stand regeneration. (**a**) The relationship between number, height, and impact factors; (**b**) The relationship between number, height, and relative abundance of soil bacteria at phyla level. Note: In the figure, each point represents a grouping. The red arrows represent different influencing factors. The sharp angle indicates that two factors are positively correlated, the right angle is not correlated, and the obtuse angle is negatively correlated. The smaller the angle, the higher the correlation. The percentages in the brackets of the coordinate axes represent the proportion of variation in the raw data that can be explained by the corresponding axes. Abbreviations of soil bacterial communities at phyla level are as follows: Proteo., Proteobacteria; Actino., Actinobacteria; Acido., Acidobacteria; Chlo., Chloroflexi; Rok., Rokubacteria; Gem., Gemmatimonadetes; Verru., Verrucomicrobia; Bac., Bacteroidetes; Firm., Firmicutes; Nitro., Nitrospirae; Pates., Patescibacteria; Lates., Latescibacteria; Plact., Planctomycetes; Dep., Dependentiae; Ento., Entotheonellaeota.

The results of the RDA analysis between "flora-forest regeneration" were presented in Figure 5b. The bacterial species contributing more than 5% to forest regeneration were Nitrospirae > Latescibacteria > Acidobacteria > Actinobacteria > Patescibacteria > Entotheonellaeota > Dependentiae > Firmicutes (Table S6). Among them, the relative abundance of Nitrospirae (−0.423), Latescibacteria (−0.186), Dependentiae (−0.002), and Firmicutes (−0.157) were negatively correlated with stand regeneration. The relative abundance of Acidobacteria (0.045), Actinobacteria (0.03), Patescibacteria (0.395) and Entotheonellaeota (0.23) showed a positive correlation with stand regeneration (Table S7). The results from the RDA also showed that the simplicity of the soil bacterial community composition structure is more conducive to regeneration.

3.5. Soil Bacterial Community Diversity, Composition and Structure: SEM Analysis

On the basis of RDA, a structural equation model was developed for all factors whose contribution was >9% and highly correlated (Figure 6). The modified model was determined according to the model fitting criteria. Among these parameters, the chi-squared degrees of freedom ratio (c2/df) < 3, *p*-value > 0.05, and the mean squared error of approximation (RMSEA < 0.05) were within the desired range. In the baseline comparison, the parameter ranges of the goodness of fit index (GFI), normal fit index (NFI), relative fit index (RFI), incremental fit index (IFI), non-constant fit index (TLI), and comparative fit index (CFI) were considered "best" in the range of 0.9 to 1.0. Therefore, the conclusions drawn from the modified model are feasible and realistic (Table S8).

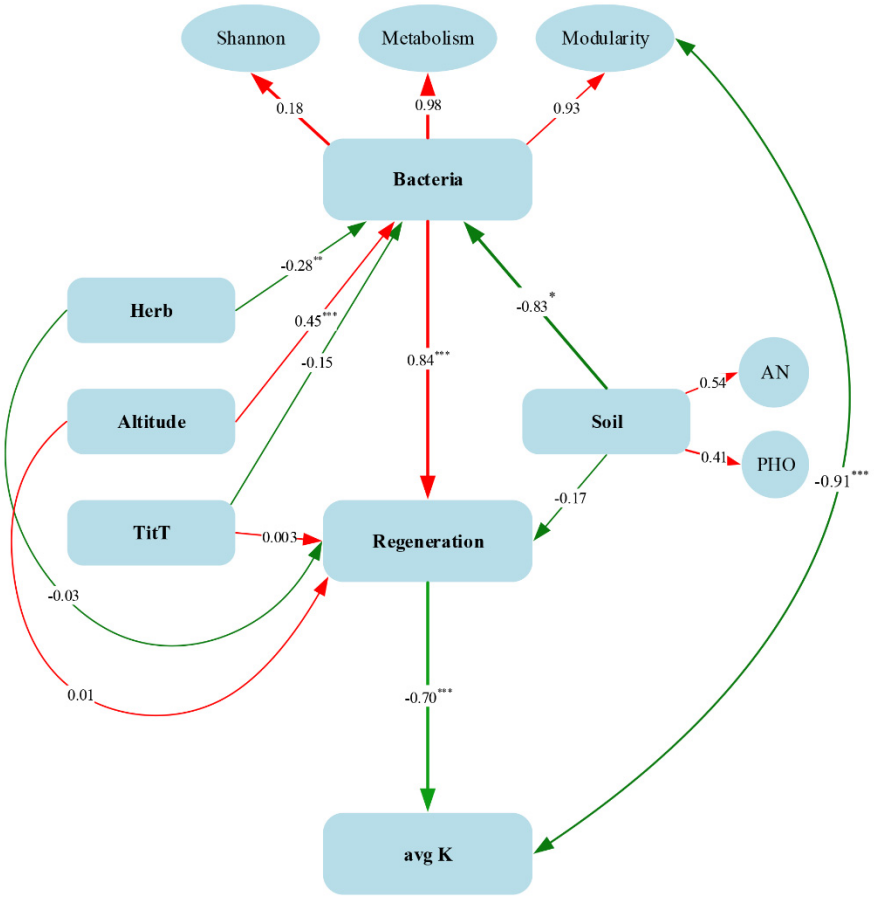


Figure 6. A structure equation model established to explore the significant effects of bacterial community composition (Shannon index, metabolism, modularity), complexity of co-occurrence network (avg K), herb diversity (H. Simpson), Altitude, Dead branch and leaf layer (Tit T), Soil nutrients (AN, PHO) and stand regeneration (the number of regenerated plants). Note: *** represents a significant correlation of $p < 0.001$. ** represents a significant correlation of $p < 0.01$. * represents a significant correlation of $p < 0.05$.

The chi-square value indicates the fit of the correlation matrix of the variables in the overall model to the correlation matrix in the actual situation. In this model, Chi-square = 95.882 and Chi/DF = 1.88 (<3), indicating a good fit of the model to the actual situation. The degree of influence of the explanatory variables on the dependent variable can be directly reflected by the magnitude of the absolute value of the standardized path coefficient (Std. PC). The number of stand regeneration was used to respond to regeneration. Soil bacterial community diversity index, degree of modularity and the most represented metabolic functional pathways were used to respond to soil bacterial community composition. The average degree of the co-occurrence network was used to respond to the complexity of soil bacterial community. Soil AN and PHO were used to respond to soil nutrients. H. Simpson index was used to respond to herb diversity. Soil bacterial community composition and altitude had positive effects on renewal, with total effects of 0.84 and 0.39, respectively. The total effect of soil nutrients, litter thickness, and herbaceous diversity on regeneration was negative, with total effects of −0.87, −0.12, and −0.27, respectively. Regeneration significantly reduced soil bacterial community

complexity, with a coefficient of −0.70. Soil nutrients had a significant negative effect on soil bacterial community composition, with a coefficient of −0.83 (Figure 6; Table S9).

4. Discussion

4.1. The modularity of the Soil Bacterial Community Co-Occurrence Network and the Metabolic Functional Potential It Possesses Are the Main Factors Influencing the Natural Regeneration of L. principis-rupprechtii Plantations

Microbial communities are built from complex assemblages of highly interacting species and understanding the relationships in microbial communities are critical for predicting the effects of microbial communities on the natural regeneration in plantations [46]. For soil bacterial communities, the co-occurrence network structure varied significantly between stands with different degrees of regeneration in *L. principis-rupprechtii* plantations. Although the increased richness and diversity of soil bacterial communities favored regeneration, it was not significant. RDA analysis showed that the network modularity degree of soil bacterial communities was highly significant and positively correlated with the proportion of microflora metabolism pathway (0.922). Our research has shown that highly regenerated stands had the highest degree of modularity and proportion of microflora metabolism pathway (Figure 3; Table S2). The production of metabolites released from soil bacterial communities can be utilized by vegetation and contribute directly or indirectly to the natural regeneration of forests [19,22,45].

This study is the first report showing the effect of co-occurrence network of soil bacterial communities on stand regeneration in *L. principis-rupprechtii* plantations in temperate China. Community interaction network and RDA analyses showed that Patescibacteria was one of the core bacteria in highly regenerated *L. principis-rupprechtii* plantation soils. The relative abundance of Patescibacteria was significantly and positively correlated with stand regeneration, indicating that Patescibacteria was closely associated with regeneration. Natural regeneration in *L. principis-rupprechtii* plantations was closely associated with an increase in the relative abundance of the dominant clade Proteobacteria, Actinobacteria, and Acidobacteria in the soil bacterial community. Proteobacteria are resistance systemic level genes carried by plasmids, known for multiple genetic and physiological mechanisms that establish physiological resistance to low nutrient soil conditions [47,48], which in turn facilitate *L. principis-rupprechtii* regeneration. Actinobacteria are also organisms that can constitute a large proportion of the population in a limited nutrient environment [49]. Soil bacterial networks in four stand regeneration degrees have different keystone species composition, further confirming that ecological niche differentiation of different bacterial taxonomic species influences the regeneration of *L. principis-rupprechtii*. Our results highlight the different patterns of bacterial networks along the degree of regeneration in *L. principis-rupprechtii* plantation ecosystems.

4.2. The Complexity of the Soil Bacterial Community Gradually Decreases as the Amount of Forest Regeneration Increases

As the amount of forest regeneration increases, the competition of seedlings for resources such as soil, water and nutrients also intensified. A wide variety of substrates produced by soil bacterial community accompanied by an increase in root inputs, which lead to a decrease in potential competition and complexity among bacterial communities. This can disrupt the balance of soil microhabitats, making the soil bacterial community structure simpler (Figures 3 and 5; Table S2) and more vulnerable to environmental stress [23,24,50,51]. The increase in stand regeneration exacerbates the stand occupancy, which leads to a decrease in herbaceous diversity in the stand (−0.350). The fact that vegetation diversity has decreased, and the vegetation structure has become more simplistic can also make the soil bacterial community more vulnerable to environmental stress. The results of the potential functional potential of the bacterial community showed that the proportion of the soil environmental information pathway was lower in highly regenerated stands (2.02%) than in moderately regenerated stands (3%), low regeneration (3.03%), and non-regenerated stands (3.03%). This also verifies the above discussion. Seedlings and saplings are more

vulnerable to environmental stress than adult trees, especially without the shelter of a dense canopy [52,53]. This vulnerability may seriously hinder forest regeneration.

4.3. Soil Bacterial Communities Mediated the Effects of Soil Nutrients, Altitude, Litter Thickness, and Herbaceous Diversity on Stand Regeneration in Northern Chinese Larch Plantations

Soil bacteria can select suitable soil properties according to their characteristics, and changes in soil properties can also have an impact on soil bacterial communities [31]. Soil nutrients such as AN, AP, PHO can reduce soil bacterial community richness and diversity [24,54]. It may be due to the input of N and P that acidifies the soil and thus limits the growth of soil microorganisms [33]. AK in soils can reduce soil bacterial community richness and diversity [31]. In our study, the increasing altitude and the lifting of the physical barrier formed by litter layer were responsible for promoting increased diversity, modularity of soil bacterial communities and enrichment of metabolic pathways [55–58], which in turn promoted vegetation regeneration indirectly [59,60]. The coupling of herbaceous diversity with soil prokaryotic diversity [61], which increases bacterial enrichment in genetic information processing pathways and decreases bacterial enrichment in metabolic functions. In addition, herbaceous cover inhibits seedling growth and forest regeneration, which is consistent with other studies [2,16,62]. Reduced light intensity with increasing numbers of regenerate seedlings reduced the diversity of light-related soil bacterial communities such as Chloroflexi, a key photosynthetic bacterium in forest stand soils, significantly [27,30,63].

4.4. Soil Nutrient Inputs Limit Bacterial Community Enrichment for Metabolic Functions Related to Vegetation Growth

Soil nutrients such as AN, AP, AK, and PHO are significant predictors of vegetation growth and reestablishment [64]. In contemporary boreal ecosystems, N is considered to be the most limited nutrient for plant growth [65], and the common limitation of plant growth by N-induced phosphorus has been confirmed [66]. PHO is involved in carbohydrate metabolism as an important extracellular enzyme. SEM analysis showed that soil nutrients including AN, AP, PHO input reduced the modularity degree of soil bacterial communities and inhibited the enrichment of metabolically functional bacteria, which in turn inhibited natural regeneration of *L. principis-rupprechtii*. The reduced carbohydrate metabolic function in highly regenerated stands was related to the inhibitory effect of PHO input on vegetation growth [67]. Sunlight had no significant effect on the regeneration density and growth of seedlings [2].

5. Conclusions and Management Implications

Based on high-throughput sequencing technology and bioinformatics analysis, this study systematically analyzed the main factors affecting the natural regeneration of *L. principis-rupprechtii*. plantations in Northern China from the perspective of the soil bacterial community, especially the changes in functional taxa of soil bacterial community were predicted and demonstrated by functional annotation tools. Based on molecular ecology network analysis, the network topology characteristics and modular communities of soil bacterial species were resolved. The mechanisms of soil bacterial communities affecting natural regeneration in *L. principis-rupprechtii*. plantations were elucidated by combining RDA and SEM analysis tools. It was concluded that the diversity and abundance of soil bacterial communities had no significant effect (coefficient of 0.18) on the amount of natural regeneration, while soil bacterial community co-occurrence network characteristics were the most significant factors affecting the natural regeneration of *L. principis-rupprechtii*. plantations. The degree of modularity of the soil bacterial community co-occurrence network and the metabolic potential function were the most significant factors affecting regeneration (coefficients of 0.93 and 0.98, respectively). Soil AN, PHO was a minor factor influencing tree regeneration (total effect of −0.87). The increased stand regeneration significantly reduced the complexity of the soil bacterial community (coefficient of −0.70). The effects of altitude, herbaceous diversity, and thickness of dead wood layer on regeneration were

mainly derived from the effects on soil bacterial community production. This has important implications for predicting the response of soil bacterial communities to sustainable forest development for plantations.

Therefore, in forestry practices, interventions to enhance the aforementioned significant factors, i.e., planting at middle to high altitudes, proper weeding and appropriate removal of the leaf litter fall etc. means for managers, which can indirectly increase the modularity of the soil bacterial community and promote the enrichment of metabolically functioning bacteria, with the resulting higher production of metabolites that can stimulate tree regeneration and maintain the health and sustainability of plantations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10091771/s1, Figure S1. Rarefaction curves of ASVs. Figure S2. Dissimilarity distance showing the differences in soil bacterial community structure between N group and H, M, L groups. Table S1. Analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) based on Bray Curtis distance with different regeneration degree. Table S2. Soil bacterial community co-occurrence network characteristics with different regeneration degrees. Table S3. Topological properties of potential functional co-occurrence networks of soil bacterial communities with different regeneration degrees. Table S4. Percent variance explained of the degree of regeneration by each environmental factor. Table S5. Correlation analysis of several environmental factors with regeneration. Table S7. Correlation analysis of soil bacterial communities at phyla levels with regeneration. Table S8. SEM model fit indices for all factors. Table S9. Indirect, direct, and total effects between the observed variables in the SEM (standardized results).

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