



Article Tea Plantation Intercropping Legume Improves Soil Ecosystem Multifunctionality and Tea Quality by Regulating Rare Bacterial Taxa

Ting Wang, Yu Duan, Xiaogang Lei, Yu Cao, Lefeng Liu, Xiaowen Shang, Menghe Wang, Chengjia Lv, Yuanchun Ma, Wanping Fang * and Xujun Zhu *[®]

College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China * Correspondence: fangwp@njau.edu.cn (W.F.); zhuxujun@njau.edu.cn (X.Z.)

Abstract: Microorganisms play essential roles in soil-ecosystem multifunctionality. However, the contribution of their community assembly processes, composition, diversity, and keystone species to ecosystem multifunctionality is unclear, especially in tea-plantation ecosystems. In order to assess the effects of various intercropping patterns (tea-plant monoculture and tea plants, respectively, intercropped with soybean, soybean-milk vetch, soybean-red clover, and soybean-smooth vetch) on soil rare and abundant taxa, a field experiment was carried out. We found that tea plantation intercropping with legumes improved the soil-ecosystem multifunctionality by altering the soil environment, and ultimately benefited nutrient absorption and quality improvement of tea leaves. Whether it was in bacteria or fungi, rare taxa had a higher proportion of deterministic processes in community assembly than abundant taxa. Additionally, intercropping practices changed the soil environment, and rare bacterial taxa were assembled and shifted from variable selection to homogeneous dispersal. Intercropping practices significantly changed the bacterial and fungal communities' composition, and rare taxa had higher α -diversity than abundant taxa. Increasing legume species in intercropping practice enhanced community dissimilarity to the tea monoculture by affecting soil pH, ammonium nitrogen, and nitrate nitrogen. Rare bacterial and fungal β-diversity exhibited stronger positive relationships with ecosystem multifunctionality (both average and multithreshold approaches) compared to the corresponding abundant taxa. Furthermore, ecosystem multifunctionality under different intercropping practices was closely related to the keystone rare operational taxonomic units, especially rare bacterial species of Chloroflexi. Our results emphasize the disparate feedbacks of rare and abundant taxa to diverse intercropping practices, as well as the important connection between rare bacterial taxa and ecosystem multifunctionality.

Keywords: rare bacterial taxa; intercropping legume; ecosystem multifunctionality; tea-plantation soil

1. Introduction

Belowground organisms comprise a large fraction of terrestrial biodiversity and are simultaneously influential in multiple ecosystem functions (ecosystem multifunctionality) [1], such as nutrient transformation, plant production, residual decomposition, and climate-altering gas release [2–5]. These functions are closely linked to food supply, water filtration, climate regulation, and other ecosystem services. Soil organisms include larger soil invertebrates and the smaller soil microbiome [1]. Soil bacteria and fungi, as the major groups of microorganisms that contribute most to the soil microbial-biomass pool, can activate soil nutrients through energy flow in the soil food web and fundamentally maintain ecosystem multifunctionality [6]. Both soil bacteria and fungi commonly exhibit unbalanced distributions, containing a great many low-abundance species ("rare taxa") and a small quantity of high-abundance species ("abundant taxa") [7]. Traditional studies focused on abundant soil microorganisms because of their main contributions to microbial biomass and nutrient transformation. Rare taxa have higher biodiversity and functional



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Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). redundancy than abundant taxa and can serve as "functional insurance" in the microbial community [8]. Therefore, the ecological significance of the rare biosphere has been the subject of recent correlative investigations.

The microbial community composition changes with environmental parameters, and the relative abundance of specific species with functional characteristics contributes to the soil ecosystem multifunctionality [9]. In addition, increasing evidence from large-scale observational surveys and field control experiments [10,11] shows that soil biodiversity can enhance ecosystem multifunctionality. Recently, network analysis has been usually applied to reveal complex intra- or inter-kingdom interactions of rare or abundant taxa. Despite their low abundance, certain species play essential roles in ecological networks, such as hubs and connections, and can be classified as keystone species based on their network scores. Keystone species of soil microorganisms are inextricably linked to ecological functions [12]. The rare and abundant groups of soil microorganisms have different structures and functions. To better comprehend the existing microbial community, it is crucial to clarify the processes by which the rare and abundant community assembly works. Deterministic and stochastic processes affect species assembly in communities. Deterministic processes are related to nonrandom and niche mechanisms in which environment filtering and interspecific interactions decide the existence and relative abundance of species [13]. On the other hand, stochastic processes are related mainly to random changes, including random birth, death, and dispersion [14]. A previous study has shown that environmental changes significantly affect the rare and abundant microbial assembly. Agricultural management continuously changes the soil environment, thereby altering the assembly and composition of rare and abundant taxa, leading to uncertain results for ecosystem multifunctionality.

Tea plants (*Camellia sinensis* L.) are a crucial cash crop in various developing countries [15]. The popularity of tea has increased recently because of its unique flavor and health functions, which are attributed to secondary metabolites, including catechins, Ltheanine, caffeine, and terpene volatiles [16]. The synthesis of secondary metabolites in fresh tea leaves is affected by local climate and soil conditions, as well as the cultivation method of tea plantations. To obtain sufficient nutritional components and stable production, tea-plantation soil frequently receives excessive chemical nitrogen fertilizer input, which leads to severe soil-structure damage, soil acidification, and nutrient losses [15,17,18]. Recent research has shown that the change of soil environment caused by excessive nitrogen fertilizer application can cause a drastic decline in microbial diversity. As the proportion of organic fertilizers replacing chemical fertilizers increase, the Chao 1 and Shannon index of soil microbial α diversity increased significantly [19]. In turn, this change can negatively affect ecosystem multifunctionality and transformation of soil nutrients [12]. Developing multiple cropping systems is an effective approach for hindering the degradation of teaplantation soil and ensuring tea-leaf yield and quality [20]. Intercropping is an agricultural management practice in which multiple species are cultivated simultaneously in the same field [21]. Intercropping provides multiple benefits, including maintaining the stability of soil fertility [22], promoting the rhizosphere to improve soil nutrient availability [23], increasing crop productivity [24], and enhancing various agro-ecosystem services [25]. Many legumes, such as soybean (*Glycine max* (L.) Merrill), milk vetch (*Astragalus sinicus* L.), faba bean (Vicia faba L.), and chickpea (Cicer arietinum L.) are increasingly grown as intercropped plants in diversified cropping systems, since they can convert atmospheric nitrogen to plant-available nitrogen due to their capacity to develop symbiotic connections with nitrogen-fixing microorganisms residing in their roots. In addition, the agricultural practice of incorporating legume plants into soil is an effective method to maintain soil productivity [26]. The incorporation of legume plants can alter the soil environment of tea plantations, which in turn influences the microbial community composition and functions, and subsequent nutrient transformations of either newly added or native organic matter [27,28]. Thus, more experimental proof concerning how the agricultural practice of intercropping patterns influence the soil rare and abundant microorganisms and ecosystem

multifunctionality, and their links to tea plant performance is essential for developing sustainable tea plantation cultivation.

We hypothesize that the rare and abundant groups in tea-plantation soil will respond differently to soil property changes caused by intercropping, and the rare species will play more roles in soil ecosystem multifunctionality. To verify these conjectures, a field experiment involving different tea-plantation practices (tea-plant monoculture, tea intercropping with soybean, tea intercropping with soybean—milk vetch, tea intercropping with soybean—red clover, and tea intercropping with soybean—smooth vetch) were carried out. The goals of the research were to: (1) compare the responses of soil properties, ecosystem multifunctionality, and tea-plant performance to different tea-plantation intercropping practices; (2) compare the reactions of rare and abundant taxa community assembly process, composition, diversity, and keystone operational taxonomic units (OTUs) to different tea-plantation intercropping practices; and (3) analyze how the soil multifunctionality in tea plantations is affected by both rare and abundant microbial groups.

2. Materials and Methods

2.1. Experimental Site and Sampling

The field experiment of the tea plantation was located in Hengxi sub-district (31°43' N, 118°44' E), Jiangsu Province, China. The region is characterized by a subtropical monsoon climate, with annual average temperature and precipitation of 16.1 °C and 1116.3 mm, respectively. The soil at this site is classified as Alfisols, developed from weathered granite material. At the initiation of the experiment, the basic soil had pH of 4.59, organic matter of 27.42 g kg⁻¹, total nitrogen (TN) of 2.01 g kg⁻¹, and available phosphorus (AP) of 0.12 g kg⁻¹. Tea plants have been cultivated for 20 years, the variety is Huangshan population, with row spacing of 0.8 m. In the experimental tea plantation, 750 kg ha⁻¹ compound fertilizer was applied in autumn, and 300 kg ha⁻¹ nitrogen was applied before spring tea leaves harvest. Five treatments (each with four replications) were imposed since 2017: tea plant monoculture (TM), tea intercropping with soybean (TI1), tea intercropping with soybean—milk vetch (TI2), tea intercropping with soybean—red clover (Trifolium pratense L.) (TI3), and tea intercropping with soybean—smooth vetch (Vicia villosa var.) (TI4), and each plot was 3 m \times 6 m = 18 m². The varieties of soybean, milk vetch and smooth vetch are Nannong 47, Mingzi 5 and Guangye smooth, respectively. Summer green manure (soybean) was sown in mid-June and incorporated into the soil in late-July (soybean full-bloom stage), and winter green manure (milk vetch, red clover, and smooth vetch) was sown in late-October and incorporated into the soil in late-March of the following year (winter manure soybean full-bloom stage). Before sowing legume green manure, both sides of tea plants were pruned, and two trenches about 5 cm deep should be dug in the soil between the tea-plant rows for sowing green manure seeds. The sowing density of all legumes was 120 kg ha $^{-1}$. Legume green manure should be irrigated in the time after sowing, and the growth period has not fertilized. The picking period of fresh tea leaves in tea plantations is concentrated in spring. Tea leaves and soil were sampled on 7 May 2021, one month after winter legume plants had been incorporated into the soil. One hundred grams of fresh tea leaves with one bud and two leaves were collected, fixed by an 800 W micro-wave oven for 60–80 s, and dried at 60 $^{\circ}$ C to a constant weight. For the following chemical analysis, the dried tea leaves were crushed into a fine powder and stored in a vacuum-drying container. Five soil cores (0–15 cm depth) were collected in each plot according to the five-point sampling method, and then mixed to get a single soil sample per plot. A piece of each soil sample was maintained at 80 °C to extract DNA, a portion was kept at 4 °C to measure enzyme activity and soil inorganic nitrogen, and the last component was air-dried to determine other soil indices.

2.2. Analysis of Tea Plant Performance and Soil Properties

The concentration of total carbon and total nitrogen in tea leaves was determined by elemental analyzer (multi EA 5000, Jena, Germany), and total phosphorus concentration of leaves was measured using H_2SO_4 -digestion and the molybdate-ascorbic acid method [29]. High performance liquid chromatography (GB/T8313-2008) was used to measure the amount of catechins and caffeine, and ninhydrin colorimetry (GB/T8314-2013) was used to assess the amount of amino acids. A pH meter (Five Easy Plus, Mettler Toledo, Switzerland) was used to test the pH of the soil, using a soil:water ratio of 1:2.5 (wt/vol). Soil total carbon (TC) and TN were measured using the elemental analyzer. Following H₂SO₄ digestion, the amount of total phosphorus (TP) in the soil was measured using a photometer [29]. Ammonium nitrogen (NH₄⁺-N) and nitrate nitrogen (NO₃-N) from the soil were extracted with KCl and measured with a continuous flow analyzer (Seal Auto Analyzer AA3, Norderstedt, Germany). NH₄F-HCl was used to extract soil AP, and molybdenum-blue colorimetry was used to determine it [30].

2.3. Approaches for Assessing Ecosystem Multifunctionality

The ability of an ecosystem to perform numerous functions at once is known as ecosystem multifunctionality [10]. To better reflect the effects of intercropping practice on multiple soil processes, seven ecosystem functions related to soil organic matter and nutrient cycling were evaluated for quantifying ecosystem multifunctionality using the average and multi-threshold approaches. In order to better represent the impacts of intercropping practice on various soil processes, seven ecosystem functions linked to soil organic matter and nutrient cycling were assessed for evaluating ecosystem multifunctionality using the average and multi-threshold methodologies. These functions included carbon (α -1,4-glucosidase, β -1,4-glucosidase, β -D-cellobiohydrolase, and β -1,4-xylosidase), nitrogen (leucine amino peptidase and β -1,4-N-acetyl-glucosaminidase) and phosphorus (phosphatase) cycling enzyme activities. As previously mentioned, the fluorescence approach was used to measure the activity of soil enzymes, with a 2.75 g fresh soil [31]. An average ecosystem multifunctionality index was obtained by averaging the Z-score normalized values of each soil enzyme activity [10]. Additionally, the multi-threshold method assesses the relationship between biodiversity and the number of functions that concurrently surpass a critical threshold (from >5% to >90% of the maximum observed level of functioning for a given function) [6].

2.4. Soil DNA Extraction, High-Throughput Sequencing, and Bioinformatic Analysis

The total soil DNA was extracted from 0.5 g of soil samples using a DNeasy PowerSoil Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's protocol, and then stored at -20 °C. Total DNA was sent to Biozeron Co. Ltd. (Shanghai, China) for high-throughput sequencing on an Illumina HiSeq2500 PE250 platform. For bacteria, the V3–V4 region of the 16S rRNA gene was amplified using the primer pair 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). For fungi, the ITS1–ITS2 region amplification primer pair was ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3'). After filtering out singletons and removing chimeras, sequences with similarity >97% were clustered into OTUs. The representative sequence of each OTU was matched to the RDP classifier's reference sequence. The bacteria and fungi information came from the SILVA and UNITE databases, respectively. All unprocessed sequencing data has been uploaded to the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) with accession numbers PRJNA880773 for bacteria and PRJNA881081 for fungi.

2.5. Definition of Rare and Abundant Taxa

We divided all OTUs into six categories, including always abundant taxa (AAT), conditionally abundant taxa (CAT), always rare taxa (ART), conditionally rare taxa (CRT), moderate taxa (MT), and conditionally rare and abundant taxa (CRAT), in order to better understand how rare and abundant communities responded to the practice of intercropping [32]. AAT, CAT, and CRAT were present in the abundant species, while ART and CRT were present in the rare species.

2.6. Statistical Analysis

IBM SPSS 26.0 was used to conduct a one-way analysis of variance to confirm significant variations in enzyme activities, tea-plant performance, soil properties, ecosystem multifunctionality, abundance of phylum/class level bacterial/fungal taxa, and microbial diversity under different intercropping practices. To investigate relationships between tea-plant performance, soil characteristics, and ecosystem multifunctionality, Pearson's correlation analysis was used. For bacterial and fungal communities, the beta nearest taxon index (NTI) and the Raup-Crick metric (RC_{brav}) were employed to assess determinism and stochasticity processes in the rare/abundant taxa [32]. Using the "Picante" package in R, the β NTI between two communities was computed to evaluate the significance of deterministic and stochastic processes: β NTI > 2 denotes a substantially greater than anticipated phylogenetic turnover, which is considered as variable selection for deterministic processes, while $\beta NTI < -2$ denotes a substantially lower than anticipated phylogenetic turnover, which is understood as homogeneous selection for deterministic processes. When β NTI is between -2 and 2, stochastic processes are primarily responsible for the turnover between two communities. $RC_{bray} > 0.95$ and $RC_{brav} < -0.95$ represent the relative influences of dispersal limitation and homogeneous dispersal, respectively. RC_{brav} of -0.95 to 0.95 indicates an undominated process [33]. The "vegan" package in R was used to perform Canonical Correspondence Analysis (CCA), which evaluated relationships between soil characteristics and rare/abundant populations of bacteria and fungi [34]. The Bray–Curtis dissimilarity of each community and the Euclidean distance of ecological multifunctionality were correlated using the Mantel method [35]. Utilizing OTU abundance correlations, the co-occurrence network was built to show the internal organization of the microbial community and the relationships between microbial groups. Additionally, Spearman's rank correlation coefficients between OTUs were determined using the "Hmisc" package in R, and only correlation coefficients with absolute values larger than 0.8 and p < 0.01were considered to be relevant for the network [36]. Gephi 0.9.2 was used to display the co-occurrence networks and to gather topological data on the pertinent node, edge, and average degree values. In terms of within-module connectivity (Zi) and among-module connectivity (Pi), the keystone OTUs in the co-occurrence network were identified. This included network hubs (highly connected nodes in the whole network, Zi > 2.5 and Pi > 0.62), module hubs (highly connected nodes inside the module, Zi > 2.5 and Pi < 0.62), and connectors (highly connected nodes between the two modules, Zi < 2.5 and Pi > 0.62) [37].

3. Results

3.1. Ecosystem Multifunctionality and Correlations with Tea Plant Performance and Soil Properties

Intercropping practices have a considerable impact on the soil's characteristics. Intercropping legumes in this tea plantation increased soil TC, TN, NH_4^+ -N, and NO_3^- -N, and reduced soil pH, TP, and AP. For soil TC and TN, TI4 had the highest value, which significantly differed from that for TM. Additionally, NH_4^+ -N and NO_3^- -N were significantly higher for TI2 and TI3 than TM and TI1 treatment (Table S1). Compared with TM, tea intercropping with legumes improved multiple enzyme activities, involving carbon, nitrogen, and phosphorus cycling. Compared with TM, Treatments TI2 and TI3 significantly increased the activities of the carbon-cycling enzymes β -1,4-glucosidase and β -D-cellobiohydrolase as well as the nitrogen- and phosphorus-cycling enzymes (Figure S1). Furthermore, intercropping legumes also affected tea-plant performance. Treatment TI2 had the greatest improvement effect on total carbon, total nitrogen, and total phosphorus in tea leaves. The amount of total catechins and amino acids in tea leaves was clearly raised by both TI2 and TI3 (Figure S2). The agricultural practice of intercropping legumes in this tea plantation enhanced soil ecosystem multifunctionality. Moreover, with the increase of intercropping species number, ecosystem multifunctionality also improved (Figure 1A). According to Pearson's correlation analysis, ecosystem multifunctionality was related positively to total carbon, total nitrogen, catechins, and amino acids in tea leaves (Figure 1B). In



addition, ecosystem multifunctionality was found to be positively associated to soil TN, NH_4^+ -N, and NO_3^- -N, but negatively with pH and soil TP (Figure 1C).

Figure 1. Ecosystem multifunctionality and its correlated indexes: (**A**) soil ecosystem multifunctionality (*Z*-scores) in different treatments. Pearson correlations of ecosystem multifunctionality with (**B**) tea-plant performance and (**C**) soil properties. Different letters represent significant differences between treatments (p < 0.05). Treatments include tea-plant monoculture (TM), tea intercropping with soybean (TI1), tea intercropping with soybean—milk vetch (TI2), tea intercropping with soybean—red clover (TI3), and tea intercropping with soybean—smooth vetch (TI4). Significant correlations were labeled as follows: ** p < 0.01; * p < 0.05.

3.2. Assembly Processes of Rare and Abundant Taxa

In the bacterial community, 90.55% of the OTU number was rare taxa, accounting for 39.05% of the total sequence number, and the abundant taxa was limited, accounting for 0.34% of the total OTU number and 11.04% of the total sequence number. Similarly with the bacterial community, the OTUs of fungal communities were dominated by rare taxa, only 3.45% of OTUs were classed as abundant taxa and accounting for 72.31% of the total sequences (Figure S3). There were 31.58% of rare bacterial taxa with an $|\beta$ NTI| value more than 2 and 68.42% with an $|\beta NTI|$ value less than 2, showing that stochastic and deterministic mechanisms both had a role in how the community was assembled. All β NTI values for abundant bacterial taxa were between -2 and 2, indicating that stochastic mechanisms were used to build the community. The percentage of BNTI values between -2 and 2 in rare and abundant fungal communities were 94.21% and 96.32%, respectively, showing that stochastic processes were primarily responsible for assembling rare and abundant fungal communities. (Figure 2A). The contributions of five key ecological processes were afterwards assessed with regard to the assembly of rare and abundant species. Variable selection and undominated processes both had a significant impact on the rare bacterial population, but undominated processes had a stronger control for the abundant bacterial community. Undominated mechanisms were largely in charge of both rare and abundant fungal populations. (Figure 2B). Intercropping practices in the tea plantation increased homogenizing dispersal of rare bacteria, and at the same time reduced the proportion of variable selection in ecological processes (Figure S4A). In the groups of abundant bacteria, rare fungi, and abundant fungi, the dispersal limitation effect of TI2 and TI3 treatments was significantly enhanced compared with TM treatment (Figure S4B–D). Mantel tests were performed to investigate the soil characteristics that influenced how rare and abundant taxa of microbes were assembled. For abundant bacteria, TP and $NO_3^{-}-N$ both had high positive correlations with β NTI, and β NTI increased as soil TP and NO₃⁻-N increased. The pH had a significant positive correlation with β NTI for the rare fungal community, indicating that pH change could significantly affect the assembly process. In the abundant fungi community, similar results were observed between soil TC and β NTI, and between soil NH₄⁺-N and β NTI (Table 1).



Figure 2. Assembly processes of microbial rare and abundant taxa: (**A**) Violin plot showing β NTI values of all pairwise rare and abundant bacterial and fungal communities. The lower and upper fine dotted lines represent first and third quantiles, respectively, and the thick dotted lines represent the median; and (**B**) The percentage of rare and abundant bacterial and fungal communities with different assembly processes.

Table 1. Mantel test for the effects of soil properties on β NTI of rare and abundant microbial communities.

	Bacteria				Fungi			
	Rare		Abundant		Rare		Abundant	
Factors	R	р	R	p	R	p	R	р
pН	0.0256	0.3660	0.1068	0.1337	0.2030	0.0438	0.0694	0.2343
TC	-0.0597	0.6840	-0.0194	0.5372	0.1702	0.0878	0.2129	0.0327
TN	-0.0753	0.6789	0.0391	0.3324	0.0200	0.4124	0.0481	0.3234
TP	-0.0884	0.8088	0.2279	0.0161	0.0179	0.4133	-0.0447	0.6592
NH4 ⁺ -N	0.0237	0.3480	0.1358	0.0660	0.0761	0.1903	0.1826	0.0221
NO ₃ ⁻ -N	0.0729	0.1777	0.2798	0.0021	0.0252	0.3508	0.1050	0.0973
AP	-0.0072	0.4873	0.0763	0.1826	0.1188	0.1122	0.0497	0.2760

Note: TC, soil total carbon; TN, soil total nitrogen; TP, soil total phosphorus; NH_4^+ -N, soil ammonium nitrogen; NO_3^- -N, soil nitrate nitrogen; and AP, soil available phosphorus.

3.3. Community Composition and Diversity of Rare and Abundant Taxa

Proteobacteria, Chloroflexi, Actinobacteriota, and Acidobacteriota accounted for 27.17% of all bacterial sequences among the rare bacterial phyla. (Figure 3A). Proteobacteria also predominated in abundant taxa, making up 6.26% of all bacterial sequences (Figure 3B). Compared to TM, TI2 and TI3 treatments considerably reduced the relative abundances of the rare Gemmatimonadota and Myxococcota, but TI4 dramatically increased the relative abundances of Proteobacteria, Bacteroidota, and Patescibacteria (Figure 3A). Compared with TM, intercropping two legume species considerably raised the relative abundance of Chloroflexi abundant bacteria, and also decreased the relative abundance of Actinobacteriota (Figure 3B). Sordariomycetes was dominant in both rare and abundant fungal communities and accounted for 6.66% and 34.29% of the total fungal sequences, respectively (Figure 3C,D). We found a significantly lower abundance of rare Agaricomycetes and *Pucciniomycetes* in intercropping practice compared with TM (Figure 3C). Intercropping two kinds of legumes greatly enhanced the relative abundance of Archaeorhizomycetes for abundant fungal taxa, but significantly lowered the relative abundance of *Pucciniomycetes* (Figure 3D). For bacteria and fungi, rare taxa had larger OTU numbers, Shannon index, and Bray–Curtis dissimilarity values than abundant taxa did (Figure 3E–G). Compared with TM, TI2 and TI3 treatments significantly reduced the rare bacterial OTU numbers, and TI3 significantly decreased the Shannon index. In contrast, intercropping practices obviously enhanced the OTU numbers and α -diversity of abundant bacterial taxa. The intercropping practice did not change the α -diversity of fungal communities as strongly as bacteria, and only the TI4 treatment significantly increased abundant fungi α -diversity of OTU numbers and Shannon index compared with TM (Figure 3E,F). The intercropping practice had stronger effects on Bray–Curtis dissimilarity for the bacterial communities than the fungal communities. Treatments TI2 and TI3 in rare/abundant bacterial taxa had

the highest Bray–Curtis dissimilarity with their corresponding TM treatments. The higher dissimilarity of rare compared to abundant fungal groups indicated that rare fungal groups were more affected by intercropping practices (Figure 3G). The CCA analysis was carried out to look at the soil factors that affect the bacterial and fungal communities. For the rare bacterial, abundant bacterial, rare fungal, and abundant fungal taxa, respectively, two axes of the CCA explained 72.90%, 98.76%, 70.23%, and 68.47% of the total variation (Figure 4). In the rare bacterial taxa, samples from TM and TI1 treatments clustered separately from TI2 and TI3 (Figure 4A). In the abundant bacterial taxa and rare/abundant fungal taxa, the TM treatment was well separated from the intercropping practices (Figure 4B–D). The relationships between soil characteristics and rare/abundant populations of bacteria and fungus were discovered using the Monte Carlo permutation test. Soil pH, NH4⁺-N, and NO3⁻-N had a considerable impact on communities of bacteria and fungi that were both rare and abundant (Table S2).



Figure 3. Composition and diversity of rare/abundant microbial taxa in different treatments. Relative abundance at phylum level (>0.5%) in (**A**) rare; and (**B**) abundant bacterial communities. Relative abundance at class level (>0.1%) in (**C**) rare; and (**D**) abundant fungal communities. Rare and abundant bacterial and fungal communities α -diversity: (**E**) number of observed OTUs; and (**F**) Shannon index in different treatments; and (**G**) pairwise Bray–Curtis dissimilarities between communities in each intercropping practice and monoculture were calculated. Bacterial phylum or fungal class with significantly higher or lower abundance in intercropping practices than monoculture are colored red or blue, respectively (p < 0.05). Boxplot shows median, first and third quartiles, and whiskers are maximum and minimum values. Different letters represent significant differences between treatments (p < 0.05). Treatments include tea-plant monoculture (TM), tea intercropping with soybean—milk vetch (TI2), tea intercropping with soybean—red clover (TI3), and tea intercropping with soybean—smooth vetch (TI4).



Figure 4. Canonical correspondence analysis (CCA) of soil properties and (**A**) rare and (**B**) abundant bacterial; and (**C**) rare and (**D**) abundant fungal community patterns. Treatments include tea-plant monoculture (TM), tea intercropping with soybean (TI1), tea intercropping with soybean—milk vetch (TI2), tea intercropping with soybean—red clover (TI3), and tea intercropping with soybean—smooth vetch (TI4).

3.4. Relationship between Ecosystem Multifunctionality and $\beta\text{-}Diversity$ of Rare and Abundant Taxa

We investigated the association between ecosystem multifunctionality (average approach) and rare/abundant populations of bacteria and fungi using Mantel testing. Rare taxa had a stronger correlation between β -diversity dissimilarity and multifunctionality dissimilarity than abundant taxa did (Figure 5A-D). Ecosystem multifunctionality significantly correlated positively with the rare bacterial (R = 0.549, p < 0.001), abundant bacterial (*R* = 0.432, *p* < 0.001), and rare fungal communities (*R* = 0.405, *p* < 0.001) (Figure 5A–C). This finding suggested that changes in microbial rare and abundant taxa may had varied effects on soil ecosystem multifunctionality. In order to further support our findings, a multi-threshold approach was used to comprehend the connection between rare and abundant microbial taxa and ecosystem multifunctionality. The results of the multi-threshold approach were similar to those of the average approach. The correlation between the β -diversity and the number of functions over threshold were stronger for bacteria than fungi, and stronger for rare than abundant species (Figure S5A–D and Table S3). Rare bacterial taxa had positive correlations with wider functional thresholds (25-95%), whereas abundant bacterial taxa had positive correlations with narrower functional thresholds (25–70%) (Figure S5A,B and Table S3). In contrast, rare fungal taxa only had a significant positive correlation with the number of functions above threshold when the threshold was 30%, 90%, and 95% (Figure S5C and Table S3).



Figure 5. Mantel correlation between ecosystem multifunctionality dissimilarity and (A) rare and (B) abundant bacterial; and (C) rare and (D) abundant fungal community β -diversity dissimilarities. The solid lines represent significant linear regressions, and the dotted line represents the 95% confidence interval of the fit.

3.5. Co-Occurrence Networks and Keystone OTUs of Rare and Abundant Taxa

To evaluate the various co-occurrence patterns of bacteria, fungi, and bacteria-fungi, co-occurrence networks were built. The findings demonstrated that bacteria (3.259) and bacteria-fungi (3.421) had substantially higher average degrees than fungi (2.559) (Figure 6A-C). This indicated that the fungal populations in the tea plantation were less complicated than the bacterial and bacterial-fungal populations. The bacterial network has 508 rare and 10 abundant OTUs, and the positive and negative edges accounted for 84.36% and 15.64% of total edges, respectively (Figure 6A). In the fungal network, there were 157 rare and 22 abundant OTUs, and the proportions of positive and negative edges were 86.46% and 13.54%, respectively (Figure 6B). The network includes 581 bacterial and 193 fungal nodes in the bacterial-fungal community co-occurrence, most of which were rare OTUs. The positive and negative correlations among OTUs accounted for 85.65% and 14.35%, respectively (Figure 6C). Zi-Pi plots were created in order to ascertain the topological properties of bacterial, fungal, and bacterial-fungal network nodes. Module hubs and connections were detected in the bacterial and bacterial-fungal co-occurrences, but network hubs were not detected in any of the three co-occurrences (Figure S6A–C). In the bacterial cooccurrence network, all four keystone OTUs were rare, three nodes (BROTU573, BROTU776, and BROTU3046) were categorized as module hubs, and BROTU160 was identified as a connector (Figure S6A). There were ten rare OTUs and only one abundant OTU in the bacterial-fungal co-occurrence network, eight nodes (BROTU73, BROTU235, BROTU776, BROTU1167, BROTU2071, BROTU3046, FROTU256, and FROTU405) were classified as module hubs, BROTU2899 and BAOTU42 were allocated as connectors (Figure S6C). Intercropping practices obviously changed the relative abundance of keystone OTUs. Treatments TI2 and TI3 had similar OTU relative abundance presentation patterns, which significantly differed from those of TM and TI1. Compared with TM and TI1, TI2 and TI3 had enrichment of BROTU235, BROTU776, BROTU2899, FROTU256, and most keystone OTUs of *Chloroflexi*. As the same time, reduced relative abundance of BROTU1167, BROTU573, BAOTU42, and FROTU405 (Figure 6D). All the rare keystone OTUs discovered by Pi and Zi values were highly connected with ecological multifunctionality, with the exception of the single abundant keystone OUT, suggesting that rare species had a considerable impact on ecosystem multifunctionality. The considerable positive correlation between the OTUs with high relative abundance in TI2 and TI3 and ecosystem multifunctionality suggests that these OTUs may be essential for promoting ecosystem multifunctionality. Significantly negative associations between ecosystem multifunctionality and the OTUs BROTU1167, BROTU573, and FROTU405 were found, and these OTUs had no necessary impact for increasing soil-ecosystem multifunctionality (Figure 6D).



Figure 6. Keystone OTUs and their relationship with ecosystem multifunctionality and soil properties. The co-occurrence networks, average degree, number of nodes, and number of edges in (**A**) bacterial; (**B**) fungal; (**C**) bacterial–fungal communities; and (**D**) Bubble plot showing the relative abundance and taxonomy of the keystone OTUs in each treatment. Spearman correlations between keystone OTUs and ecosystem multifunctionality and soil properties are shown in the heatmap. Node size is proportional to the number of connections (degree), and the keystone OTUs are represented by square nodes. The red edges indicate positive interactions between two nodes, and green edges indicate negative interactions. The bubble size represents the relative abundance. Treatments include tea-plant monoculture (TM), tea intercropping with soybean—red clover (TI3), and tea intercropping with soybean—smooth vetch (TI4). The BROTU, BAOTU, and FROTU represent rare bacterial, abundant bacterial, and rare fungal OTUs, respectively. The colors in the heat map represent Pearson's correlation coefficients, with red representing positive and blue representing negative. Significant correlations are labeled as follows: ** *p* < 0.01; * *p* < 0.05.

4. Discussion

4.1. Intercropping Practices Affect Soil Ecosystem Multifunctionality and Tea Plant Performance

Our results showed that intercropping legumes significantly enhanced the soil multifunctionality of a tea plantation and improved the nutrient content and quality characteristics of fresh tea leaves. This is consistent with previous findings that intercropping patterns have important effects on multiple ecosystem functions in field soil and can improve the yield and quality of main crops [38]. Intercropping legumes can affect ecosystem multifunctionality by changing the soil environment. We found that intercropping legumes reduced the pH of tea-plantation soil from 4.96 to 4.41, which remained within the reasonable range of healthy growth of tea plants and could directly or indirectly affect ecosystem multifunctionality. Numerous earlier research studies have demonstrated the critical roles that soil pH plays in controlling the soil ecosystem multifunctionality, but there is still substantial disagreement over whether this has positive or negative impacts. [10,39]. Our results suggest that a reduction in soil pH contributes to enhanced ecosystem multifunctionality, possibly due to the adaptability of microorganisms in tea-plantation soil to acidic habitats. Legumes can provide nitrogen to the soil through biological nitrogen fixation during their growing period, thus increasing the nitrogen-supply capacity of the soil to the main crop [40]. In addition, legumes decompose rapidly when incorporated into the soil, increasing the contents of fresh organic matter and many nutrient elements in soil [41]. Therefore, the nutrients added by intercropping may be responsible for the increased ecosystem multifunctionality. Many agroecosystem experiments have shown that increasing crop biodiversity can enhance various ecosystem functions [42,43]. This conclusion is in line with our findings; that is, with the increased number of intercropped legumes, the soil ecosystem multifunctionality was also enhanced. The mechanism of this biodiversity effect is resource complementarity and niche partition, which occurs when species in an intercropping system have unique traits and requirements and leads to greater resource capture than that of a monoculture species [43]. In addition, if there is too much overlap between the resource requirements of intercropped legumes and tea plants, this may have a lower promoting or even an inhibitory effect, which also partially explains why the improvement effect of TI2 and TI3 on ecosystem multifunctionality was stronger than for TI4. Carbon, nitrogen, and phosphorus are essential elements for tea-plant performance, among them, carbon is essential for the growth and development of plants [44], nitrogen can promote amino acid synthesis and endow tea infusions with "umami" taste [45], and phosphorus is conducive to the formation of tea polyphenols and has antioxidant capacity [46]. Our data indicated that intercropping practices could improve the nutrient content and quality of fresh tea leaves by enhancing the soil ecosystem multifunctionality and can increase amino acid and catechin contents while reducing caffeine, which is a sustainable tea-plantation management practice.

4.2. Intercropping Practices Alter the Assembly of Rare and Abundant Taxa

Understanding the existence of rare and abundant bacterial and fungal populations in tea plantations requires calculating the potential contribution of deterministic and stochastic mechanisms to microbial assembly. Our findings showed that the assembly process of rare bacterial taxa was dominated by variable selection and undominated processes, while those of abundant bacterial, rare fungal, and abundant fungal taxa were entirely dominated by undominated processes. This outcome is in line with other research demonstrating that rare bacterial and fungal taxa are more controlled by deterministic processes than abundant bacterial and fungal taxa [47]. Compared to TM, stochasticity predominated in the construction of the rare bacterial population in the tea plant and legume intercropping, and the additional input of nutrients in the intercropping system boosted the proportion of stochastic processes in the rare bacterial assembly. This is consistent with previous reports that stochastic processes became more prominent as soil-resource availability increased [48]. Both rare and abundant taxa showed greater dispersal limitations in fungal than bacterial communities, and this can result from the variations in the bodies and lifestyles of bacteria and fungus. Soil fungi are unicellular or multicellular organisms that are much larger than bacteria [49], and it might reduce their ability to disseminate in the soil environment. In order to better comprehend the process by which microbial communities are assembled, work must be done to determine the relative contributions of environmental elements. We discovered that the soil properties related to community assembly of stochastic and deterministic processes differed for the rare and abundant bacteria and fungi. The TP and NO₃⁻-N were linked to the β NTI values of abundant bacteria. The possible explanation is that most rare bacteria in the present study had enough intracellular nitrogen and phosphorus storage, and thus were insensitive to nitrogen and phosphorus nutrients. Additionally, compared to other soil variable, changes in pH and TC were more significantly related with the β NTIs of rare and abundant fungi. This suggests that ecological niches of rare and abundant fungi are not the same, and abundant fungi could be resistant to environmental pH and tolerant of large variations. In conclusion, diverse environmental factors had an impact on how rare/abundant taxa gathered in bacterial and fungal populations.

4.3. Intercropping Practices Alter Composition and Diversity of Rare and Abundant Taxa

Previous studies showed a significant difference between the rare and abundant microbial populations under plant residue returned to soil and intercropping [50]. Similarly, our results indicated that intercropping practices resulted in a remarkable shift in rare/abundant taxa for bacterial and fungal communities. We found that regardless of rare or abundant taxa, Proteobacteria, Chloroflexi, and Actinobacteriota were the main bacterial phyla in tea-plantation soil, similar to previous results [12,27]. Compared with TM, treatment TI4 significantly increased the relative abundance of Proteobacteria in rare and abundant bacterial communities. Many studies have shown that microorganisms with nitrogen-fixation ability belong to Proteobacteria [51], and increasing the amount of these bacteria in soil is crucial to raising the amount of nitrogen that tea plants can use. Actinobacteriota have the ability to decompose soil organic matter and play an essentla role in soil carbon cycling, but their effect on plant residue decomposition is more prominent in less fertile soils [52], which may explain their lower abundance in the abundant bacterial community under the intercropping practices. In addition, *Chloroflexi* are phototrophic bacteria, which could participate in degrading plant residue via starch and cellulose degradation pathways [53]. As a result, it is not surprising that tea-plant intercropping with two legumes in our study increased *Chloroflexi* in the abundant bacterial community. Similarly, the intercropping practice also significantly changed the composition of the rare and abundant fungal communities. In the tea plantation, Sordariomycetes was the main class of rare and abundant fungi. The relative abundance of Agaricomycetes and Pucciniomycetes (both belong to Basidiomycota) decreased significantly during intercropping practices. Previous investigations have shown that *Basidiomycota* play a significant part in the degradation of lignin-rich plant residue [54]. As fresh organic matter, incorporated legumes will reduce the proportion of lignin in tea-plantation soil, which may further limit *Basidiomycota* growth. Archaeorhizomycetes belongs to Ascomycota, and most members are saprotrophic decomposers of organic matter and play important roles in nutrient and carbon cycling [55]. Therefore, intercropping may greatly improve the relative abundance of *Archaeorhizomycetes* and enhance the soil carbon, nitrogen, and phosphorus cycles. Previous studies found that rare bacterial and fungal populations had significantly higher levels of α -diversity than abundant populations [56]. In comparison to TM, the bacterial communities of the intercropped tea plant and soybean—milk vetch/red cover showed the biggest alterations, which may have been caused by changes in the soil's pH, TP, NH_4^+ -N, and NO_3^- -N levels. The CCA analysis and Monte Carlo permutation test between soil characteristics and bacterial populations supported that the abundant bacterial populations was more impacted by soil pH and the nitrogen and phosphate levels. Compared to the rare bacterial population, the abundant bacterial population was more sensitive to changes in the soil environment brought on by intercropping practices. Similar findings from earlier research showed that

rare bacteria had broader environmental adaptation than abundant bacteria [57]. Previous research has found that the rare fungal community exhibited potentially narrow environmental adaptation compared to the abundant community [58]. In line with earlier findings that the rare fungal community was more sensitive to the soil environment than the abundant fungal community, our results showed that the Bray–Curtis dissimilarity of the rare fungal community varied more between TM and intercropping practices than that of the abundant community. At the same time, CCA analysis and Monte Carlo permutation test results in our study also showed that pH, NH_4^+ -N, NO_3^- -N, and AP had a greater impact on rare compared to abundant fungal communities. Previous study showed the critical roles of pH and NO_3^- -N in the regulation of fungal community in tea plantations [3].

4.4. Rare Taxa Play a More Important Role in Soil Ecosystem Multifunctionality of Tea Plantation

Given that the composition and diversity of the rare and abundant microbial communities differs, we explored the relative contributions to ecosystem multifunctionality of these communities for bacteria and fungi. The correlation between ecosystem multifunctionality and rare bacterial and fungal communities was found to be stronger than that between abundant communities using both average and multi-threshold methods. Previous studies also found that rare bacteria and fungi with lower abundance played an over-proportional role in multiple ecosystem functions and were identified as the main drivers of ecosystem multifunctionality [59]. Many rare species have similar functions and are not necessary to maintain ecosystem functions, therefore, the functional importance of the rare microbiome may be due to its "insurance effect" [8], enabling them to respond quickly to external environment changes and so maintain ecosystem function. Moreover, rare species of microorganisms may become important in providing unique functions to ecosystems under favorable conditions. For example, in this study, intercropping practices did not increase α -bacterial diversity, possibly because of functional redundancy, but did enhance ecosystem function. Rare microorganisms, as a reservoir of genetic resources, can provide additional and special functions under intercropping practices. We found that intercropping practices enhanced ecosystem multifunctionality and were associated with the evolution of some keystone OTUs. In this study, 12 keystone OTUs were identified, of which only BAOTU42 was abundant, and the others were all rare. These rare species were significantly positively correlated with ecosystem multifunctionality, further demonstrating that rare, rather than abundant species, played an irreplaceable role in ecosystem multifunction [60]. According to the distribution of keystone OTUs under different treatments, tea-plantation intercropping could trigger the variation in specific keystone OTUs. For instance, the intercropped tea plants with soybean—milk vetch/red cover led to the enrichment of four keystone OTUs, BROTU73, BROTU160, BROTU2071, and BROTU3046. They are all members of the *Chloroflexi* and which all showed a strong positive correlation with ecological multifunctionality. There have been several reports of *Chloroflexi* species (important species in carbon cycling) enrichment in soil from tea plantations [53]. Additionally, there were notable positive associations between the abundances of 12 keystone OTUs and soil TN, NH_4^+ -N, and NO_3^- -N, but a negative connection with pH. These results suggest that intercropping practice affected soil pH and nitrogen, activating the growth of keystone OTUs associated with ecosystem multifunctionality. More focus should be put on the research of rare taxa in relation to ecosystem functions as it is still unknown what ecological role rare microbial communities play in tea plantations.

5. Conclusions

In conclusion, we investigated the impacts of intercropped tea plantations on soil rare and abundant microbial communities. The findings confirmed that tea intercropping with soybean—milk vetch and with soybean—red clover was more conducive to enhancing ecosystem multifunctionality and improving tea-plant performance. The intercropping practice, by changing soil properties, could determine the assembly processes of rare and abundant taxa, and result in taxonomic and functional transformation. Both average and multi-threshold approaches showed that the rare taxa played a more important role in determining ecosystem multifunction than abundant taxa. The significant correlations between ecosystem multifunctionality and keystone OTUs indicated that intercropping practice shifting the abundance of rare keystone OTUs was the vital driver in shaping ecosystem multifunctionality in tea-plantation soil. Altogether, adopting tea plant intercropping with legumes has potential to enhance soil ecosystem multifunctionality in tea plantations, where the underlying microbial mechanisms are dominated by rare taxa (especially rare bacteria).

Supplementary Materials: The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/agronomy13041110/s1, Figure S1: Levels of multiple soil functions based on enzyme activities in the different treatments; Figure S2: Heat map showing the changes in tea performance (Min-Max normalization value) including total carbon content, nutrient content, and secondary metabolite content in tea leaves under different treatments; Figure S3: Proportion of six categories of microbial taxa to the overall OUTs and sequence numbers; Figure S4: The percentage of rare and abundant bacterial, rare and abundant fungal communities with different assembly processes.; Figure S5: The relationships between the number of functions beyond threshold and rare/abundant bacterial/fungal community Bray–Curtis dissimilarities.; Figure S6: Zi-Pi plot shows the distribution of OTUs based on their topological roles for bacteria, fungi, and bacteria-fungi; Table S1: Soil properties under different intercropping practices in tea plantations; Table S2: Impacts of edaphic factors on rare and abundant communities (Monte Carlo permutation test); Table S3: R^2 and p values corresponding to the regressions in Figure S5.

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