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Dynamic Changes of Rhizosphere Soil Microbiome and Functional Genes Involved in Carbon and Nitrogen Cycling in Chinese Fir Monoculture

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Abstract: We used metagenomics to investigate the rhizosphere microbial community assembly and functions associated with different nutrient cycles in Chinese fir at different monoculture times and growth stages. Mantel test results indicated significant positive correlations between soil TP contents and bacterial communities. The concentrations of soil AP also exhibited a significantly positive association with the fungal community. The relative abundance of ko00720 and ko00680 increased from young-old stands to mature stands. It then decreased in over-mature plantations (45 years) and had a recovery in 102-year-old stands. The potential degradation pathway of cellulose had the highest abundance in 26-year-old stands than the other aged plantations. Potential N cycling processes were dominated by assimilatory nitrate reduction to ammonium and dissimilatory nitrate reduction to ammonium pathways. The variation-partitioning analysis revealed that three forms of N contents (NH_4^+-N, NO_3^--N, and DON) comprised 7%, whereas the other soil properties constituted 15.6% variation in the relative abundance of the genes involved in N cycling. Thus, metagenomics elucidates the evolution characteristics of rhizomicrobial composition and their functional changes at different developmental stages of Chinese fir plantations, providing a suitable reference for the potential utilization of carbon and nitrogen properties.

Keywords: Chinese fir plantations; rhizosphere; monoculture; metagenome; functional gene; C and N cycling; stand age

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Citation: Wang, S.; Chen, W.; Gao, Q.; Zhou, C. Dynamic Changes of Rhizosphere Soil Microbiome and Functional Genes Involved in Carbon and Nitrogen Cycling in Chinese Fir Monoculture. *Forests* **2022**, *13*, 1906. https://doi.org/10.3390/f13111906

Academic Editor: Donald L. Rockwood

Received: 27 September 2022
Accepted: 10 November 2022
Published: 14 November 2022

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1. Introduction

The planting area and forest stock of Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook.) account for 17.33% and 4.69% of all forested areas in China [1,2], respectively. Chinese fir is widely monocultured in the subtropical forests of southeast China [3]. It plays an important role as a carbon sink and is essential in timber production due to its fast growth, high yield and great timber quality [4,5]. Zhu et al. [5] demonstrated that the increased limitation of fine root carbon availability to the decreases nutrient foraging ability in older stands, which is one of the main causes of yield decline at the later growth stages of Chinese fir. Additionally, the nutrient return of the Chinese fir litter fall does not offset the biomass uptake demand of the plantations, despite the increasing total nutrient stores and nutrient return of the litter fall as the plantation ages. Thus, the total soil nutrients decrease with the increase in stand age [4]. Moreover, higher beta diversity of soil bacterial, fungal and archaeal communities was observed in natural forests than Chinese fir plantations [6]. Hence, elucidating the underlying mechanism of productivity decline is crucial for maintaining long-term site quality and sustainable management of Chinese fir plantations.

Rhizosphere microbiota is critical for plant–soil feedback processes, and are influenced by soil type, host development stage, and genotype [7,8]. The complex signal exchange between rhizosphere microbes and plants dictates the growth and adaptation of plants to nutrient absorption, stress tolerance and resistance to pathogens [9,10]. Harbort et al. [11]

reported that the iron-limiting soils induce the coumarins secretion from plant roots, which alters the iron absorption and immune regulation mediated by the root microbiome.

Rhizodeposits, accounting for about 10%–30% of plant net photosynthate production, cause rapid carbon (C) and nitrogen (N) cycling in the soils [7,12]. The plant rhizosphere recruits microbial communities from the bulk soil and endosphere [13]. Copiotrophs (common for r-strategists), such as *Bacteroidetes* and *Proteobacteria*, are dominant rhizobacteria phyla, characterized by fast growth and the ability to form highly modular but unstable rhizobacterial networks [12]. Rhizobacterial diversity is affected by substrate resource availability [12], since available soil nutrients mediate the balance between the stress tolerance and nutrient cycling of the rhizosphere microbes [13]. Ling et al. [12] showed that the Shannon diversity index of rhizobacteria in the coniferous forests is greater than the bulk soil. The current study, for example, with respect to soil depth and forest stand density, expands on our understanding of soil microbial community composition of Chinese fir [14,15]. For instance, Islam et al. [16] explored the soil microbial community compositions in the depth profiles along a chronosequence from 5 to 40 years of Chinese fir plantations. However, the general pattern of the Chinese fir rhizosphere microbiota at the taxonomic and functional levels at the different development stages, especially the over-mature plantations, remain largely unexplored.

Relative abundance changes of the key functional genes reveal how variations in soil properties affect microbial processes correlating with ecosystem functions in the soil microbial community [17]. The extraction and sequencing of microbial metagenomes have enabled the recovery of the total microbial genomes and have largely circumvented the cultivating limitations of the soil microbial community [18], unlocking potential functions of the soil ecosystem. The abundance of microbial functional genes drives the conversion and degradation of soil organic matter (SOM) through enzyme-catalyzed reactions [19]. A study by Yang et al. [20] indicated that the relative abundance of enzyme-active genes encoding 3-hydroxyacyl-CoA dehydrogenase (K01782) and phosphomannomutase (K15778) positively correlated with the soil salinity of the Yellow River Delta, China. Furthermore, the microbial genes involved in recalcitrant carbon metabolism and defense and stress responses were increased after the reforestation of the reclaimed mine sites [17]. Thus, metagenomics can expand our understanding of the factors regulating the compositional and functional assembly of rhizosphere microbial communities.

Here, we used metagenomics to explore the community assembly of rhizosphere bacteria, fungi and archaea along a chronosequence of Chinese fir monoculture plantations. We hypothesized that (i) the relative abundance of rhizomicrobial composition and functional genes are closely related to the developmental stages of Chinese fir plantations; (ii) nutrient properties of rhizosphere soil, especially available nutrient content, play an important role in rhizosphere microbial assembly. This study aimed to identify the characteristics of the microbial compositions, their functional genes and enrichment in the rhizosphere of Chinese fir plantations. The study also evaluated the association between the relative abundance of microorganisms and functional genes with the ecosystem functions and soil properties. Therefore, the findings of this study expound on the carbon distribution patterns and nitrogen cycling processes in the rhizosphere of long-term plantation sites and how the plantations cope with soil quality changes. The findings also demonstrate the potential of harnessing the beneficial rhizomicrobiome attributes for biocontrol in order to improve the function and to develop sustainable management of the Chinese fir plantation ecosystem.

2. Materials and Methods

2.1. Site Description

The study was conducted at a small watershed in Wangtai Tower, Fujian Province, China (26°38′–26°42′ N, 117°54′–117°57′ E). The detailed information about the climate, soil types, understory vegetations, and the planting history of the study field was modeled according to the previous studies [21,22] and the basic information about the sampling point had been provided Li et al. in [22]. In October 2020, we selected five representative stands with natural age gradients of 6 years (young-aged), 17 years (middle-aged), 26 years (mature-aged), 45 years

(over mature-aged), and 102 years (over mature-aged). For each stand, three replicate sites with similar altitude, topography, soil texture, and parent material were selected [21].

2.2. Soil Samples Collection and Chemical Property Analysis

A plot (20 m × 20 m) was set, and five Chinese firs with similar growth conditions along an S-shape were selected from each replicate site and were combined to form a composite sample. Thereafter, the rhizosphere soil which was attached tightly to the fine roots of the plants (diameter < 2 mm) was brushed into the sterile bags [23]. The fresh soil samples were passed through a 2 mm sieve to remove, gravel and plant debris and then divided into three parts. One part was stored at −20 °C determining the soil moisture content (SMC), the ammonium nitrogen (NH_4^+-N), the nitrate nitrogen (NO_3^--N), the dissolved organic carbon (DOC), and the total soluble nitrogen (TSN) contents. The other part was stored at −80 °C for metagenomic sequencing, while the remaining part was sieved and air-dried in the shade for measuring the pH, total carbon (TC), and total nitrogen (TN) concentrations. For the pH and available phosphorus (AP) determination, the soil was sieved using a 2 mm sieve, while a 0.149 mm nylon sieve was used for measuring the TC and TN.

Soil pH was determined in a 1:2.5 volumetric suspension of air-dried soil and distilled (CO_2-free) ultrapure water. Soil TN and TC content analyses were performed by the combustion method with an elemental analyzer (Vario Macro Cube, Elementar, Germany) and their contents were indicated by the C/N ratio. Soil NH_4^+-N and NO_3^--N contents were measured using 2M KCl via the continuous flow analyzer (SKALA, SAN++, Netherlands). Conversely, 2M KCl was used to determine the soil DOC and TSN concentration using a total organic carbon (total nitrogen) analyzer (TOC-VCPH/CPN Analyzer, Shimadzu, Japan). Dissolved organic nitrogen (DON) was calculated from the differences between the TSN and total inorganic N (the concentration summation of NH_4^+-N and NO_3^--N) concentrations [24]. Soil AP was measured with 0.03 M NH_4F and 0.025 M HCl, and its content was determined using the molybdenum blue colorimetry method. The SMC was determined by oven-drying its samples to a constant weight at 105 °C.

2.3. Metagenomic Sequencing of Rhizosphere Microbiota

We subjected 15 soil samples (5 samples × 3 replicates) to metagenomic sequencing at Beijing Allwegene Technology Co., Ltd. (Beijing, China). Briefly, genomic DNA was extracted using the Power Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) and Qubit was used to accurately quantify the DNA concentration. The DNA samples were randomly broken into ~350 bp fragments by the Covaris ultrasonic processor and were subjected to end-repair, poly-A-tailing, sequencing adapter ligation, purification, and PCR amplification for the library construction. Thereafter, qPCR was used to accurately quantify library concentration (effective concentration >3 nM). Raw reads with sequencing adapters, an N (unknown bases) content ratio greater than 1% and low-quality base ($Q \leq 20$) content greater than 50% were eliminated to obtain clean reads for further analysis.

MEGAHIT (v1.0.6) assembly tool [25] was used to assemble the reads (Hong Kong, China; Tokyo, Japan), and the fragments below 500 bp in the assembly were removed. Subsequently, the prodigal [26] was used to predict the open reading frames (ORFs) of the assembled contig sequences, and the CD-HIT [27] was used to remove redundant sequences of the predicted genes with a similarity of 0.95 in order to obtain non-redundant data. The sequencing data were compared with the non-redundant gene set using Bowtie [28]. Thereafter, the abundance of a single gene in different samples was calculated and normalized to obtain a gene abundance table. Bowtie was used to compare the sequencing data with the non-redundant gene set, and the abundance information of genes in different samples was counted.

The taxonomic composition of the microbial community (bacteria, fungi and archaea) was determined by searching the reads against the NCBI-NR database using DIAMOND [29] and MEGAN 6 [30] for taxonomic analysis. Functional annotation was conducted by searching the non-redundant gene against the KEGG database (https://

www.kegg.jp/ (accessed on 2 May 2019)) using KOBAS (v2.1.1). The non-redundant genes were also searched against the CAZyme (Carbohydrate-active enzyme) database using HMMER [31] for CAZyme annotation.

The relative abundance of individual genes represented their hits and was normalized to the sum number of the sequencing reads in each sample, begore then being multiplied by 10^6 to reflect gene hits in 10^6 of the aligned sequencing reads. This was to eliminate the bias arising from the sequencing depth difference among the samples [32]. We selected some specific genes for comparison with those in the NCBI-NR database to identify the microbial taxonomy. The potential functions involved in nitrogen cycling [33] and the decomposition of soil carbon-containing compounds [34,35] were calculated as the total relative abundance of special functional genes.

2.4. Data Analysis

Unless otherwise specified, all data processing was conducted in R (4.1.3). The most abundant functions of the rhizosphere microbial community for KEGG levels 2 and 3 were presented as the mean values of the relative abundance transformed by the Z-score, and their heatmap plots were generated using the "pheatmap" package. We performed Shapiro–Wilk and Levene's tests to check the normality and homogeneity of variance assumptions, respectively, followed by a one-way analysis of variance (ANOVA). In cases where ANOVA could not be performed, we applied the non-parametric Kruskal–Wallis test with Bonferroni-adjusted p values using the "agricolae" (v1.3-5) package. The soil nutrient contents, Shannon index of the microbial communities, the relative abundance of the functional genes, and relevant functional potential were presented as mean value ± standard error (SE). The ANOVA was conducted using the IBM SPSS (v19), followed by least significant difference (LSD) multiple comparisons tests.

Redundancy analysis (RDA) was conducted to test the potential connections between soil nutrient contents and the relative abundance of functional genes involved in nitrogen cycling processes. The analysis used the "vegan" package (2.6.2) and removed the explanatory variables with VIF > 10 (variance inflation factor). The abundance of the functional genes was subjected to a "Hellinger" transformation. Moreover, the variation partitioning analysis (VPA) was performed with the "vegan" package, using the "varpart" function to separate the effects of soil nutrient factors on the relative abundance of functional genes in nitrogen cycling processes transformed by the "Hellinger" method [36].

We calculated the Shannon indexes of the microbial communities based on the number of species using the "vegan" package. A Mantel test was performed to determine the connection between microbial compositions and soil properties using the "ggcor" package [37]. Additionally, the "random forest" (RF) analysis was performed using the "random forest" package to explore the key predictors of the soil nutrient fractions driving the changes in soil microbial abundance. The significance of each predictor and that of the RF model, were determined using the "rfPermute" and "A3" package, respectively [38]. The partial least-squares path model (PLS-PM) was conducted through the "plspm" package to estimate the effects of stand ages, soil factors, and microbial community compositions on soil C and N cycling process. The variables with VIF > 10 and loading values <0.7 were removed from each block [39].

3. Results

3.1. Analysis of Soil Nutrient Properties

The acidic soil pH of the Chinese fir-aged groups ranged from 4.70 (26 years) to 5.15 (45 years). Notably, the soil pH, NH_4^+-N, and AP decreased, while the soil DOC concentration increased from 6–26 years of Chinese fir plantations. Soil DON showed a decreasing trend from 17 to 102 years of the Chinese fir plantation. Nevertheless, SMC decreased significantly with the increasing plantation age and had a minimum value (16.15%) at 45 years ($p < 0.05$) (Table 1).

Table 1. Rhizosphere soil nutrient concentrations in different stand ages of Chinese fir plantations in subtropical China.

Stand Ages (Years)	6	17	26	45	102
pH	5.09 ± 0.07 a	4.77 ± 0.09 b	4.70 ± 0.01 b	5.15 ± 0.07 a	4.71 ± 0.07 b
DOC (mg kg^{-1})	242.65 ± 13.17 ab	288.95 ± 30.27 a	303.14 ± 29.28 a	186.80 ± 4.52 b	218.68 ± 10.52 b
DON (mg kg^{-1})	51.55 ± 4.76 a	66.91 ± 6.82 a	58.19 ± 5.06 a	49.53 ± 3.95 a	48.79 ± 5.46 a
NH_4^+-N (mg kg^{-1})	10.29 ± 1.12 a	10.35 ± 1.13 a	7.94 ± 0.95 a	8.58 ± 1.07 a	9.56 ± 2.82 a
NO_3^--N (mg kg^{-1})	5.29 ± 0.26 bc	4.85 ± 0.25 c	6.89 ± 0.74 ab	4.56 ± 0.28 c	7.96 ± 1.11 a
TN (g kg^{-1})	1.20 ± 0.10 a	1.03 ± 0.15 a	1.13 ± 0.07 a	0.90 ± 0.10 a	1.27 ± 0.12 a
TC (g kg^{-1})	18.37 ± 2.02 a	15.40 ± 2.12 ab	18.73 ± 1.62 a	10.83 ± 1.11 b	17.83 ± 2.09 a
C/N	15.54 ± 0.09 a	14.80 ± 0.38 ab	16.04 ± 0.60 a	12.19 ± 0.17 c	14.07 ± 0.62 b
SMC (Soil moisture content)	0.31 ± 0.02 a	0.26 ± 0.02 ab	0.26 ± 0.01 bc	0.16 ± 0.01 d	0.22 ± 0.00 cd
AP (mg kg^{-1})	3.21 ± 0.5 ab	2.46 ± 0.24 ab	1.91 ± 0.51 b	3.89 ± 1.11 ab	3.98 ± 0.50 a

All data are presented as means ± standard error (n = 3). Means with different lowercase letters in a row indicate significant differences in rhizosphere soil nutrient concentrations along the stand age gradient ($p < 0.05$).

3.2. The Abundant Functional Groups Based on The KEGG Database

Most functional pathways at KEGG level 2, including carbohydrate and energy metabolism pathways, were enriched in mature forest stands (26-year-old stands). Two functional pathways involved in parasitic and viral infectious diseases (human diseases group) were enriched in 17-year-old stands, and their activities decreased with stand age (Figure 1a). The relative abundance of the carbon metabolism pathway (ko01200), the quorum sensing pathway (ko02024), the starch and sucrose metabolism pathway (ko00500), the oxidative phosphorylation (ko00190), citrate cycle (TCA cycle) (ko00020), cysteine and methionine metabolism (ko00270), and pentose phosphate pathways (ko00030) increased from 6-year-old to 26-year-old stands (Figure 1b).

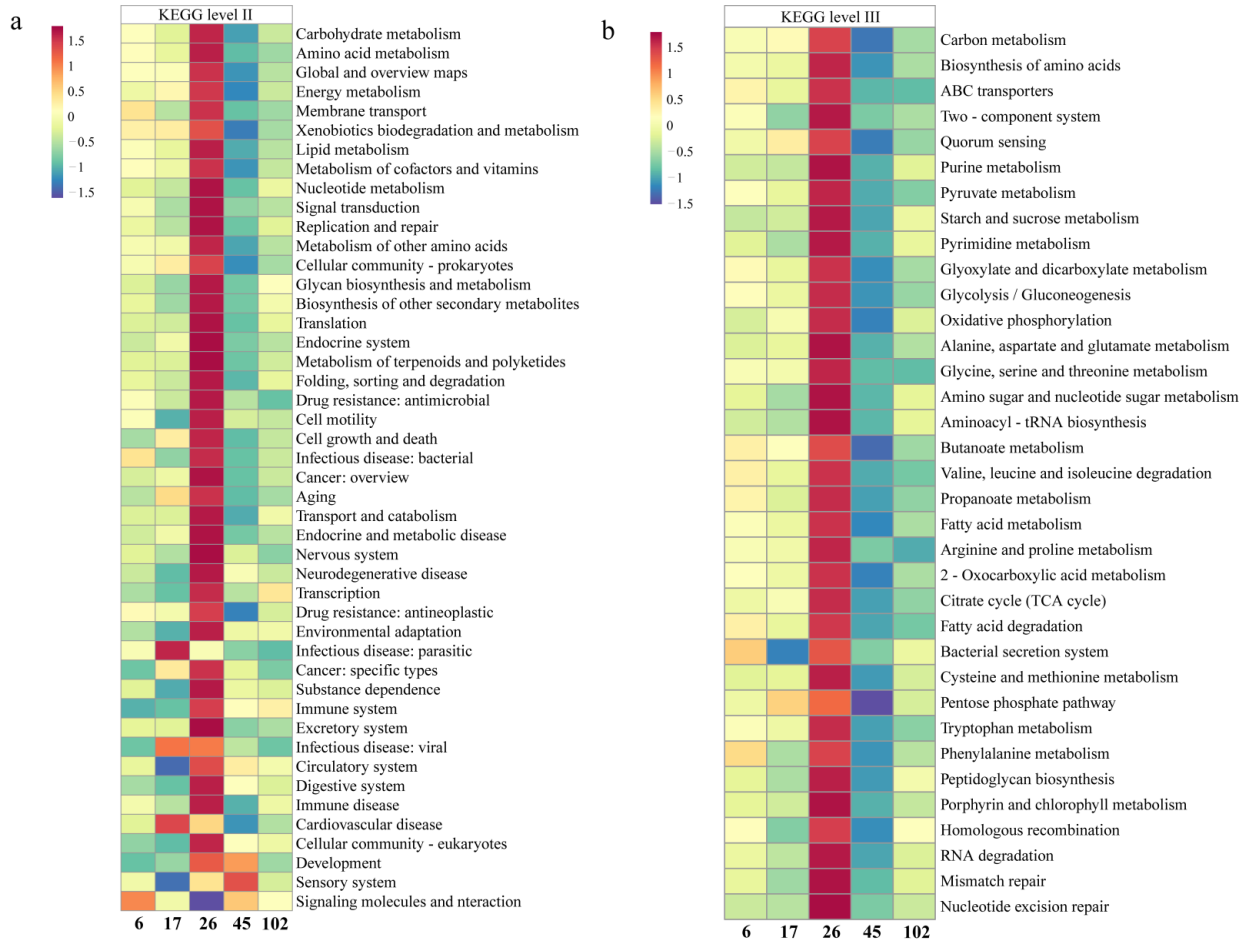


Figure 1. Heatmap of KEGG pathway function annotation (**a**) at level 2 and (**b**) dominated functions at level 3 in rhizosphere soil of Chinese fir plantations at five different stand ages.

3.3. Assemblage Characteristics of Rhizosphere Microbial Community

Generally, the compositions of the rhizosphere microbial community were closely related to the different stages of Chinese fir plantations (Figure 2). The relative abundance of *Actinobacteria* increased significantly from 6- to 26-year-old stands but reduced for 45-year-old stands, with a recovery at 102-year-old stands. The relative abundance of *Planctomycetes* was significantly high in 17-year-old stands (Figure 2b). In the fungal community, the relative abundance of *Basidiomycota* increased with the age gradient and was abundant in 45-year-old plantations (Figure 2c). *Thaumarchaeota* had the highest relative abundance in the 102-year-old Chinese fir stands among the archaeal community (Figure 2d). The relative abundance of phylum *Euryarchaeota* increased from young stands (6 years) to middle-aged stands (17 years) but decreased as the stands aged. Conversely, the relative abundance of *Crenarchaeota* reduced until 26 years of age for the plantation stands. The Shannon index of the archaeal community showed the lowest value in 102-year-old Chinese fir plantations (Table S1).

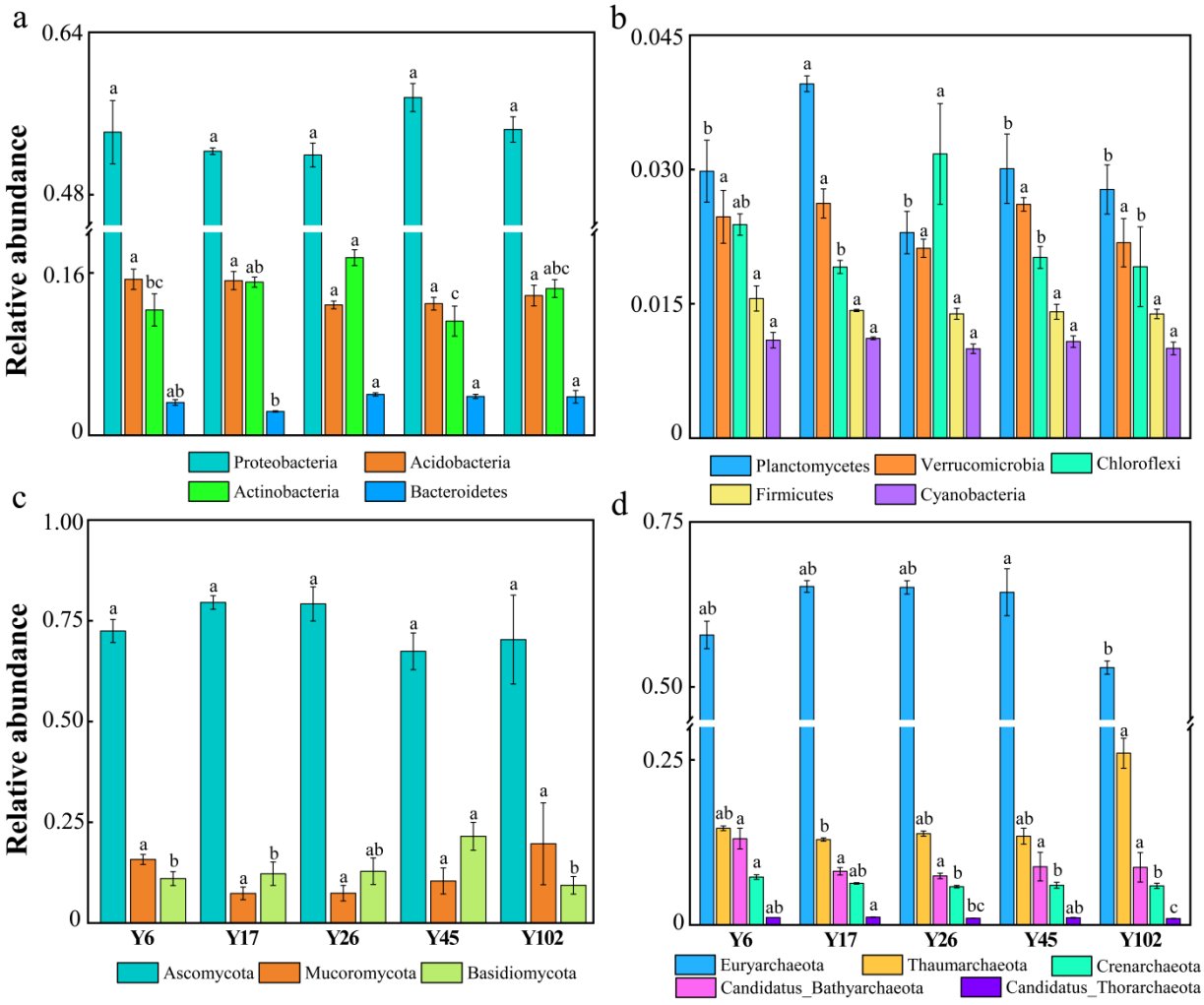


Figure 2. Relative abundance of the dominant phyla of bacterial (**a**,**b**), fungal (**c**) and archaeal (**d**) communities. Values are means, and error bars show standard error (n = 3). Different letters above the bars indicate significant differences between stand groups for each phylum ($p < 0.05$).

3.4. Function Pathways Involved in C Cycling

The functional pathways involved in the two carbon fixation pathways were ko00710 (carbon fixation in photosynthetic organisms) and ko00720 (carbon fixation pathways in prokaryotes), while ko00680 was involved in the methane metabolism pathway (Figure 3a). The green line represents the relative abundance summation of the two carbon fixation

pathways, ko00710 and ko00720. We summarized the relative abundance of the genes potentially involved in the degradation of six carbon-containing substances (lignin, pectin, cellulolytic, starch, chitin, and hemicellulose) based on the CAZyme database (Figure 3b).

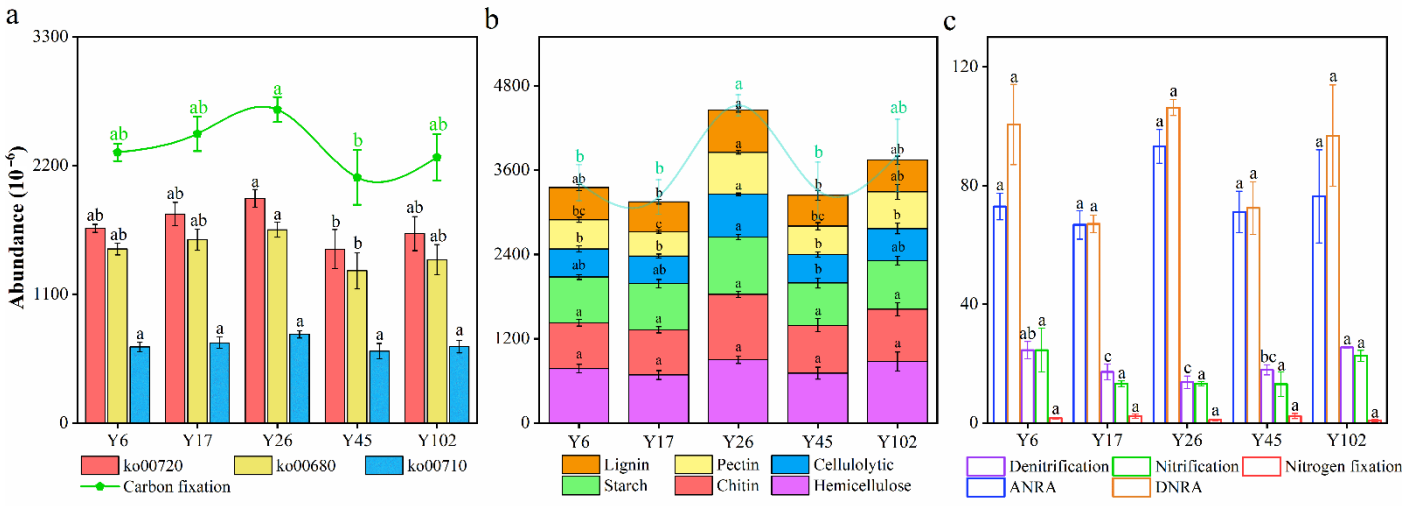


Figure 3. Abundance of the functional pathways involved in potential carbon fixation pathways and methane metabolism pathway (**a**), as well as potential degradation of carbon-containing materials (**b**) and nitrogen cycling processes (**c**).

The relative abundance of ko00720 and ko00680 increased from young stands (6 years) to mature stands (26 years) but decreased in over-mature (45 years) plantations, with a recovery at 102 years of age for the stands. The potential degradation of carbohydrates was higher in mature plantations than in other stands. For instance, the relative abundance of the potential degradation of cellulose was significantly higher in 26-year-old stands than in any other plantation group.

3.5. Functional Pathways Involved in N Cycling

We performed analyses of five potential pathways of N cycling based on abundance summations of the genes involved in relevant metabolic pathways which were documented in earlier literature (Figure 3c). The key functional genes involved in the N cycling process were also identified (Figure 4). The pathway of assimilatory nitrate reduction to ammonium (ANRA) and pathway of dissimilatory nitrate reduction to ammonium (DNRA) dominated the relative abundance of the N cycling processes. Moreover, the denitrification potential of rhizosphere microorganisms first decreased and then increased with the increasing stand age and reached a minimum value in the mature stands (26 years).

The *ureC* gene abundance (involved in ammonification) increased from 6-year-old to 26-year-old stands, but then decreased with the growth of Chinese fir plantations. The gene abundance of *gdhA* (involved in ammonia assimilation) also increased from 6-yeaar-old to 26-year-old stands, but had no statistical significance. Furthermore, the relative abundance of *nxrB* (involved in nitrification) and *norB* (involved in denitrification) decreased from 6-year-old to 26-year-old stands, but significantly increased from 26-year-old to 102-year-old stands. Gene abundance of *nrfA*, involved in the DNRA process, reached its highest values in middle-aged plantations (17 years) and decreased with the increasing Chinese fir stand ages.

The microbial phyla, harboring genes which encode the key enzymes in nitrogen-transforming processes, are shown in Figure 5. The *nrfA*-containing phyla were dominated by *Nitrospirae*, *Verrucomicrobia*, and *Proteobacteria*. *Nitrospirae* had increasing relative abundance from young-aged to mature stands, which decreased in over-mature stands (Figure 5a). For the *nirA*-harboring phyla, the main phyla were *Proteobacteria*, *Verrucomicrobia*, *Acidobacteria*, and *Planctomycetes* (Figure 5b). Three phyla, including *Proteobacteria*,

Actinobacteria and *Chloroflexi*, dominated the *ureC* genes-containing group (Figure 5c). *Proteobacteria* and *Bacteroidetes* contained *nirK* genes involved in denitrification, and the relative abundance of *Proteobacteria* increased with the growth of Chinese fir stands (Figure 5d).

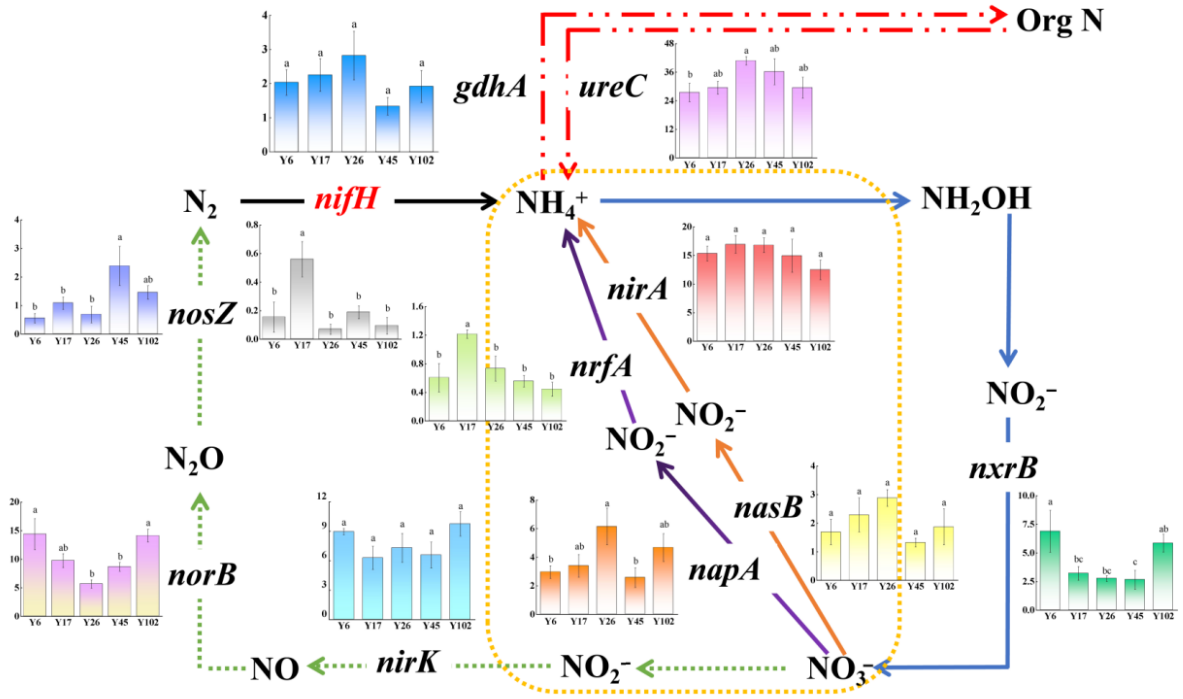


Figure 4. Nitrogen cycling processes and abundance of corresponding functional genes (10^{-6}) of rhizosphere microbial communities in Chinese fir stand ages in subtropical China. The different letters indicates significant differences at $p < 0.05$.

3.6. The Linkage between Stand Age, Soil Nutrient Contents and the Abundance of C and N Functional Pathways

Mantel test results indicated that there was a significant positive correlation between soil TP contents and bacterial community. The soil AP concentrations correlated positively with the fungal community. Similarly, soil pH, SMC and AP also showed a positive correlation with soil archaeal community ($p < 0.05$) (Table 2).

Table 2. Mantel tests (Spearman's correlation) between microbial communities and soil nutrient properties. *: $p < 0.05$.

Soil Property	Mantel r		
	Bacterial Community	Fungal Community	Archaeal Community
pH	−0.04	0.09	0.24 *
TN	−0.04	0.01	0.19
TC	−0.06	−0.03	0.04
C/N	0.05	−0.05	0.02
SMC	0.09	−0.12	0.30 *
DOC	−0.05	−0.16	−0.13
NH_4^+-N	0.01	0.10	0.09
NO_3^--N	0.05	0.14	0.12
TP	0.27 *	−0.11	0.14
AP	−0.04	0.36*	0.30 *
DON	−0.10	0.00	−0.18

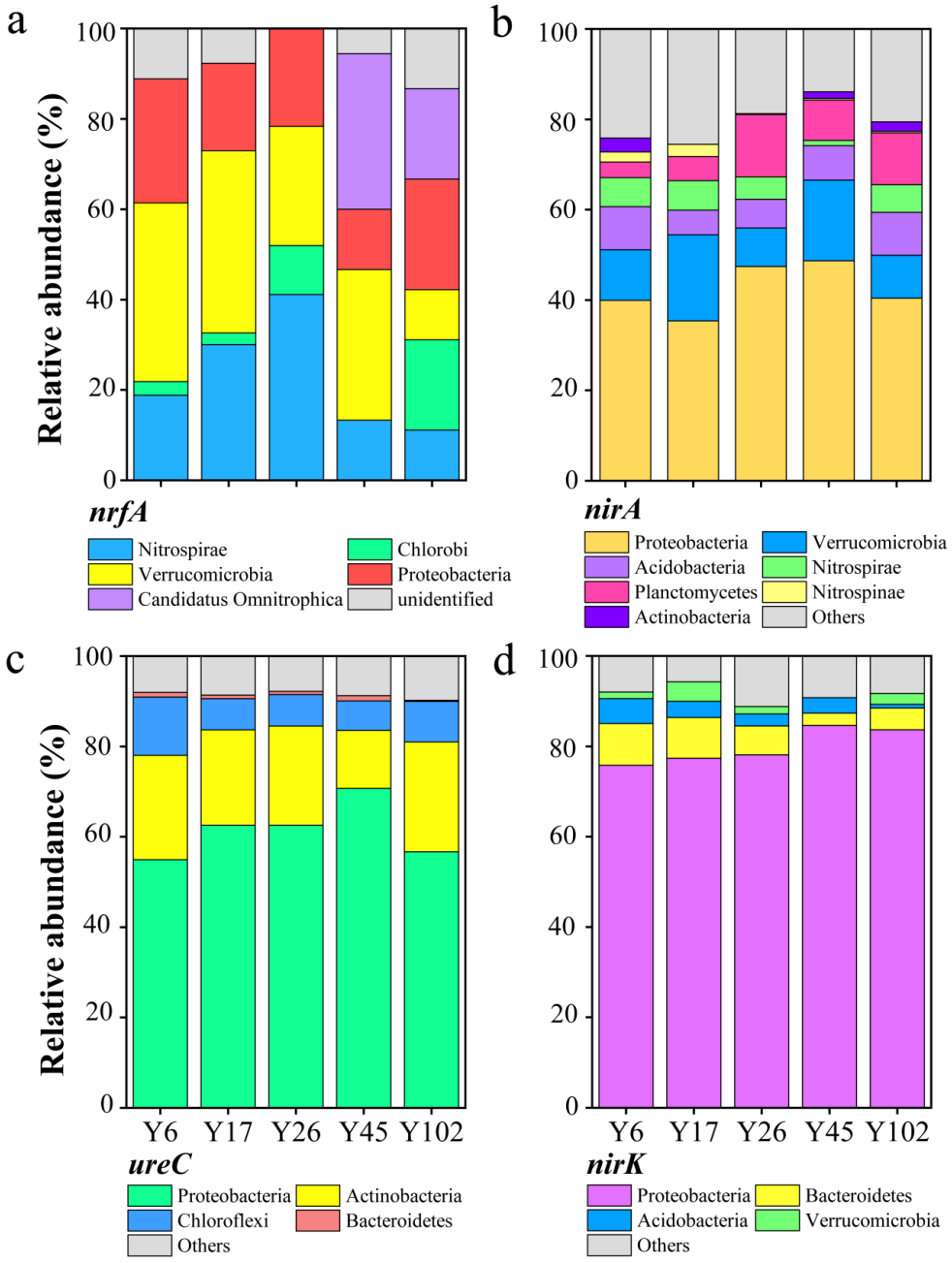


Figure 5. Phyla containing genes involved in N cycling processes in Chinese fir stand ages in subtropical China. (**a**) nrfA; (**b**) nirA; (**c**) ureC; (**d**) nirK.

We explored the potential correlation between soil nutrient content and the abundance of nitrogen cycling-related genes using redundancy analysis (RDA) (Figure 6a). The results showed that the gene abundance of *nirK* positively correlated with the soil TN concentrations, while *nrfA* gene abundance negatively correlated with soil TN concentrations. There was also a positive correlation between *napA* gene abundance and the soil C/N ratio; however, the soil NO_3^--N contents negatively correlated with *nifH* gene abundance. Similarly, the gene abundance of *nasB* and *norB* were negatively correlated with soil NH_4^+-N concentrations and soil DON contents, respectively. The VPA revealed that three forms of N contents (NH_4^+-N, NO_3^--N, and DON) accounted for 7%, and the other soil properties accounted for 15.6% of the relative abundance variation of the genes involved in N cycling (Figure 6b).

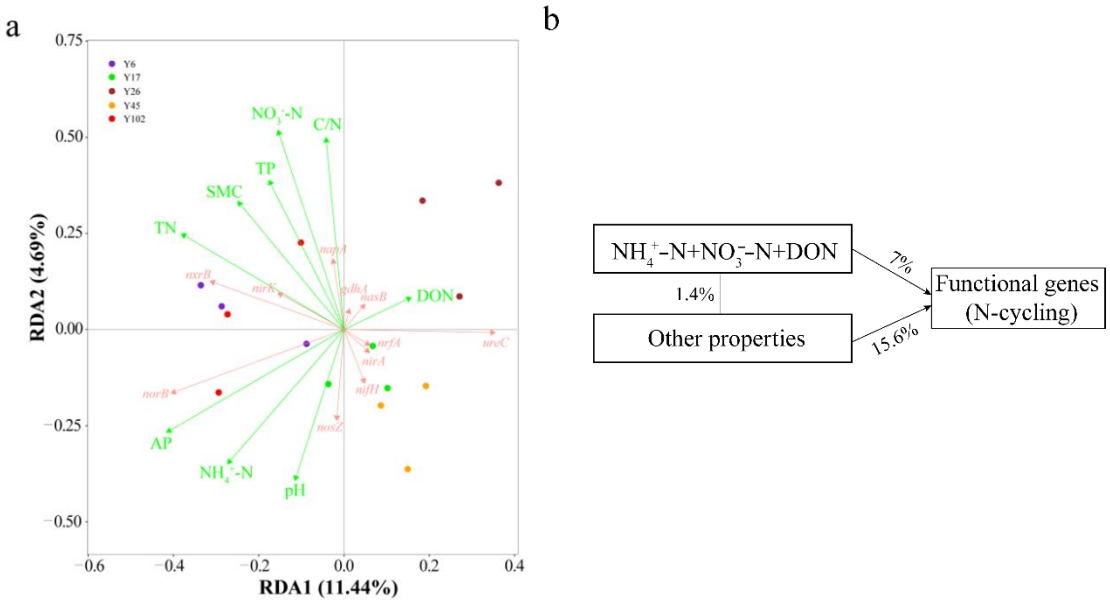


Figure 6. Redundancy analysis (RDA) ordination plots of soil properties and abundance of genes involved in N cycling (**a**) and its variation-partitioning analysis (**b**).

We also used PLS-PM to further explore the possible association by which stand age, soil nutrient and microbial community influence the function pathways involved in C and N cycling processes in rhizosphere soil of Chinese fir plantations (Figure 7). Our model generated a GOF (goodness-of-fit) value of 0.706, showing that the age of Chinese fir stands had direct negative effects on soil nutrient contents (SMC, DOC and TP) (path coefficient = −0.65, $p < 0.01$) and had positive effects on archaeal phyla (*Thaumarchaeota*) abundance (path coefficient = 1.12, $p < 0.001$). Similarly, the soil factors showed direct negative effects on the relative abundance of fungal phyla (*Basidiomycota*) (path coefficient = −0.80, $p < 0.05$). The bacterial community (*Planctomycetes* and *Verrucomicrobia*) also exhibited direct negative effects on the N cycling (ANRA and DNRA) (path coefficient = −0.81, $p < 0.001$) and C cycling (lignin, hemicellulose and cellulolytic) (path coefficient = −0.67, $p < 0.01$) processes.

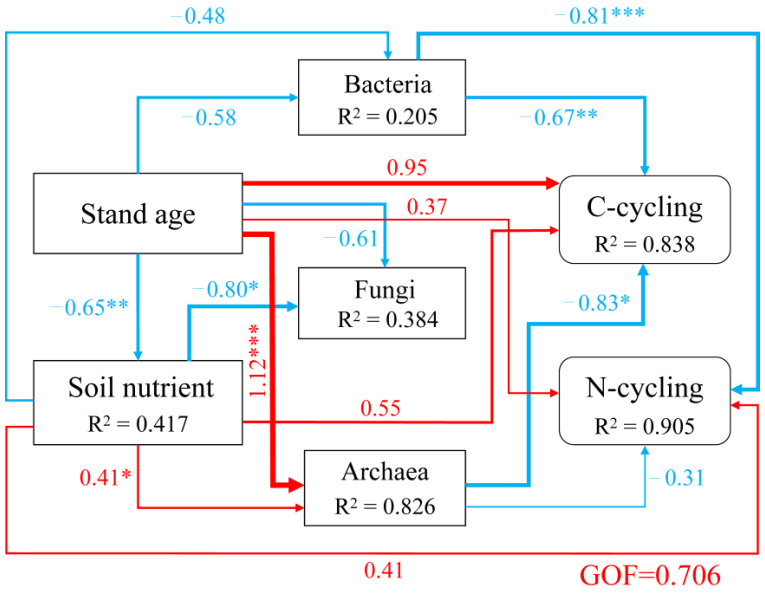


Figure 7. Partial least-squares path models (PLS-PM) showing the effects of stand ages, nutrient contents and microbial communities on potential C and N processes. *: $p < 0.05$; **: $0.001 < p < 0.01$; ***: $p < 0.001$.

4. Discussion

4.1. Functional Pathways Characteristics of Rhizosphere Microbial Community in Chinese Fir Plantations at Different Developmental Stages

Our study provides a metagenomic profile of a 102-year chronosequence and sheds light on the underlying mechanisms of the microbe-driven functional changes in rhizosphere carbon and nitrogen cycling processes. Trees in monocultures with similar ageing trends increase the susceptibility to particular pathogens [40]. Two functional pathways involved in parasitic and viral infectious diseases (human diseases group) were enriched in 17-year-old stands (Figure 1a), which might reveal the adverse effect of the rhizosphere environment on microbes [41]. Moreover, our findings indicated that the functional pathway involved in plant-pathogen interaction (ko04626) decreased in 17-year-old stands (Table S2), showing that the middle-aged forests of single species stands are more vulnerable to biotic damage [40]. We also found that the abundance of the genes with the potential to degrade carbon-containing materials was significantly smaller in the middle-aged plantation than in the mature plantations but had a higher microbial diversity index without statistical significance (Figure 3b; Table S1). Yuan et al. [42] found that the proportion of organic carbon protected within micro-aggregates of the middle-aged stands was significantly higher than that in the young and mature Chinese fir stands. The middle-aged Chinese fir plantations have strong natural pruning and tree differentiation, higher litter accumulation [43], more root allocation [42], and higher fine root biomass [21] during the fast-growing stage. Therefore, diverse microbes may be selectively attracted to colonize in the rhizosphere (beneficial and harmful).

Neighboring plants alter the root exudates and rhizosphere community assemblage of the focal plants [44]. Moreover, the understory vegetation positively affects soil C cycling in the over-mature vegetation stands owing to its additive effect on the overall fine root biomass, which attributes to natural thinning and re-initiation of understory canopy opening [21]. Therefore, the relative abundance of the functional pathways involved in carbon cycling increased from young to mature plantations (Figure 3a), but decreased in over-mature stands, and its balance level was restored thereafter.

Rhizosphere soil nutrient content, especially soil available nutrient content, played an important role in degrading carbon-containing materials. Our finding established a significantly positive association between soil DOC and NO_3^--N contents and the potential degradation of starch (Figure S1). This indicated that the ability of rhizosphere soil to provide available C and N nutrients might provide substrate support for related metabolic activities.

4.2. Microbial Composition and Assemblage in the Chinese Fir Rhizosphere

Soil properties alternation dominated the microbial assemblage during the secondary succession [45]. Our research found that soil pH, TP and AP concentrations were the most important indicators of *Actinobacteria* abundance (Figure S2b). The soil pH had a significantly negative correlation with the relative abundance of *Actinobacteria* (Figure S3a), consistent with Tayyab et al. [46]. *Actinobacteria* play pivotal roles in soil nutrient cycling and plant growth and can colonize the rhizosphere by antagonizing and competing with other microbes [47]. The relative abundance of *Streptomyces*, the dominating genus of phylum *Actinobacteria*, increased from young stands (6 years) to mature stands (26 years) (Table S3). *Streptomyces* secrete various hydrolases to degrade cellulose, chitin and other insoluble macromolecular carbon-containing organic compounds from plants and fungi, while producing several biologically active secondary metabolites (such as antibiotics) [47]. Neutral effects predominated the interactions between *Actinobacteria* and other microbes under eutrophic conditions of red soil [48]. Thus, *Actinobacteria* composition increased from young-aged to mature-aged stands of Chinese fir plantations, indicating that their antibiotics production likely increased competitive advantage.

Furthermore, we found a significant correlation between the archaeal community and soil properties. The archaeal community positively correlated with soil pH (Mantel

r = 0.24, $p < 0.05$) and SMC (Mantel r = 0.30, $p < 0.05$) (Table 2). Similar results from a previous study showed that the copy numbers of the 16s rRNA gene of the archaeal community positively correlated with the topsoil pH [49]. The study by Angel et al. [50] revealed that the water content significantly correlated with the bacterial and archaeal community structures. The domain variation of the archaeal phyla, including *Euryarchaeota* and *Thaumarchaeota*, was closely related to the soil ecological processes. Our study found that the relative abundance of *Thaumarchaeota* positively correlated with soil NO_3^--N concentrations ($p < 0.01$) (Figure S3b). *Thaumarchaeota* play an important role in nitrogen and carbon cycling, since most are considered to be ammonia oxidizers of the terrestrial ecosystem [51]. The ammonia-oxidizing archaea community (AOA) is related to soil nitrate leaching, leading to nitrogen loss from soil [52]. We also found that the relative abundance of *Thaumarchaeota* was higher in the 102-year-old stands (Figure 2d). Meanwhile, the potential nitrification in the rhizosphere of Chinese fir plantations was higher in 102-year-old stands (Figure 3c). The relative abundance of *Euryarchaeota* increased from 6 to 17 years and was accompanied by an increase in methane metabolism potential (ko00680) (Figure 2d; Figure 3a). However, the relative abundance of *Methanosarcina* and *Methanobacterium* were lower in 102-year-old stands than at the other growth stages of Chinese fir (Table S4). Methanogens, such as *Methanobacterium* and *Methanosarcina*, are obligate anaerobic microorganisms belonging to the phylum *Euryarchaeota*, which can reduce CO_2-type or methyl-type substrates to methane [53]. Plant rhizosphere can provide a special habitat representing anoxic or oxygen-depleted micro-niches for the archaeal community [51], enabling phyla such as *Euryarchaeota* to degrade organic matter in anoxic environments [51]. Many anaerobic protozoa contain hydrogenosomes that can produce H_2, CO_2 and acetate, serving as electron and carbon donors for methanogens [51]. The SMC decreased significantly from 26 to 45 years, which may provide fewer niches for obligate anaerobic microorganisms, therefore, decreasing the potential of methane metabolism.

As avid rhizosphere colonizers, the phylum *Acidobacteria* are key players in the C and N cycling processes occurring in acidic soil conditions of conifers [54,55]. The relative abundance of *Acidobacteria* had a significant positive correlation with soil NH_4^+-N contents (Figure S3c), which also contained nitrate reductase gene *nirA* (Figure 5b). This indicated an important role of phylum *Acidobacteria* in the nitrogen cycle. For instance, Domeignoz-Horta et al. [56] reported an *Acidobacteria* strain that carries genes essential for amino acid, ammonium, and nitrate uptake.

4.3. Nitrogen-Cycling Processes in Rhizosphere of Chinese Fir Plantation

Metagenomic analysis is the key to identify soil microbial composition and functional profile. For example, Pang et al. [57] applied metagenomics to investigate the changes of functional genes involved in N cycling processes in the rhizosphere of sugarcane under continuous cropping. The bioavailable forms of nitrogen, such as NH_4^+-N and NO_3^--N, rely on nitrogen-transforming processes that are mainly catalyzed by the corresponding enzymes encoded by several genes of the functional microbes [58,59]. Moreover, the abundance of microbial genes in the environment is related to the functional rates they mediate, reflecting the potential biogeochemical cycling processes to some extent [60]. However, it is important to note that the analysis of metagenomic sequencing provides functional potentials instead of actual microbial activities. Additional data confirmed that the functional genes involved in the DNRA process were the most abundant in soil N cycling of the Chinese fir rhizosphere environment (Figure 3c), and were positively correlated with the contents of soil NO_3^--N (Figure S1). This was similar to the findings by Minick et al. [61], which showed a significantly positive relationship between DNRA and soil NO_3^--N concentrations. DNRA is a vital N retention pathway in the acidic forest ecosystem, which reduces NO_3^--N leaching and nitrous oxide (N_2O) losses [61]. The *nrfA* gene was used as a marker gene to investigate the nitrite reduction in the DNRA process [62]. We found that the relative abundance of the *nrfA* gene was higher in the middle-aged forest (17 years) and then decreased with the stand ages (Figure 4). Therefore,

the potential nitrogen retention process of the DNRA decreased in the rhizosphere with the development of Chinese fir plantations.

N_2O is a powerful greenhouse gas and ozone-depleting agent. Although N_2O is only released at the fluxes of 12 Tg nitrogen yr^{-1} from the terrestrial ecosystem, it has a profound impact on the earth [58]. Our result found that the gene abundance of *norB* decreased from 6 to 26 years, after which it increased with the stand age (Figure 4). *NorB* gene encodes nitric oxide reductase, which reduces NO to N_2O during the denitrification process [63]. Hence, the potential emission of N_2O was lowest in the mature-stage forest. The ammonia assimilation process was impacted by the gene *gdhA* encoding glutamate dehydrogenase [64]. Moreover, *UreC*, encoding urease, is involved in the ammonification process [65]. The gene abundance of *gdhA* and *ureC* increased from 6-year-old to 26-year-old stands (Figure 4), indicating the increase in potential ammonia assimilation and ammonification processes.

Distinct processes of nitrogen cycling were mediated by microorganisms at different abundances. The *nirK* gene (encoding copper-containing nitrite reductase) is widely used as a gene maker for denitrification [58]. In our study, the predominant *nirK*-containing phyla were *Proteobacteria* and *Bacteroidetes* (Figure 5), which is consistent with the findings of Helen et al. [66]. Furthermore, our results indicated there was a positive correlation between *nirK* gene abundance and soil TN contents (Figure 6a). Bu et al. [67] also found that soil TN concentration is the most important indicator of the *nirK* gene. However, there was a negative correlation between soil NO_3^--N contents and *nifH* gene abundance (Figure 6a). *NifH* gene, encoding dinitrogenase reductase, is an important marker in the potential process of soil N fixation [68]. Since the assimilation process of soil NO_3^- competes with nitrogen fixation for carbon sources, it inhibits the progress of nitrogen fixation [69].

5. Conclusions

The study of a 102-year scale of monocultured Chinese fir plantations provides insight into how the composition and functions of rhizosphere microbial community change along with temporal dynamics. Our findings highlight the underlying mechanisms of microbial carbon and nitrogen cycling processes in a long-term chronosequence through a metagenomic profile. The soil nutrient contents had positive effects on carbon and nitrogen cycling processes. Positive correlations and copiotroph microbes were found in the co-occurring networks of the rhizomicrobial community. The concentrations of soil AP had a significant positive correlation on the fungal community. Moreover, soil pH, SMC, and AP showed positive correlations with soil archaeal community. The abundance of most functional pathways, including, carbon fixation and methane metabolism, increased from young-aged stands to mature forest and decreased in over-mature stands, with a recovery in 102-year-old Chinese fir plantations. The functional pathways involved in parasitic and viral infectious disease were enriched in middle-aged stands, but this decreased as the stands aged. The relative abundance of N cycling processes was dominated by assimilatory nitrate reduction to ammonium (ANRA) and dissimilatory nitrate reduction to ammonium (DNRA) pathways. The *nrfA* gene abundance (involved in the DNRA process) reached the highest values in middle-aged plantations and reduced with the age of Chinese fir stands. Thus, extending the rotation and mixing of the stands properly enables complete utilization of the growth factors, thereby realizing more sustainable and higher rates of growth and yield.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f13111906/s1. Table S1: Shannon diversity index of rhizosphere soil microbial communities in different stand ages of Chinese fir plantations in subtropical China; Table S2: Relative abundance of the pathway ko04626 (plant-pathogen interaction); Table S3: Relative abundance of the genus *Streptomyces*; Table S4: Relative abundance of the genus of *Methanosarcina* and *Methanobacterium*; Figure S1: Heatmap of correlation (Pearson's rank) between the relative abundance of the potential functional pathways and soil properties; Figure S2: Random forest (RF) analysis was performed to explore the soil indicators of *Acidobacteria* (a) and *Actinobacteria* (b); Figure S3:

Linear regression analysis between soil nutrient concentrations and the relative abundance of selected microbial phyla.

Author Contributions: S.W.: Formal analysis, Investigation, Validation, Data Curation, Writing—Original Draft; W.C.: Validation; Q.G.: Investigation; C.Z.: Conceptualization, Visualization, Funding acquisition, Resources, Supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the National Natural Science Foundation of China (32071746) and Forestry Peak Discipline Construction Project of Fujian Agriculture and Forestry University (72202200205).

Data Availability Statement: The data is available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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