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Soil Microbial Biomass and Bacterial Diversity Enhanced through Fallow Cover Cropping in Rice–Fish Coculture

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Abstract: Traditional rice production is often reliant on the unsustainable practice of utilizing intensive inputs in monoculture cropping systems. Alternatives fallow cover cropping and rice–fish coculture (RFC) offer promising solutions. However, the potential of fallow cover cropping in RFC remains underexplored, and its impact on soil microbes is poorly understood. In this study, assessments of soil–plant–microbe interactions were conducted across three cover cropping systems: Chinese milk vetch (*Astragalus sinicus* L.) single cropping (CM), Rapeseed (*Brassica napus* L.) single cropping (RP), and a combination of Chinese milk vetch and rapeseed intercropping (CM_RP). These systems were evaluated with and without nitrogen (N) addition, encompassing both the RFC and rice monoculture (RMC) systems. The findings indicate a notable increase in soil microbial biomass nitrogen (MBN) with CM. Soil microbial biomass carbon (MBC), influenced more by N-fertilizer than crop species, decreased with N addition. In the RFC system, the soil bacterial co-occurrence network exhibited more connections, yet negative links increased. CM_RP displayed similarities to CM without N but shifted closer to RP with N addition. N addition in intercropping significantly increased the root–shoot ratio (R/S) of *A. sinicus*, associated with decreased aboveground biomass and total root length. Compared to RMC, RFC with N addition reduced the relative abundance of *Anaerolineaceae* in CM while increasing *Bacillus* and *Pontibacter* across cover cropping systems. Overall, with N addition, both RFC and RMC showed decreased soil bacterial diversity indices. Changes in soil bacterial diversity correlated significantly with soil MBC, MBN, and plant R/S. Continuous fallow cover cropping altered soil microbial biomass and affected cover crop biomass distribution, impacting bacterial composition in paddy soil. These results shed light on how bacterial communities respond to N addition and fallow cover cropping in RFC and RMC systems, offering insights for sustainable nutrient management in paddy systems.

Keywords: cover crop; intercropping; nitrogen addition; root–microbe interaction; paddy system

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1. Introduction

Sustainable agricultural practices are crucial for conserving the biodiversity of agro-ecosystems [1,2]. However, compared to natural ecosystems, the species diversity and stress resistance of agro-ecosystems are low due to the excessive use of chemical fertilizers and pesticides, leading to soil aggregate structure degradation and decreases in the levels of beneficial organisms, such as earthworms and plant-growth-promoting rhizobacteria [3]. To preserve biodiversity and increase the resilience of agro-ecosystems, traditional ecological agricultural models, such as the rice–fish coculture system (RFC), have gained popularity [4,5]. Previous studies have highlighted RFC's potential to meet carbon peak and neutrality goals by reducing chemical inputs, curbing nutrient loss, and optimizing

resource allocation during the summer rice season [6]. However, the impact of RFC on crop production and agro-ecosystem biodiversity during the winter season remains unclear.

Fallow cover cropping, especially intercropping, represents a viable approach to enhancing agro-ecosystem species diversity. In intercropping systems, the roots of different plant species directly interact, producing root exudates that contain higher concentrations of organic compounds like sugars, amino acids, and carboxylic acids [7]. Higher concentrations of organic nutrients provide adequate carbon sources for the reproduction of rhizosphere microflora and enhance the metabolic activity of soil microbes, thus promoting the diversification of soil microbial community [8]. Microbial functionalities such as carbon fixation pathways in prokaryotes, the citrate cycle of bacteria, and wood saprotrophs of fungi are upregulated in intercropping systems [9]. Among various intercropping models, the Chinese milk vetch–rapeseed intercropping system exhibits a significant improvement in sustainable productivity in the middle and lower reaches of the Yangtze River in China. Previous rice monoculture-based studies have reported that intercropping with Chinese milk vetch reduces the carbon utilization rate and microbial metabolic activity in the rhizosphere soil of rapeseed, altering its microbial community structure and reducing diversity [10,11]. Additionally, rapeseed intercropping offers notable advantages, displaying root exudates akin to those observed in rapeseed monocultures [12,13]. While the mechanisms governing crop interactions in intercropping systems have been widely reported, the specific role of intercropping in the RFC system and the impacts of cultivation patterns, nitrogen (N) addition, and crop species remain inadequately understood. A thorough understanding of how fallow cover cropping and N addition impact soil microbes in the RFC system is pivotal, as it not only reveals intricate soil–plant–microbe interactions but also expands the applications of cover crops in the RFC system. By addressing the knowledge gap on the soil legacy effect and exploring the mechanisms governing soil–plant–microbe feedback during winter fallow, this study provides novel insights for sustainable paddy field management. Optimizing fallow cover crop configurations and implementing artificial interventions like N addition offer practical approaches to bolster the stability and sustainability of the RFC system.

In the RFC system, investigating the effects of cover cropping and N addition on crop production and biodiversity conservation might entail augmenting agro-ecosystem root–microbe interactions. Recently, specific root–microbe interactions have been identified in several cover crop species within natural systems [14–16]; however, comprehending the intricate community interactions and trophic relationships among living organisms in agro-ecosystems is essential. Insights into the impacts of intercropping systems and N addition on root–microbe interactions are critical for improving food production sustainability and maintaining productivity. The functional attributes of plant roots and soil microorganisms are pivotal in anticipating agro-ecosystem responses to human interventions. Plants exhibit mutualistic relationships with various organisms, including fungal endophytes, mycorrhizal fungi, and growth-promoting bacteria, which are all pivotal drivers of root–microbe interactions [17–19]. While species-specific soil pathogens and root herbivores might curtail crop yield in agro-ecosystems, they facilitate plant succession and biodiversity maintenance [20,21]. Long-term excess tillage and fertilization in agro-ecosystems reduce microbial biomass and disrupt interaction networks, resulting in soil nitrogen leaching and detrimental feedback on plant productivity [4,22]. While many studies have focused on the impact of root–microbe interactions on crop productivity, the correlation between soil microbial transformation and root morphogenesis, along with the effects of tillage practices on root–microbe interactions, remains unclear. Therefore, comprehending the pivotal role of soil organisms in influencing root–microbe interactions' dynamics and strength is vital for leveraging these organisms as efficient management tools within the RFC system.

The objective of this study was to assess and compare the impacts of fallow cover cropping and N addition on soil microbial biomass, soil bacterial diversity, and the distribution of aboveground and belowground cover crop biomass in the rice–fish coculture (RFC) and rice monoculture (RMC) systems. A comprehensive analysis of root–microbe

interactions in three cropping systems was conducted, employing two soil cultivation practices (RFC and RMC) with and without N-fertilizer addition. The hypotheses were as follows: (1) soil microbial biomass without N addition would surpass that with N addition, and the variation will be influenced by diverse fallow cover cropping methods in the RFC system; (2) cover crop management practice is expected to exert more pronounced effects on shaping soil bacterial communities in RFC compared to RMC, considering its multifunctionality in improving soil conditions; and (3) interspecific belowground interactions will play a pivotal role in altering soil bacterial diversity and reshaping spatial distribution characteristics under cover cropping. The findings illustrate that implementing cover cropping and optimizing N fertilizer management in paddy fields not only reduces reliance on chemical inputs but also enhances agro-ecosystem service functions, thereby contributing significantly to biodiversity conservation and sustainable agriculture. Additionally, potential application scenarios are provided for farmers to develop adaptable coculture models and employ diverse cover cropping techniques, promoting more resilient and environmentally friendly farming practices.

2. Materials and Methods

2.1. Experimental Design

A mesocosm experiment was performed in a greenhouse to analyze the impacts of distinct soil sources, intercropping, and N fertilization on soil composition, nutrient availability, bacterial diversity, and root morphology. Three cropping systems were established: Chinese milk vetch (*Astragalus sinicus* L.) single cropping (CM), Rapeseed (*Brassica napus* L.) single cropping (RP), and Chinese milk vetch and rapeseed intercropping (CM_RP). In the single cropping approach, *A. sinicus* or *B. napus* were planted at a density of 6 or 3 plants per pot, respectively. For intercropping, *A. sinicus* and *B. napus* were planted at densities of 4 and 2 plants per pot, respectively. The plant density was set considering field planting habits and aiming to make optimal use of the limited mesocosm area. Two distinct concentrations of N-fertilizer were applied: N0 (0 mg kg^{-1}) and N1 (100 mg kg^{-1}), which corresponded to 0 kg N hm^{-2} and 225 kg N hm^{-2}, respectively, in accordance with local agricultural practices. Twelve treatment combinations were established from the three factors, and each treatment comprised three replicates.

The experiment was conducted in a greenhouse located at Zhuanghang Experimental Station of Shanghai Academy of Agricultural Sciences (SAAS), Shanghai, China (30°88′ N, 121°38′ E). The greenhouse was maintained at temperatures ranging from 20 to 25 °C during the day and 6 to 11 °C at night, with the air relative humidity at around 60%–80%. Throughout the growth period, there was exposure to daylight for 10.5–11.2 h per day, with a light intensity of approximately 400 μmol m^{-2} s^{-1}. Soil samples (calcareous alluvial soil; soil depth: 0–20 cm) were collected from the standard rice rooting zone at a long-term rice rotation experiment site situated on Chongming Island, Shanghai, China (31°77′ N, 121°26′ E), in October 2020 during the rice harvest season for both rice–fish coculture (RFC soil) and conventional rice monoculture (RMC soil). Upon arrival at the SAAS, the samples were immediately air-dried. Subsequently, the soil was crushed using an industrial mill, sieved with a mesh size of 2 mm to break macro aggregates, and the remaining stones were removed prior to milling. Microcosms were prepared in circular pots (16.5 cm diameter at the top, 10 cm at the bottom, and 15.2 cm high) filled with 1.5 kg of air-dried soil. The initial RFC soil parameters were as follows: pH, 8.05; total organic carbon (TOC) content, 10.2 g kg^{-1} soil; available N (AN) content, 66.8 mg kg^{-1}; available P (AP) content, 22.5 mg kg^{-1}; available K (AK) content, 180.2 mg kg^{-1}; total dissolved salt (TDS), 1.98%; and 16S rRNA gene copies, 2.95×10^9 g^{-1} soil. The initial RMC soil parameters were as follows: pH, 7.82; total organic carbon (TOC) content, 9.54 g kg^{-1} soil; available N (AN) content, 102.0 mg kg^{-1}; available P (AP) content, 38.7 mg kg^{-1}; available K (AK) content, 161.3 mg kg^{-1}; total dissolved salt (TDS), 1.86%; and 16S rRNA gene copies, 2.24×10^9 g^{-1} soil. The application concentrations of P and K in all treatments were set at 112.5 kg hm^{-2} and 225 kg hm^{-2}, respectively. N, P, and K fertilizers were applied

in the form of calcium nitrate tetrahydrate (N% = 11.86), monopotassium phosphate (P% = 22.79 and K% = 28.68), and potassium sulphate (K% = 44.83), respectively. Additionally, to ensure adequate nutrient supply for plant growth, the soil samples were fertilized with the following basal nutrients (mg pot^{-1}): $Ca(NO_3)_2{\cdot}4H_2O$, 1687; K_2SO_4, 200; $MgSO_4{\cdot}7H_2O$, 65; Fe-EDTA, 8.78; $MnSO_4{\cdot}H_2O$, 10; $ZnSO_4{\cdot}7H_2O$, 15; $CuSO_4{\cdot}5H_2O$, 3; H_3BO_3, 2; and $Na_2MoO_4{\cdot}5H_2O$, 0.25.

The seeds of *A. sinicus* and *B. napus* were obtained from Shanghai Nongle Planting Co., Ltd. (Shanghai, China). To ensure sterility, the seeds underwent surface sterilization using a 30% v/v H_2O_2 for 20 min. They were then rinsed thoroughly with deionized water, immersed in a $CaSO_4$-saturated solution for 12 h, and subsequently placed on wet filter paper within Petri dishes for 1 day at 25 °C to facilitate germination. All pots were arranged in a completely randomized design and rerandomized weekly throughout the experiment. Soil moisture was maintained at 18% (w/w), determined gravimetrically by daily weighing of each pot during the experiment period [23].

2.2. Soil Sampling

After 70 days of continuous incubation in the greenhouse, plants were harvested, and soil samples were collected. Roots were meticulously extracted from the pots, gently shaken to eliminate loose soil, and the remaining tightly adhering soil around the roots was defined as rhizosphere soil. Approximately 5.0 g of rhizosphere soils was collected by shaking roots gently for 3 min into a bag and mixing thoroughly. Simultaneously, around 1.0 kg of bulk soils from the pots was collected and homogenized by sieving through a 4 mm mesh. The collected soil samples were then separated for further analysis: (1) bulk soil samples were air-dried for analysis of their basic physicochemical properties, and (2) rhizosphere soil samples were stored at −20 °C for subsequent high-throughput gene sequencing and real-time quantitative polymerase chain reaction (qPCR) analysis.

2.3. Measurements

2.3.1. Plant Biomass Estimation and Root Analyses

Plant shoots and roots were harvested and separated 70 days after planting. Before preparing for the biomass measurement, roots were washed in deionized water and scanned using an EPSON root scanner at a 400 dots-per-inch resolution (Epson Expression 1600 pro, Seiko Epson Corporation, Nagano, Japan). Total root length (TRL) was calculated using the Win-RHIZO 2017a (Regent Instruments Inc., Quebec, QC, Canada) [24]. Shoots and roots were oven-dried at 105 °C for 30 min, followed by an additional drying period at 65 °C for 3 days, and they were ultimately weighed to determine dry biomass.

2.3.2. Soil Physicochemical Properties

Soil chemical properties, including TOC, nitrate nitrogen (NIN), ammonium nitrogen (AMN), AP, AK, TDS, and pH, were assessed using the methods described by Lu [25]. TOC content was determined using the potassium dichromate method. NIN content was determined using the KCl extraction–ultraviolet spectrophotometry method. AMN content was determined using the indigo colorimetric method. AP and AK contents were determined using spectrophotometry. Soil pH was determined in a 1:2.5 (soil/water) aqueous suspension using a pH meter (FE28, Mettler Toledo, Giessen, Germany). Soil TDS was determined using the gravimetric method. Soil microbial biomass carbon (MBC) and nitrogen (MBN) were measured using the chloroform fumigation method [26].

2.3.3. Soil DNA Extraction and Bacterial Community Analysis

Bacterial DNA was extracted from around 0.5 g of the rhizosphere soil samples using the FastDNA Spin Kit for Soil, and stored at −20 °C. The V3–V4 region of the 16S rRNA gene was amplified from the isolated bacterial DNA using the primer set 338F/806R in an ABI GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction (PCR) was performed as follows: 3 min of denaturation at 95 °C, 27 cycles of

30 s at 95 °C, 30 s of annealing at 55 °C, and 45 s of elongation at 72 °C, and a final extension occurred at 72 °C for 10 min. PCR was performed in triplicate with a reaction mixture (20 µL) containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA. Quality and concentration of the extracted DNA were assessed using 1% agarose gel electrophoresis and the NanoDrop™1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Illumina pair-end library preparation, cluster generation, and 250 bp pair-end sequencing were conducted by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

2.3.4. qPCR

The impact of intercropping and N-fertilizer on bacterial abundance in the soil samples was assessed via qPCR. The qPCR mixture (25 µL) comprised 12.5 µL of Maxima SYBR green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania), 1 µL each of both primers (338F/806R, 5 µM), 5 µL of template DNA, and 5.5 µL of ddH_2O. The specificity of the qPCR amplicons was confirmed through melting curves and gel electrophoresis. For all the experiments, negative controls without template DNA were subjected to the same qPCR procedure. Gene abundance in each reaction was calculated based on the constructed standard curves and converted to copies per gram of soil, assuming 100% DNA extraction efficiency.

2.3.5. Structural Equation Modeling (SEM) Equations

SEM analysis was conducted to elucidate the direct and indirect pathways through which abiotic and biotic soil properties influenced plant growth and its response to N-fertilizer, following the expectations outlined in a priori models. Maximum likelihood estimation was employed to fit SEM using IBM SPSS Amos 21.0 software (Amos Development Corporation, Chicago, IL, USA). Models were considered to fit well when $0 \leq$ Chi-squared/df ≤ 2, as well as when the p-value > 0.05 [27].

2.3.6. Statistical Optimization and Biometric Analysis of Sequencing Data

Purified amplicons were equimolarly pooled and subjected to paired-end sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) following standard protocols provided by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Raw FASTQ files were quality filtered using Trimmomatic (version 0.33) and merged using FLASH (version 1.2.7). Operational taxonomic units (OTUs) were clustered using a 97% similarity threshold in the UPARSE pipeline (version 7.1). After high-throughput sequencing and optimization, 2,261,796 sequences with 939,596,193 bps were obtained for the twelve treatments (n = 36); the average sequence length was 415.4 bp. The OTU representative sequences for the bacteria were identified using the Silva (SSU123) 16S rRNA database (https://www.arb-silva.de/, accessed on 22 February 2021). Sequence data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP415876.

2.4. Statistical Analysis

A one-way analysis of variance was used to examine the effects of soil cultivating patterns, N-fertilizer levels, and cropping systems on the shoot biomass, N uptake, and root morphology (including root biomass and root length) of RP or CM. Post hoc mean comparisons were conducted using the least significant difference (LSD) test at a 5% probability level ($p \leq 0.05$) via SPSS statistical software (SPSS version 21.0, IBM SPSS Inc., Chicago, IL, USA). Following the verification of data normality through the Shapiro–Wilk test, all data were normalized for other analyses using z-score transformation. Student's *t*-tests were employed to compare data between *A. sinicus* or *B. napus* treatments with and without N addition. QIIME (1.7.0) software was used to calculate the alpha and beta diversity of bacteria. Principal co-ordinates analysis (PCoA) was performed using the vegan data package in R software (version 3.5.1, Lucent Technologies, Holmdel, NJ, USA).

3. Results

3.1. Soil Physiochemical Properties

The analyses revealed that cultivation patterns, N addition, and fallow cover cropping systems significantly affected NIN, MBC, and MBN (microbial biomass nitrogen). The interaction among soil cultivation patterns, N addition, and cropping systems had a more pronounced effect on MBN (F = 177.378, $p \leq 0.001$) and NIN (F = 43.890, $p \leq 0.001$) compared to MBC (F = 8.917, $p = 0.006$) and TOC (F = 0.618, $p = 0.440$). In the RFC system, soil MBN significantly increased in both CM and CM_RP treatments without N addition (Table 1, Table S1). Soil NIN notably increased in both RFC and RMC systems with N addition. Soil AP was significantly higher in RFC than in RMC.

Table 1. Effects of different treatments on soil carbon and nitrogen in paddy soil.

Source	Variable	F	*p*	Source	Variable	F	*p*	Source	Variable	F	*p*
Soil (S)	NIN	128.328	**<0.001**	S ∗ C	NIN	667.168	**<0.001**	S ∗ C ∗ N	NIN	43.890	**<0.001**
	AMN	0.597	0.447		AMN	89.684	**<0.001**		AMN	2.963	0.098
	MBC	16.510	**<0.001**		MBC	14.408	**<0.001**		MBC	8.917	**0.006**
	MBN	142.914	**<0.001**		MBN	92.677	**<0.001**		MBN	177.378	**<0.001**
	TOC	65.733	**<0.001**		TOC	1.264	0.301		TOC	0.618	0.440
Crop (C)	NIN	188.467	**<0.001**	N ∗ C	NIN	1257.417	**<0.001**				
	AMN	17.639	**<0.001**		AMN	59.995	**<0.001**				
	MBC	43.835	**<0.001**		MBC	18.746	**<0.001**				
	MBN	309.256	**<0.001**		MBN	31.657	**<0.001**				
	TOC	7.437	**0.003**		TOC	4.350	**0.024**				
Nitrogen (N)	NIN	6122.326	**<0.001**	S ∗ N	NIN	4817.469	<0.001				
	AMN	44.391	**<0.001**		AMN	26.534	**<0.001**				
	MBC	109.748	**<0.001**		MBC	7.213	**0.013**				
	MBN	243.445	**<0.001**		MBN	142.935	**<0.001**				
	TOC	27.439	**<0.001**		TOC	1.759	0.197				

Note: General linear model (*p*-values) for determining the effects of rice cultivating pattern (S), nitrogen addition (N), and cover cropping system (C) on physiochemical properties of the paddy soil. Significant *p*-values (≤0.05) are shown in bold. NIN, nitrate nitrogen; AMN, ammonium nitrogen; MBC, microbial carbon; MBN, microbial nitrogen; TOC, total organic carbon.

3.2. Characterization of Bacterial Communities and Co-Occurrence Networks

The bacterial community composition exhibited no significant variation between the RFC and RMC soils. In both soil types, the predominant phyla, including Proteobacteria, Actinobacteria, Chloroflexi, and Acidobacteria, collectively accounted for 78.55–81.40% of all sequences (Figure S1). PCoA revealed that both cultivation practices and N addition had more substantial influences on soil bacterial composition than the type of cropping system. The bacterial composition was akin to that between CM and CM_RP soils without N addition (Figure S2), suggesting that CM was more competitive than RP without N addition. However, with N addition, the inter-specific competition between CM and RP shifted, resulting in a closer resemblance of the CM_RP microbial community composition to that of RP.

Detailed analysis at the genus level revealed greater differences among the three cover cropping systems (Figure 1). In the RFC system, the abundance of the genus norank_f_Anaerolineaceae in CM_RP was 59.28% and 17.33% higher than that in CM and RP, respectively, with N addition. Similar trends were observed for the genus Pontibacter. Without N addition, norank_f_Anaerolineaceae increased significantly in CM (5.35%), while in RP and CM_RP, it increased up to 2.91% and 2.70%, respectively. In the RMC system without N addition, the abundance of norank_f_Anaerolineaceae and norank_f_Actinobacteria in CM and CM_RP was significantly higher than in the RP samples.

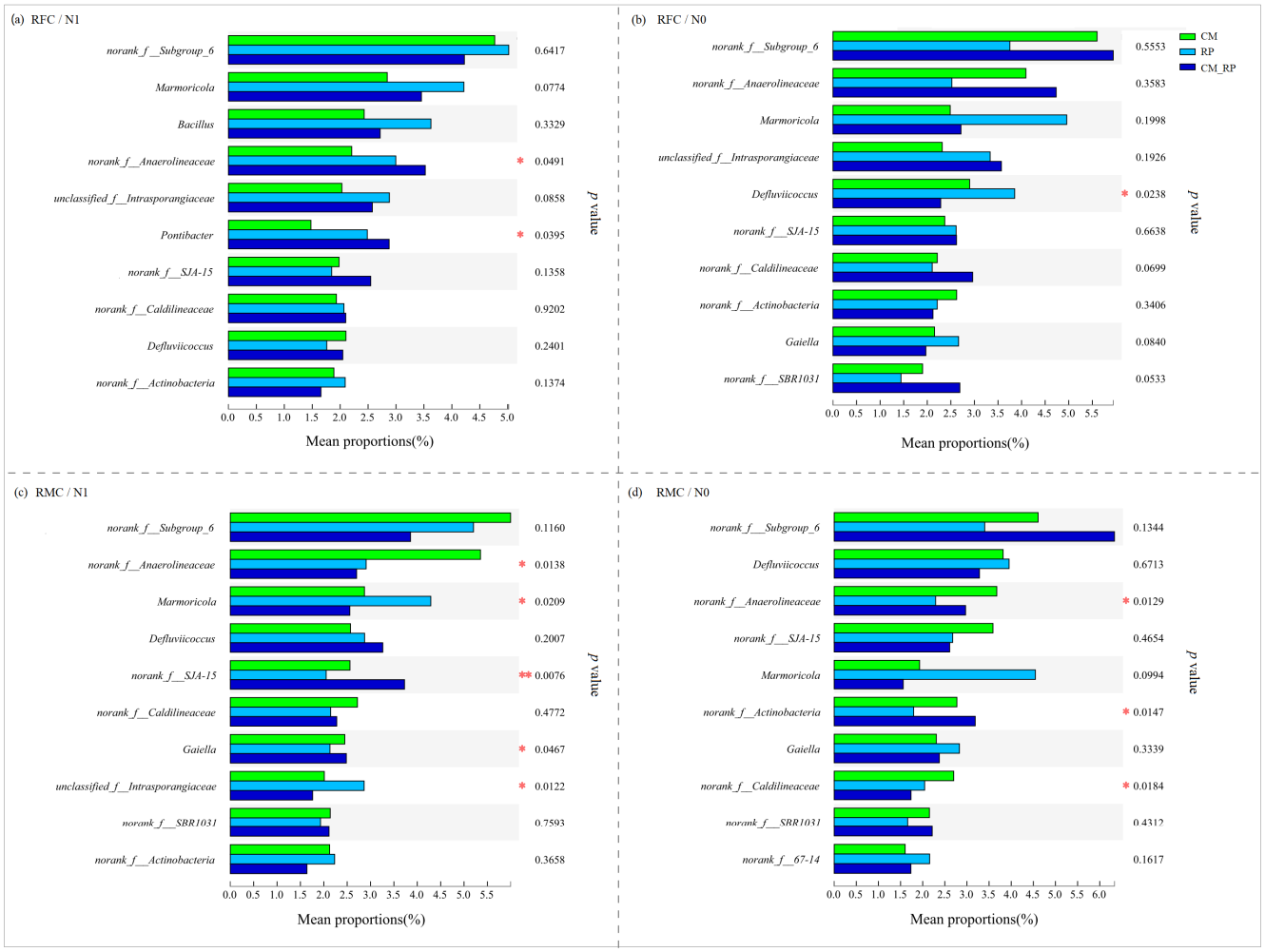

Figure 1. Comparative analysis of the bacterial community at genus level in soils with different cultivation systems (subgraph (**a**): rice–fish coculture soil with N addition; (**b**): rice–fish coculture soil without N addition; (**c**): rice monoculture soil with N addition; (**d**): rice monoculture soil without N addition; legend CM: single cropping of *Astragalus sinicus*; RP: single cropping of *Brassica napus*; CM_RP: intercropping of *A. sinicus* L. and *B. napus* L.; *, $p \leq 0.05$; **, $p \leq 0.01$).

We constructed bacterial association networks to assess overall effects on soil bacterial interactions in both RFC and RMC. The feedback response had a significant impact on these networks. In the RFC network, total bacterial edges were 42.7% higher compared to the RMC network, indicating enhanced connections and closer relationships (Figure 2). The feedback response also led to notable changes in the ratio of positive (red line) to negative (green line) correlations. The number of positive correlations exceeded the number of negative correlations in both networks, yet the positive links in RFC were 23.97% fewer than those in RMC. These results suggested that while soil bacterial associations were more closely linked in RFC, competitive inhibition was higher. Additionally, N addition and intercropping significantly affected the alpha diversity of bacterial communities in both RFC and RMC systems (Figure 2). Specifically, the Shannon diversity index of soil bacteria in RFC decreased with N addition, showing varied suppression effects among different cover cropping systems. Moreover, the Chao1 diversity index indicated that CM exhibited significantly higher diversity than RP and CM_RP without N addition ($p \leq 0.05$).

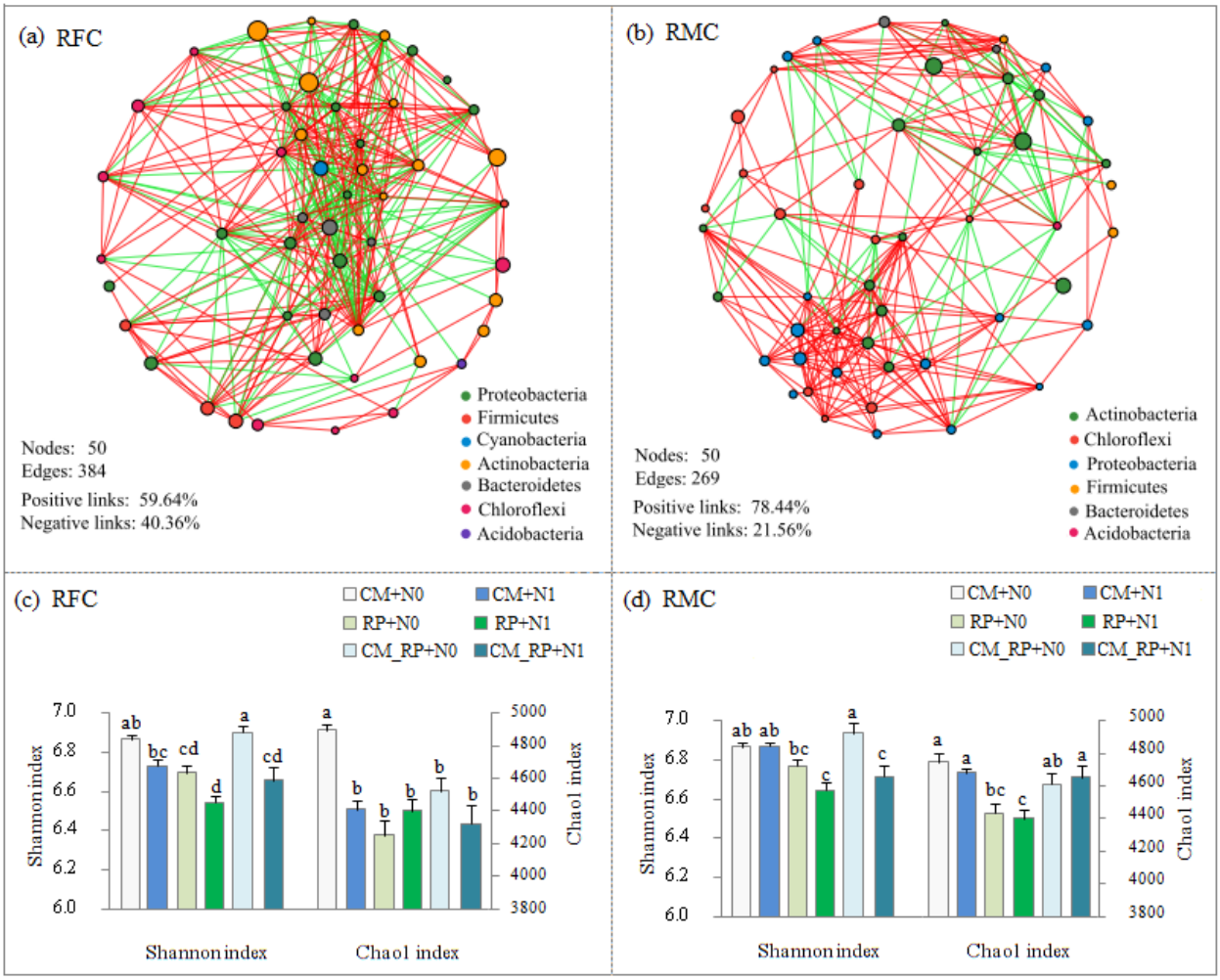

Figure 2. Co-occurrence networks and diversity indices of soil bacterial communities (subgraph (**a**): co-occurrence networks of rice–fish coculture soil; (**b**): co-occurrence networks of rice monoculture soil; (**c**): conventional soil with nitrogen (N) addition; (**d**): conventional soil without N addition; legend CM + N0, single cropping of *Astragalus sinicus* without N addition; CM + N1, single cropping of *A. sinicus* with N addition; RP + N0, single cropping of *Brassica napus* without N addition; RP + N1, single cropping of *B. napus* with N addition; CM_RP + N0, intercropping of *A. sinicus* and *B. napus* without N addition; CM_RP + N1, intercropping of *A. sinicus* and *B. napus* with N addition. A connection represents a strong correlation for the rice–fish coculture soil and conventional soil fractions. The co-occurring networks are colored by phylum. For each panel, the size of the node is proportional to the degree of connections. Red lines indicate a positive interaction between two individual nodes, while green lines indicate a negative interaction. Lowercase letters denote significant difference between treatments).

3.3. Plant Biomass and Root Morphology

N addition in both RFC and RMC systems inhibited the development of belowground parts of A. sinicus (Figure 3), with significantly lower root dry biomass compared to conditions without added N ($p \leq 0.05$). However, no significant effect of N addition was observed on the dry biomass of the aboveground parts of A. sinicus in both the RFC and RMC systems ($p > 0.05$). Interestingly, intercropping mitigated the inhibition of A. sinicus belowground growth caused by N addition. The observed trends in the RFC system were consistent with those in the RMC system.

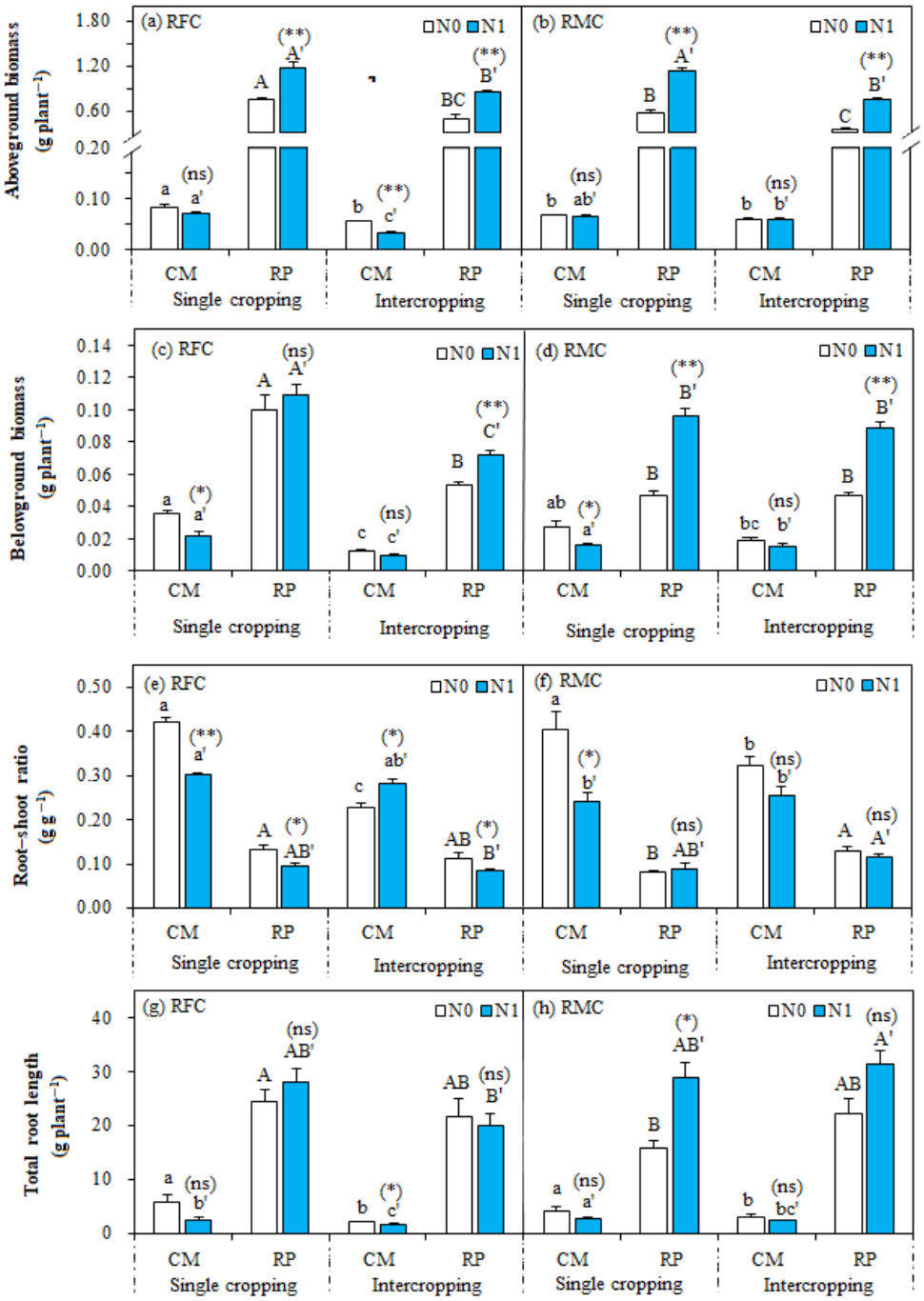

Figure 3. Effects of nitrogen (N) addition and cover cropping system on crop growth. (subgraph (**a**,**c**,**e**,**g**): crop aboveground biomass, belowground biomass, root–shoot ratio and total root length for rice–fish coculture soil; subgraph (**b**,**d**,**f**,**h**): crop aboveground biomass, belowground biomass, root–shoot ratio and total root length for rice monoculture soil. CM: *Astragalus sinicus*; RP: *Brassica napus*. Each value is the mean of four replicates (+SE). Two-factor randomized block analysis was used to analyze the significant differences in plant aboveground biomass of *A. sinicus* or *B. napus* between single cropping and intercropping. Capital letters denote significant differences among *B. napus* in single cropping or intercropping without N addition and capital letters with single quotes (′) denotes significant differences in *B. napus* with N addition. Lowercase letters denote significant difference in *A. sinicus* without N addition. Lowercase letters with single quotes (′) denote significant differences in *A. sinicus* with N addition. *, $p \leq 0.05$; **, $p \leq 0.01$; ns, $p > 0.05$).

The total root length (TRL) and the root–shoot ratio (R/S) were significantly influenced by the interaction of N addition and cover cropping (Figure 3). Specifically in the RFC system, B. napus had a TRL of 28.2 m plant^{-1} with N addition and 24.5 m plant^{-1} without N addition, while A. sinicus had TRL values of 2.5 m plant^{-1} with N addition and 5.9 m plant^{-1} without N addition. Furthermore, in the RFC system, the R/S of B. napus was 221.28% lower than that of A. sinicus with N addition and 218.94% lower without N addition. The R/S of plants in different cover cropping systems was significantly affected by N addition. N addition significantly reduced R/S of A. sinicus and B. napus in single cropping, but it increased in the case of intercropping. The effect of N addition on the belowground growth of A. sinicus varied between the CM_RP treatments in the RFC and RMC systems. In the RFC system, N addition proved to be more beneficial for the belowground growth of A. sinicus in CM_RP, whereas the opposite trend was observed in the RMC system. On the other hand, for B. napus, N addition stimulated aboveground growth and led to a decrease in R/S in both RFC and RMC systems.

In the RFC system, the R/S of A. sinicus and B. napus decreased by 39.40% and 40.43%, respectively, due to N addition in single cropping. However, a significant decrease in the belowground growth of plants in the CM_RP treatment was observed only in B. napus. Notably, for A. sinicus in CM_RP, there was a significant decline in aboveground biomass ($p \leq 0.05$), followed by a significant increase in R/S ($p \leq 0.05$). The result indicates that intercropping in the RFC system reduced the suppressive effect of N addition on the R/S of A. sinicus through the redistribution of aboveground and belowground biomass and the reshaping of root morphology.

3.4. Correlations between Soil Properties and Plant Growth Parameters

The correlations between soil properties and plant growth parameters exhibited considerable variability between the RFC and RMC systems (Table 2). Within the RFC system, significant correlations ($p \leq 0.05$) were observed among soil MBC and MBN, Shannon diversity index, plant R/S, and root dry weight (RDW). Notably, R/S and RDW showed strong correlations with soil AMN, pH, bacterial gene copies, and plant TRL. Conversely, in the RMC system, the previously observed negative correlation between MBN and RDW and the positive correlation between MBC and R/S were not statistically significant ($p > 0.05$). However, a significant positive correlation was found between bacterial gene copies and soil NIN content in the RFC system. Additionally, significant negative correlations were noted between bacterial gene copies and soil AMN and MBC in the RMC system ($p \leq 0.05$).

Table 2. Pearson's correlation matrix for 12 traits in soil and plant samples under rice–fish coculture cultivation (lower-left diagonal) and rice monoculture cultivation (upper-right diagonal) treatments.

Traits	NIN	AMN	MBC	MBN	pH	TOC	RDW	R/S	RTL	Shannon Index	Gene Copies
NIN	-	−0.09	−0.05	0.26	0.33	−0.45	−0.45	0.13	−0.41	0.00	0.48 *
AMN	0.52 *	-	0.29	0.21	0.37	−0.63 **	0.07	−0.40	0.21	0.34	−0.50 *
MBC	−0.51 *	0.32	-	0.29	−0.43	−0.44	−0.74 **	0.39	−0.60 **	0.57 *	−0.61 **
MBN	−0.35	0.40	0.78 **	-	0.20	−0.61 **	−0.22	0.46 *	−0.05	0.50*	0.00
pH	−0.14	−0.36	−0.36	−0.39	-	−0.18	0.35	−0.51 *	0.39	−0.34	0.42
TOC	0.27	0.19	0.05	−0.05	−0.23	-	0.47 *	−0.25	0.28	−0.68 **	0.25
RDW	−0.34	−0.76 **	−0.56 *	−0.55 *	0.57 *	−0.44	-	−0.50 *	0.95 **	−0.53 *	0.12
R/S	0.08	0.67 **	0.70 **	0.81 **	−0.65 **	0.12	−0.84 **	-	−0.46 *	0.47 *	−0.05
RTL	−0.41	−0.72 **	−0.46 *	−0.48 *	0.61 **	−0.29	0.86 **	−0.85 **	-	−0.41	0.00
Shannon index	−0.33	0.31	0.71 **	0.57 *	−0.42	0.43	−0.62 **	0.53 *	−0.40	-	−0.34
Gene copies	−0.36	−0.42	−0.13	−0.37	0.59 **	−0.45	0.57 *	−0.46 *	0.43	−0.30	-

Note: NIN, nitrate nitrogen; AMN, ammonium nitrogen; MBC, microbial carbon; MBN, microbial nitrogen; TOC, total organic carbon; RDW, root dry weight; R/S, root–shoot ratio; RTL, root total length; Shannon index, Shannon diversity index for 16S rDNA genes; Gene copies, gene copies for 16S rDNA. *, $p \leq 0.05$; **, $p \leq 0.01$.

3.5. Mechanisms of Soil Bacterial Community and Plant Growth to N and Intercropping

To elucidate the effects of N addition and cover cropping treatments on plant growth and soil bacterial diversity, structural equation modeling (SEM) was employed. The pathways involving soil bacterial abundance and diversity, R/S, and shoot dry weight (SDW) responding to N addition and cover cropping treatments differed between the RFC and RMC systems (Figure 4a,b). Notably, the intercropping treatment exhibited a more pronounced effect on soil MBC, MBN, NIN, plant R/S, and SDW, as well as soil bacterial diversity and abundance compared to the N addition treatment. These findings aligned with the results obtained from the analysis of variance (ANOVA) (Table 1). Moreover, the standardized total effect values for cover cropping on the SDW were 0.743 and 0.794 in the RFC and RMC systems, respectively. In contrast, the effect values for N addition on the SDW were −0.215 and 0.108 in the two systems, respectively (Figure 4c,d), indicating that plant aboveground growth responded more significantly to the cover cropping treatment than to N addition in both systems.

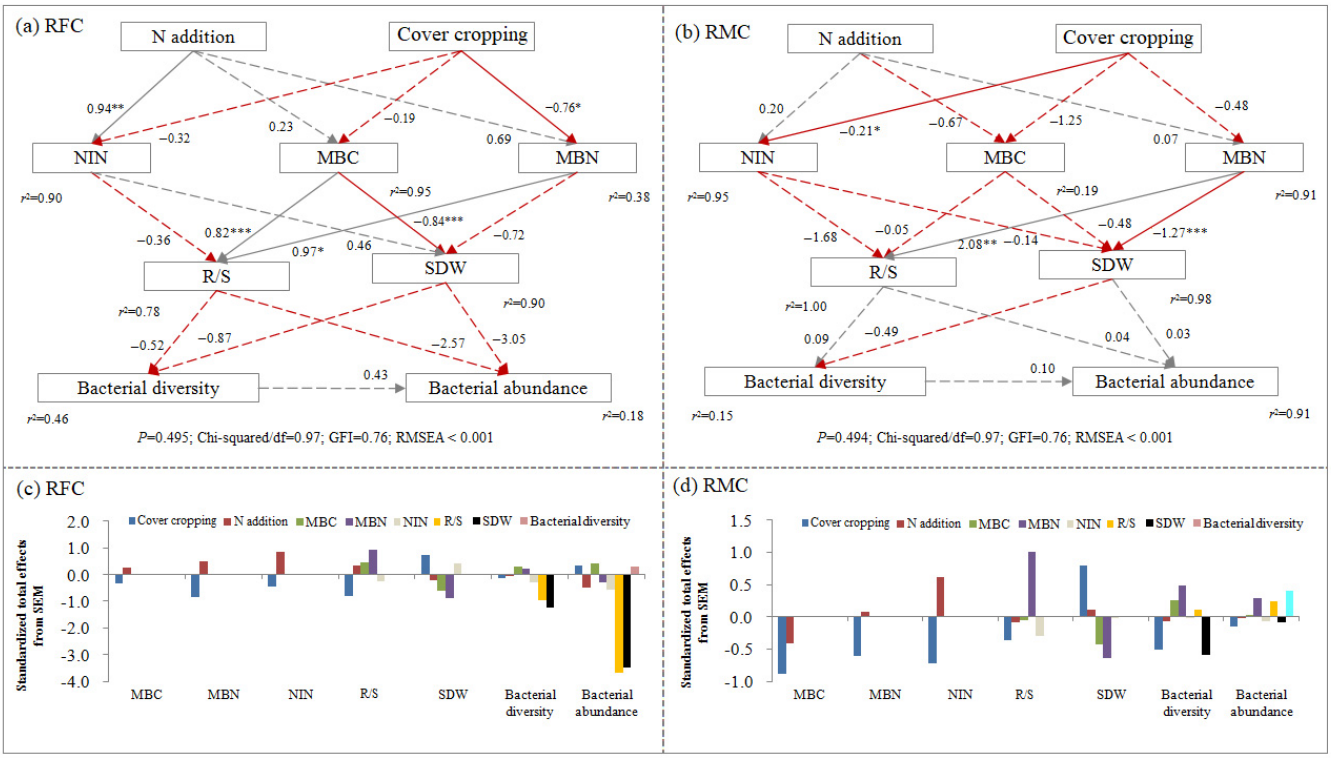

Figure 4. Structural equation models (SEMs) based on abiotic and biotic links in the rice–fish coculture soil (**a**) and rice monoculture soil (**b**); standardized direct and indirect effects from SEMs in the rice–fish coculture soil (**c**) and rice monoculture soil (**d**). Numbers adjacent to arrows represent covariances. Gray and red arrows indicate positive and negative relationships, respectively. r^2 values indicate the proportion of variance explained for each variable. Significance levels are denoted with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. The model satisfactorily fitted to the data as suggested by the Chi-squared/df values. GFI, Jöreskog's goodness of fit index; RMSEA, the root mean square error of approximation; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; NIN, nitrate nitrogen; R/S, root–shoot ratio; SDW, shoot dry weight.

The standardized total effects values of R/S on bacterial diversity and abundance were 7.79 and 14.63 times greater in RFC compared to RMC soil, respectively (Figure 4c,d). Similar trends were observed for the effect of the SDW on bacterial diversity and abundance. This indicates that plant growth had a greater influence on the soil bacterial community in the RFC system. The model elucidated 46% and 18% of the variability in bacterial

diversity and abundance within the RFC system, and 15% and 91% within the RMC system, respectively.

4. Discussion

4.1. Cover Crop Species Composition Altered Soil Microbial Biomass and Nutrient Availability in the RFC System

Intercropping brassicas with legumes has been reported to enhance brassica yield by promoting root interactions and amplifying soil microbial biomass and functional activity in the brassica rhizosphere [12,13]. However, the observations indicated that in the RFC system, intercropping did not bolster aboveground biomass accumulation for either of the two crop species (Figure 4). This study demonstrates that cover crop species differ in their abilities to increase soil nutrient and microbial biomass, suggesting that RFC might intensify interspecific competition between cover crop species. Particularly noteworthy is the substantial increase in soil MBC and MBN associated with the cultivation of *A. sinicus* (Table S1). Intriguingly, intercropping with *B. napus* reversed the inhibitory effects of *A. sinicus* on soil TOC, AP, and AK. These findings support our first hypothesis and emphasize the importance of cover crop species composition in formulating fallow cover cropping strategies for the RFC system.

Soil microbial biomass, commonly regarded as an indicator of soil biological activity, plays a pivotal role in nutrient cycling, organic matter decomposition, and mineral transformation processes [28]. Previous studies of RFC systems have demonstrated the maintenance of highly efficient nutrient transformation by increasing soil microbial biomass and ensuring microbial community stability [29,30]. The leguminous species *A. sinicus* has been widely acknowledged for its ability to enhance N utilization and prevent soil degeneration owing to its extensive root systems and legume nodules [31]. The findings confirm that cover cropping with *A. sinicus* accelerated N recycling, thereby increasing soil N availability, consistent with previous reports [32]. Previous studies have highlighted that cover crops may secure soil available nutrients by forming and stabilizing macroaggregates as well as by reducing soil nutrients leaching [33–35]. Additionally, various cover crop species may offer distinct qualities of C and N resources, resulting in discrepancies in supporting microbial growth, proliferation, and metabolic activity [36–39]. Soil C sequestration is facilitated alongside N promotion due to heightened microbial activity in the rhizosphere of *A. sinicus*. Simultaneously, the extensive root system improves soil permeability and releases organic acids, showcasing *A. sinicus*'s potential to enhance the soil availability of C, P, and K [29]. Considering the substantial root density development of *A. sinicus*, the elevated nutrient contents in paddy soil might effectively meet the nutritional requirements for cover crop growth, potentially reducing reliance on supplemental chemical fertilizers in the RFC system.

4.2. N Addition Inhibited Microbial Carbon Biomass in the RFC System

Previous studies have indicated that when soil nutrients sufficiently meet microbial stoichiometric requirements, microbial activities increased, and C and N mineralization was stimulated [40,41]. Contrary to these findings, this study observed a decrease in MBC and MBN with N addition in all cases, except for *B. napus* when used as a single cover crop. This decline is primarily attributed to the accumulation of soil N induced by N addition, resulting in an imbalanced C:N:P ratio [42,43], leading to a reduction in soil microbial community diversity, particularly affecting functional microbial communities related to the N cycle. Single cover cropping the cruciferous crop *B. napus* with N addition increased both aboveground biomass and soil MBN by meeting its high N demands. In the RFC system, it was observed that intercropping mitigated the inhibitory effect of N addition on the belowground biomass of *A. sinicus*, whereas an opposite trend was noticed for aboveground biomass. The results highlighted how intercropping cultivation management, with or without N addition, might regulate the root–shoot ratio through interspecific competition [32,44]. Compared to the RMC system, the RFC system demonstrated relatively

more stable interspecific competition concerning soil microbial biomass in intercropping with N addition, supporting our second hypothesis. While this study suggests that suitable fallow intercropping practices enhance soil nutrient availability and crop complementarity, it is crucial to consider additional parameters when deciphering root–microbe interactions. As interactions among each component rely on cropping system optimization, resource-use efficiency, disease resistance, and other related factors [19,20], future research should explore the underlying mechanisms used to improve paddy soil productivity. Enhancement in species cooperation can be achieved in the RFC system through crop rotation and intercropping. Therefore, further research endeavors can facilitate the optimization of the RFC system to restore agro-ecosystems post anthropogenic disturbance, paving the way toward ecologically sustainable and environmentally friendly rice production.

4.3. Cover Cropping Had a Greater Impact on Restructuring Soil Bacterial Communities in the RFC System Compared to the RMC System

In this study, the correlation network of the soil bacterial community for the RFC system exhibited more connections compared to the RMC system (Figure 2). Furthermore, it was observed that the soil bacterial community characteristics in intercropping were more akin to those of *A. sinicus* single cropping without N, rather than with N addition (Figure S2). These results are in accordance with previous observations, emphasizing that efficient cultivation management fosters positive plant–soil feedback interactions, temporally and spatially enhancing productivity by facilitating belowground rhizosphere microflora [14,20,24,45,46]. Investigating root–rhizosphere microbe interactions between *A. sinicus* and *B. napus* intercropping is crucial for developing strategies to manage rhizosphere, thereby enhancing crop productivity and nutrient-use efficiency in the RFC system. *A. sinicus* and *B. napus*, belonging to different green manure families, exhibit distinctive characteristics that could result in variable effects on the abundance and diversity of the rhizosphere soil bacterial community. The relative abundance of *Anaerolineaceae* and *Pontibacter* significantly differed among cover cropping patterns in the RFC system with N addition (Figure 1), while *Defluviicoccus* showed a significant difference without N addition. Prior research has highlighted the involvement of *Anaerolineaceae* in a methanogenic alkanes-degrading consortium [47]. Moreover, *Pontibacter* has been associated with potential nitrogen-fixing activity [48], while *Defluviicoccus* might face inhibition in high nitrite concentrations and compete with phosphate-accumulating organisms during anaerobic polyhydroxyalkanoates synthesis [49].

It was observed that, compared to *B. napus*, the root proliferation of *A. sinicus* was inhibited by intercropping (Figure 3), consequently leading to a reduction in soil bacterial diversity (Figure 2) and the diminishing of the advantage of root–shoot allocation (Figure 3). These observations provide evidence that root–microbe interactions between *A. sinicus* and *B. napus* were pivotal in managing intercropping, influencing the microbial composition in the rhizosphere, supporting our third hypothesis. Given the diverse effects of different cropping systems and fertilization regimes on nutrient utilization efficiency and soil microbial community diversity [41], optimizing agriculture management strategies is essential for improving the production capacity of paddy soil and exploiting positive root–microbe interactions. This finding supports that cover cropping with different green manure species during winter fallow, with or without N addition, profoundly affects rhizosphere processes related to N fixation and P activation in the RFC system. In recent decades, climate change has led to cycles of soil drying and moistening, potentially increasing nutrient loss and carbon emissions from rice paddies [50,51]. However, the effectiveness of cover cropping in mitigating climate-induced nutrient loss remains uncertain, and data on the contribution of fallow seasons to global paddy emissions are lacking. For future research, it is advisable to explore high-throughput multiomics approaches to investigate root exudate dynamics and nutrient signaling networks. Additionally, exploring rhizosphere microbial assembly, interaction, functioning, and diversification at regional and global scales is pivotal for advancing the understanding of C, N, and P cycling in agro-ecosystems.

5. Conclusions

Understanding soil biotic and abiotic factors in the rice–fish coculture system is crucial for enhancing rice field management and advocating for sustainable land utilization. This study reveals that cover crop species and N-fertilizer management have diverse effects on microbial properties under different soil conditions. Interestingly, it was observed that cover crops without N addition generally enhance root–microbe interactions by augmenting microbial biomass and bacterial diversity compared to those with N addition. Irrespective of the cover crop species, N addition emerged as a primary factor shaping the soil bacterial community and altering root–microbe interactions. These findings propose a promising model for advancing sustainable agriculture, particularly in regions practicing rice cultivation in conjunction with fish farming. Further investigation is warranted to delve deeper into the ecological implications of cover cropping on soil legacy and the underlying mechanisms governing nutrient cycling within this traditional rice–fish coculture agro-ecosystem.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14030456/s1, Table S1: Effects of different treatments on soil physiochemical properties in the rhizosphere of *Astragalus sinicus* and *Brassica napus*; Figure S1: Comparative analysis of the bacterial community at phylum level in soils with different cultivation systems; Figure S2: PCoA analysis on OUT level for the bacterial community structures in the soils using the method of unweighted_unifrac distance algorithm.

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