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Effects of Intercropping and Nitrogen Application on Soil Fertility and Microbial Communities in Peanut Rhizosphere Soil

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Abstract: The intercropping of peanuts and sugarcane is a sustainable planting model that deserves in-depth research. For this study, two variables, i.e., intercropping status (peanut monocropping or sugarcane/peanut intercropping) and the level of nitrogen fertilization (low, medium, or high), were evaluated to analyze the effects of intercropping and nitrogen application on soil fertility and microbial communities in peanut rhizosphere soil. These analyses revealed that higher nitrogen application led to increased total nitrogen (TN), available nitrogen (AN), and soil organic matter (OM) levels in rhizosphere soil for both monocropped and intercropped peanuts, with a decrease in pH. Monocropped peanuts had higher TN, total phosphorus (TP), and total potassium (TK) levels compared to intercropped peanuts at the same nitrogen level but lower AN content and pH levels. The diversity of microbial communities in the rhizosphere soil of intercropped peanuts was significantly higher than that of monocropped peanuts under high levels of nitrogen fertilizer application. Higher levels of *Gemmatimonadetes* abundance were observed in intercropping rhizosphere soil, compared to that associated with peanut monocropping under low, middle, and high levels of nitrogen fertilizer application, whereas the opposite trend was observed for *Chloroflexi* abundance. *Nitrospira* abundance levels rose gradually in the monocropping treatment group, whereas the opposite trend was evident under intercropping conditions. Further analyses of nitrogen cycle-related genes demonstrated higher levels of nitrogen conversion cycle activity in intercropping peanut rhizosphere soil under low nitrogen levels, whereas nitrogen transformation cycle activity levels were higher in monocropping peanut rhizosphere soil under high levels of nitrogen amendment. It can be concluded that intercropping and nitrogen fertilizer application change the physical and chemical properties of soil, thus affecting the diversity and function of soil microbial communities in the peanut rhizosphere. These results offer a theoretical foundation for more efficient sugarcane/peanut intercropping systems.

Keywords: peanut; intercropping; nitrogen fertilizer; soil fertility; soil microorganisms

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1. Introduction

Soil microbes are well-established as key regulators of a range of agroecological processes, facilitating the transformation of soil nitrogen, organic matter, and other nutrients to address the needs of plants [1,2]. They also play a role in material cycling and nutrient conversion within the soil microcosm [3–5]. Microorganisms with soil nitrogen-fixing, nitrifying, and denitrifying activity, for instance, serve as key mediators in the transformation and cycling of soil nitrogen through the processes of nitrogen fixation, ammoniation, nitrification, and denitrification [6–9]. Soil microbes are also capable of breaking down organic matter in the soil, facilitating its decomposition and conversion such that it can serve as a rich source of nutrients [10]. Studies to date have demonstrated that soil microbial community composition and diversity are closely associated with the physicochemical properties

of soil [11,12], with these properties, in turn, reflecting the fertility status of soil [13,14]. Thus, studies of the structural diversity and function of soil microbial communities have the potential to yield greater insight into changes in the microbiological characteristics of the soil.

Nitrogen fertilizer is widely applied in agricultural settings with the goal of increasing crop yield [15]. However, nitrogen fertilizers can have broader effects beyond the promotion of plant growth, impacting the microbial communities present within the rhizosphere. Indeed, nitrogen fertilizer amendment has been shown to alter the nutrient content and pH levels of soil [16,17], thereby altering the physicochemical properties of the soil in a manner that impacts the composition and diversity of the microbial communities therein [18–21]. Despite research focused on the effects of nitrogen fertilizer on soil microbes, information regarding the structural and functional characteristics of rhizospheric soil microflora associated with leguminous crops, and how these change in response to nitrogen fertilizer application and the intercropping of leguminous and gramineous species, remains limited.

Intercropping is a strategy where two or more crops are planted in close proximity to one another during the same growing season on the same area of land [22]. Intercropping alters land use and the functional and structural characteristics of the soil microbiota [23,24]. Prior studies have demonstrated that the intercropping of corn and peanuts can alter the community composition and diversity of rhizospheric soil microbes associated with these plants while enhancing the abundance of nitrogen-fixing microbial species within the rhizosphere [25–27]. Soil microbe functioning can also be influenced by the intercropping of soybean and sugarcane [28], while a decrease in the function and diversity of rhizospheric soil microbes associated with oilseed rape plants has been reported in response to oilseed rape intercropping with milk vetch, with corresponding shifts in microflora structure [29]. Soybean–oat intercropping resulted in an increase in nitrogen-fixing microbial abundance in the rhizospheric soil of oat plants, in addition to modulating nitrogen-fixing microflora composition [30]. The intercropping of peanuts and sugarcane can increase beneficial microbial abundance and the diversity of bacteria in rhizospheric soil [31,32]. Moreover, sugarcane–peanut intercropping, which is the same as gram–legume intercropping, is popular in the Guangxi region of China, as it allows for the intercropping of peanut plants without adversely affecting sugarcane yield efficiency, resulting in superior yield advantages that contribute to greater income for farmers. Thus, there is clear value in further exploring intercropping models with the goal of exploring how the structural and functional features of soil microflora vary in response to nitrogen fertilizer application. Such studies will inform efforts to assess the impact of intercropping models on the microbiological characteristics of intercropped soils in response to fertilizer amendments.

Here, an Illumina MiSeq high-throughput sequencing platform and metagenomics approaches were employed to analyze the impact of intercropping and nitrogen application on the structural and functional characteristics of peanut rhizospheric soil microflora. We believe that sugarcane/peanut intercropping and different levels of nitrogen fertilizer application can change the physical and chemical properties of soil, thereby regulating the structure and function of the soil microbial community. Thus, these analyses have the potential to (1) clarify the impacts of different magnitudes of nitrogen fertilizer application on the physicochemical properties of rhizospheric soil for monocropped or intercropped peanuts; (2) enable comparisons of how different levels of nitrogen fertilizer application alter the structure and function of rhizospheric microflora associated with monocropped or intercropped peanuts; and (3) clarity the associations between shifts in the peanut rhizospheric soil microbiota and the physicochemical characteristics of the soil.

2. Materials and Methods

2.1. Study Site and Soil Collection

This study was conducted in Xixiangtang District, Nanning City, Guangxi Province, China (22.8° N, 108° E). The test site has an average annual temperature of 21.8 °C, an average annual rainfall of 1286 mm, and a subtropical monsoon climate. The soil type at

the study site was latosol [33], and the total nitrogen (TN), total phosphorous (TP), and total potassium (TK) contents of the soil were 0.113%, 0.053%, and 0.240%, respectively; the available nitrogen (AN) content was 88.9 mg/kg, the available phosphorous (AP) content was 50.4 mg/kg, the available potassium (AK) content was 110 mg/kg, the soil organic matter (OM) content was 13.5 g/kg, and the pH value was 6.5. Peanut (Guihua 36) and sugarcane (Guitang 42) varieties used for these analyses were provided by the Guangxi Academy of Agricultural Sciences (Nanning, China). Two different planting methods (peanut monocropping, sugarcane/peanut intercropping) and three nitrogen application levels (low N: 15 kg/ha, intermediate N: 75 kg/ha, high N: 150 kg/ha) were tested. All fertilizer was evenly applied to the soil prior to planting, with urea being used as the nitrogen fertilizer, while phosphorus fertilizer was applied in the form of superphosphate (P_2O_5: 80 kg/ha), and potassium fertilizer was applied in the form of potassium sulfate (K_2O: 90 kg/ha). Sugarcane/peanut intercropping was achieved by alternating three rows of peanut plants and one row of sugarcane, while peanut monocropping instead entailed planting four rows of peanuts. Moreover, 4 replicates were established per condition, totaling 24 total experimental plots, each covering an area of 20 m^2. The spacing between rows is presented in Figure 1. Sugarcane and peanuts were planted in March 2022, and peanut rhizosphere soil was collected by July 2022. At the stage of pod maturity, peanut plants were uprooted in the experimental plots and loose soil was shaken off from the peanut root system, after which, the soil attached to the root surface was brushed off with a brush and collected as rhizosphere soil. About 20 peanut plants were plucked from each replicated experimental plot, and about 100 g of rhizosphere soil was collected, of which, 10 g of rhizosphere soil was stored in a refrigerator at −80 °C for high-throughput sequencing, and the other 90 g of rhizosphere soil was air-dried for the physicochemical analysis of soil.

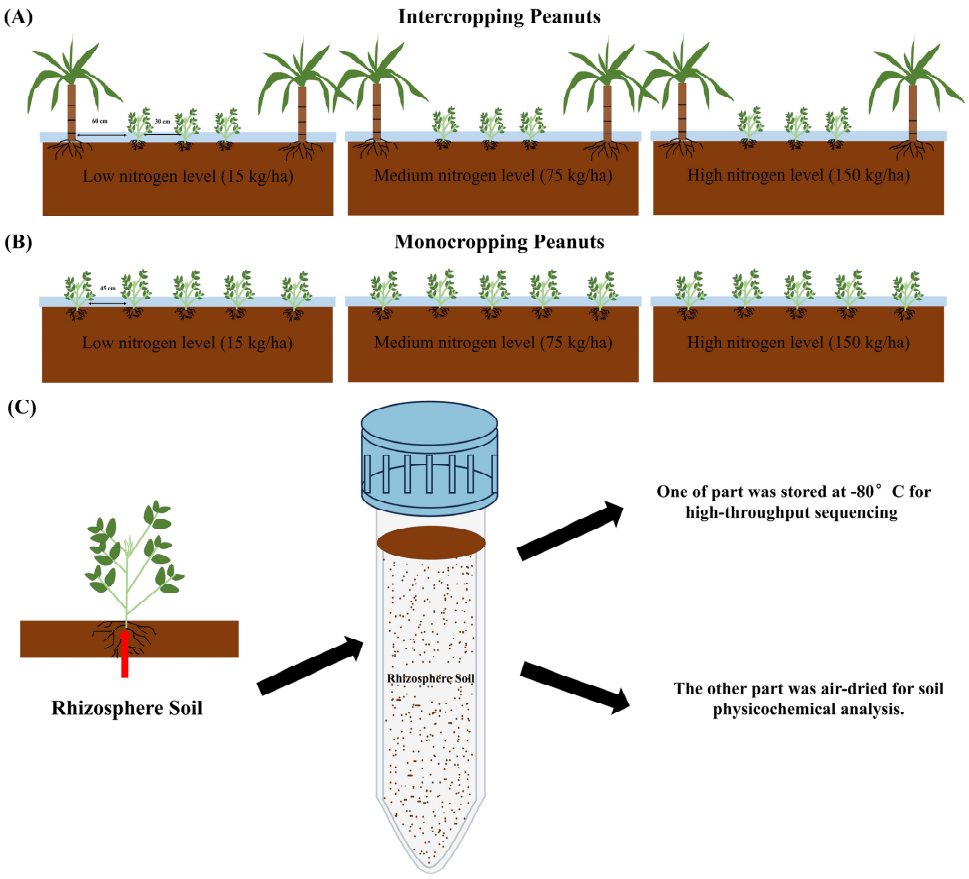

Figure 1. Schematic overview of the experimental peanut and sugarcane fields. (**A**) Intercropping peanuts; (**B**) monocropping peanuts; (**C**) sampling of peanut rhizosphere soil.

2.2. Physicochemical Characterization of Soil

Air-dried samples of rhizosphere soil were used to analyze the TN, TP, TK, AN, AP, AK, soil OM, and soil pH. TN was measured via the sulfuric acid digestion–Kjeldahl method [34], while AN was assessed with the alkaline hydrolysis diffusion method [35]. TP and AP were quantified through a sodium hydroxide melt–sulfuric acid digestion–molybdenum antimony resistance colorimetric method and a sodium bicarbonate leaching–molybdenum antimony anti-colorimetric method [36,37]. TK and AK were measured with the sodium hydroxide melt–flame spectrophotometer and ammonium acetate leaching–flame atomic absorption spectrophotometer methods [38]. OM content was assessed with the potassium dichromate volumetric method [39]. Soil pH was measured with a pH meter (a-AB23PH ZH, OHAUS) after preparing soil–water suspensions (1:2.5 w/v) [40].

2.3. Soil Metagenomic Sequencing

Soil DNA was extracted with the E.Z.N.A® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) as directed, and a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis were used to quantify the concentration of the isolated DNA and assess its integrity. S220 Focused-ultrasonicators (Corvallis, OR, USA) were used for DNA fragmentation, after which, Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) were used to purify the amplified products. The TruSeq Nano DNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA) was used for library preparation, after which, an Illumina NovaSeq 6000 sequencing instrument was used for 150 bp paired-end sequencing. Sequencing and data analyses were performed by Shanghai Ouyi Biomedical Technology Co., Ltd. (Shanghai, China).

2.4. Illumina MiSeq Sequencing and Data Analysis

Illumina MiSeq sequencing raw data underwent three processing steps. Initially, raw data in the FASTQ format were processed with Trimmomatic (v 0.36) to remove the 3′ and 5′ adaptor sequences and filter out low-quality bases to eliminate N base-containing reads. Then, these valid reads were used for the assembly of a macrogenome using MEGAHIT (v 1.1.2) [41]. Lastly, the assembled scaffolds were used for ORF predictions with prodigal (v 2.6.3) and translated into amino acid sequences. Predicted genes for all samples were used to construct non-redundant gene sets with CDHIT (v 4.5.7) [42]. Clustering was performed at 95% identity with 90% coverage between sequences, selecting the longest gene as the representative gene set sequence in each clustering set. After representative gene set sequences were obtained, clean reads were separately compared to the non-redundant gene sets with bowtie2 (v 2.2.9) at a 95% identity level to assess the abundance of genes in the analyzed samples.

Species annotations were obtained through comparisons between NR libraries and taxonomic databases based on the gene abundances of the species. Species abundance within individual samples was determined at each taxonomic level and the results were used to establish a corresponding abundance profile for that taxonomic level. Representative amino acid sequences for gene sets were compared to the NR library, KEGG, COG, SWISS-PROT, GO, and CAZy databases with DIAMOND (v 0.9.7), setting the expected e-value to 1×10^{-5} for BLAST comparisons. Annotated details regarding carbohydrate-activating enzymes corresponding to the gene were obtained through comparisons with the gene set and the CAZy database made with the hmmscan (v 3.1) tool from the CAZy database, after which, the abundance of these enzymes was calculated based on the sum of the abundance of the genes corresponding to carbohydrate-activating enzymes.

PCoA analyses and the mapping of species or functional abundance were performed with R (v 3.2.0). Redundancy analysis (RDA) and Spearman correlation analyses were conducted to identify the environmental factors that affected the microbial community structure and function in soil samples. RDA was performed using the "vegan" package in R (v 3.2.0), while the Spearman correlation analysis was performed using the "psych" package in R (v 3.2.0). Appropriate R packages were used to analyze data with ANOVA/Kruskal–

Wallis/*T* test/Wilcoxon approaches. All data were compared via one-way ANOVAs and the least significant difference (LSD) test using SPSS 20.0.

3. Results

3.1. Physicochemical Characteristics of Soil

The initial analyses of the physicochemical properties of soil revealed that as nitrogen application levels increased, corresponding rises in TN, AN, and OM levels were evident in the rhizosphere soil from the monocropped and intercropped groups, whereas pH levels decreased (Figure 2A,D,G,H).

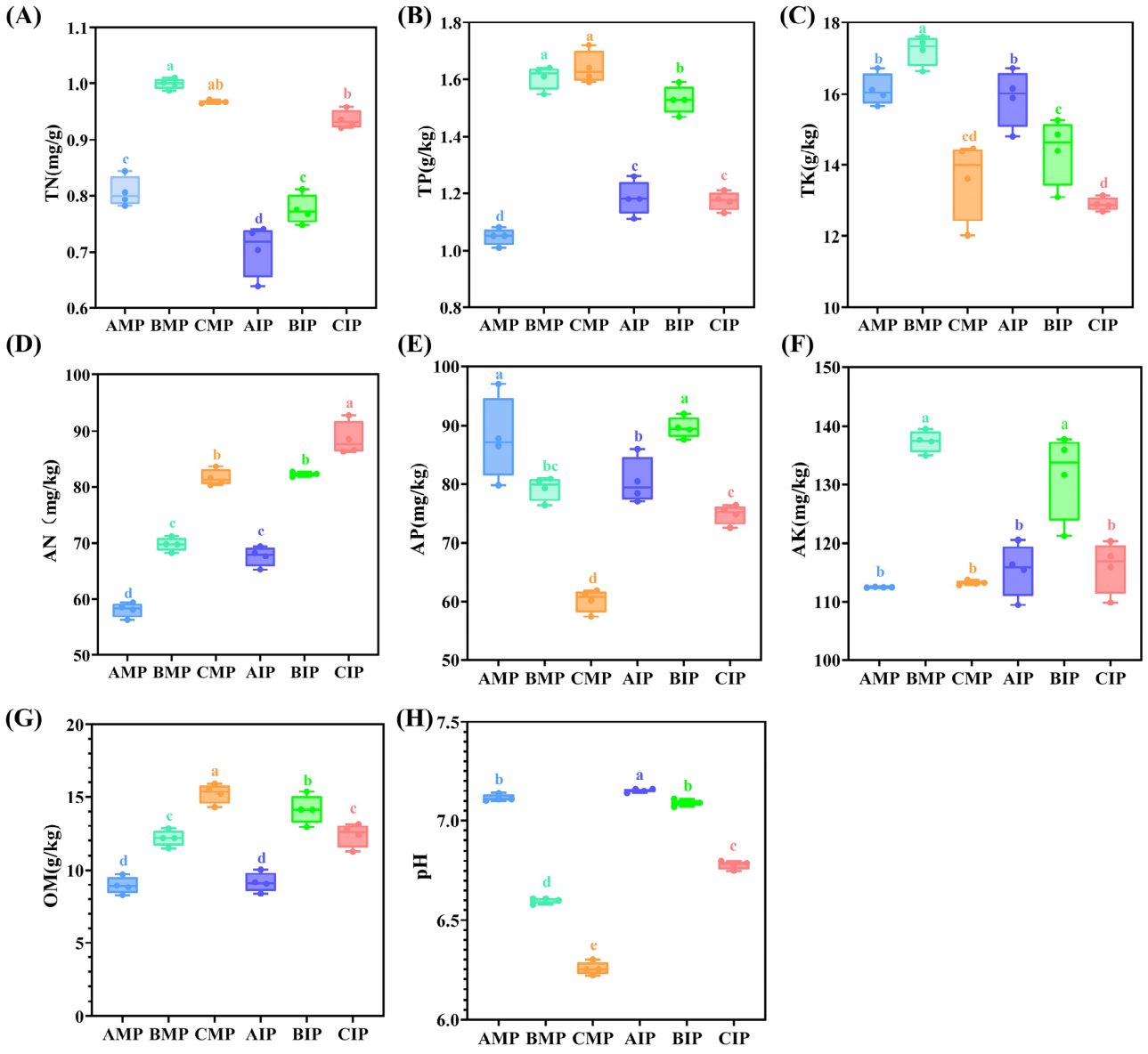

Figure 2. The physicochemical properties of peanut rhizosphere soil under monocropping and intercropping cultivation strategies. Identical lowercase letters indicate the absence of a significant difference between treatments ($p > 0.05$, LSD test). (**A**) was the total nitrogen of peanut rhizosphere soil under monocropping and intercropping, (**B**) was the total phosphorus of peanut rhizosphere soil under monocropping and intercropping, (**C**) was the total potassium of peanut rhizosphere soil under monocropping and intercropping, (**D**) was the available nitrogen of peanut rhizosphere soil under monocropping and intercropping, (**E**) was the available phosphorus of peanut rhizosphere soil under

monocropping and intercropping, (**F**) was the available potassium of peanut rhizosphere soil under monocropping and intercropping, (**G**) was the soil organic matter of peanut rhizosphere soil under monocropping and intercropping, (**H**) was the pH of peanut rhizosphere soil under monocropping and intercropping. TN, total nitrogen; TP, total phosphorus; TK, total potassium; OM, soil organic matter; AN, available nitrogen; AP, available phosphorus; AK, available potassium; AMP, monocropping peanuts with low nitrogen levels; BMP, monocropping peanuts with intermediate nitrogen levels; CMP, monocropping peanuts with high nitrogen levels; AIP, intercropping peanuts with low nitrogen levels; BIP, intercropping peanuts with intermediate nitrogen levels; CIP, intercropping peanuts with high nitrogen levels.

At a given level of nitrogen fertilizer amendment, TN, TP, and TK levels in rhizosphere soil samples from monocropped peanuts were higher than those in intercropped peanuts, with significant differences in TN levels under low and intermediate of nitrogen amendment. TP levels also differed significantly under low and intermediate nitrogen levels, while TK levels only differed significantly under intermediate levels of nitrogen application (Figure 2A–C). The AN content and pH levels of rhizosphere soil from the monocropping group were significantly less than those for the intercropping group, while the AP levels in samples of rhizosphere soil from monocropped plants were significantly lower than those for peanuts from the intercropping group at intermediate and high levels of nitrogen amendment. No significant differences in AK levels were observed when comparing the rhizosphere soil from the monocropping and intercropping groups, while significantly lower soil OM content was evident for monocropping peanuts relative to intercropped peanuts at intermediate levels of nitrogen application (Figure 2D–H).

3.2. High Throughput Sequencing Analyses

This study included 24 total samples. Following high-throughput sequencing, a total of 13.33–17.1 Gb of data were obtained per sample, with N50 contigs ranging from 242,286 bp. After the removal of redundancies, the number of ORFs in the gene catalog was 17,671,216. The longest and shortest lengths among these were 24,696 bp and 201 bp, respectively. Annotation rates for non-redundant genes were 85.89%, 65.97%, 9.99%, and 0.83% for the NR, eggNOG, KEGG, and CAZy databases, respectively (Tables S1–S3).

3.3. Analyses of the Rhizosphere Soil Microbial Composition and Diversity

To clarify the structure and diversity of rhizosphere soil microbial communities, high-throughput sequencing analyses were performed. Alpha diversity analyses revealed that as nitrogen fertilizer levels rose, Chao1 index, ACE index, and obs index values for the monocropping and intercropping treatments first increased and then decreased, whereas the Shannon index and Simpson index values trended downward. In contrast, the Shannon index and Simpson index of the intercropping group gradually increased. Under moderate and high levels of nitrogen application, Chao1 index, ACE index, and obs index values for the monocropping group were higher than those for the intercropping group, while under high nitrogen levels, the Shannon index and Simpson index values for the monocropping group were lower than those for the intercropping group. The goods_coverage of these six sample groups was close to one (Figure S1A–F).

Beta diversity analyses at the phylum, genus, and species levels were conducted, and the 95% confidence ellipses in the PCoA plots intersected for all six groups. However, the respective ANOSIM R values at these taxonomic levels were 0.692, 0.508, and 0.91, and all *p*-values were less than 0.05. Thus, these ANOSIM analyses indicated that the groupings were meaningful and that between-group differences were significantly greater than within-group differences (Figure S2A–F).

The microbes identified through the metagenomic sequencing analyses of these 24 samples of rhizosphere soil were ultimately classified into 197 phyla, 172 classes, 369 orders, 862 families, 4013 genera, and 36,360 species (Figure S3 and Tables S4–S9). The dominant phyla in these samples included *Proteobacteria*, *Acidobacteria*, *Gemmatimonadetes*, *Actinobacte-*

ria, *Chloroflexi*, *Bacteroidetes*, and *Verrucomicrobia*, which consisted of more than 71% of the analyzed microbes. *Proteobacteria* and *Acidobacteria* comprised over 48% of these microbes (Figure 3A). Increasing levels of nitrogen fertilizer application resulted in a gradual decrease in the relative abundance of *Proteobacteria* and *Verrucomicrobia* in the monocropping groups, whereas *Actinobacteria* and *Chloroflexi* abundance first rose and then declined. *Gemmatimonadetes* abundance in soil samples from the intercropping group was lower than that from the monocropping group under all three levels of nitrogen fertilizer application, whereas *Chloroflexi* abundance exhibited the opposite trend.

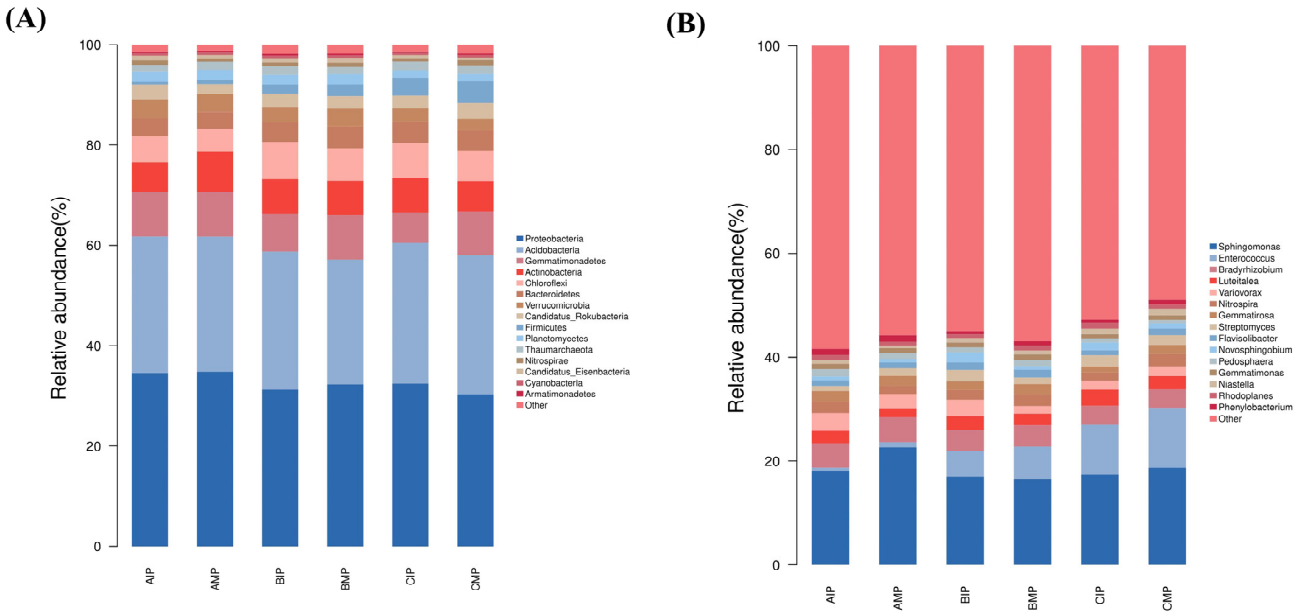

Figure 3. Analysis of the relative abundance of the dominant soil microbial community in the rhizosphere soil of peanuts. (**A**) Dominant rhizosphere soil groups at the phylum level; (**B**) dominant rhizosphere soil groups at the genus level; AMP, monocropping peanuts with low nitrogen levels; BMP, monocropping peanuts with intermediate nitrogen levels; CMP, monocropping peanuts with high nitrogen levels; AIP, intercropping peanuts with low nitrogen levels; BIP, intercropping peanuts with intermediate nitrogen levels; CIP, intercropping peanuts with high nitrogen levels.

The dominant genera identified in these analyses were *Sphingomonas*, *Enterococcus*, *Bradyrhizobium*, *Luteitalea*, *Variovorax*, *Nitrospira*, *Gemmatirosa*, and *Streptomyces* (Figure 3B). As nitrogen fertilizer application levels rose, *Enterococcus* abundance levels associated with the monocropping and intercropping groups increased gradually, with corresponding gradual decreases in *Bradyrhizobium* and *Variovorax* abundance. *Nitrospira* abundance in the monocropping group also rose gradually, whereas it exhibited the opposite trend in the intercropping treatment group.

3.4. Functional Pathways of Rhizosphere Soil Microbes

KEGG and CAZy database annotations of non-redundant genes were used to analyze and compare the functional pathways associated with rhizosphere soil microbes. In total, 1,786,065 and 147,943 genes in these soil samples were annotated with the KEGG and CAZy databases, respectively (Figures 4B and S4).

In total, 265 grade 3 KEGG functional pathways were annotated across all samples, with the most common functional pathways including the ABC transport vehicles, aminoacyl tRNA biosynthesis, two-component system, oxidative phosphorylation, ribosome, quorum sensing, purine metabolism, porphyrin, and chlorophyll metabolism pathways (Figure 4A and Table S10). With increasing nitrogen fertilizer levels, the relative abundance of functions, including cell cycle *Caulobacter*, amino sugar, nucleotide sugar metabolism,

nucleotide excision repair, oxidative phosphorylation, the two-component system, and glycerol phosphate metabolism gradually declined in rhizosphere soil microbial communities from the monocropping and intercropping groups. At the same nitrogen level, higher levels of functional relative abundance for pathways, including the ribosome, RNA degradation, cell cycle Caulobacter, nucleotide excision repair, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, oxidative phosphorylation, and nitrogen metabolism pathways, were observed among rhizosphere soil microbes from the monocropping group relative to the intercropping group. In contrast, the relative abundance of the quorum sensing function in rhizosphere soil microbes from the monocropping group was lower than that for microbes from the intercropping group at low and intermediate levels of the nitrogen application.

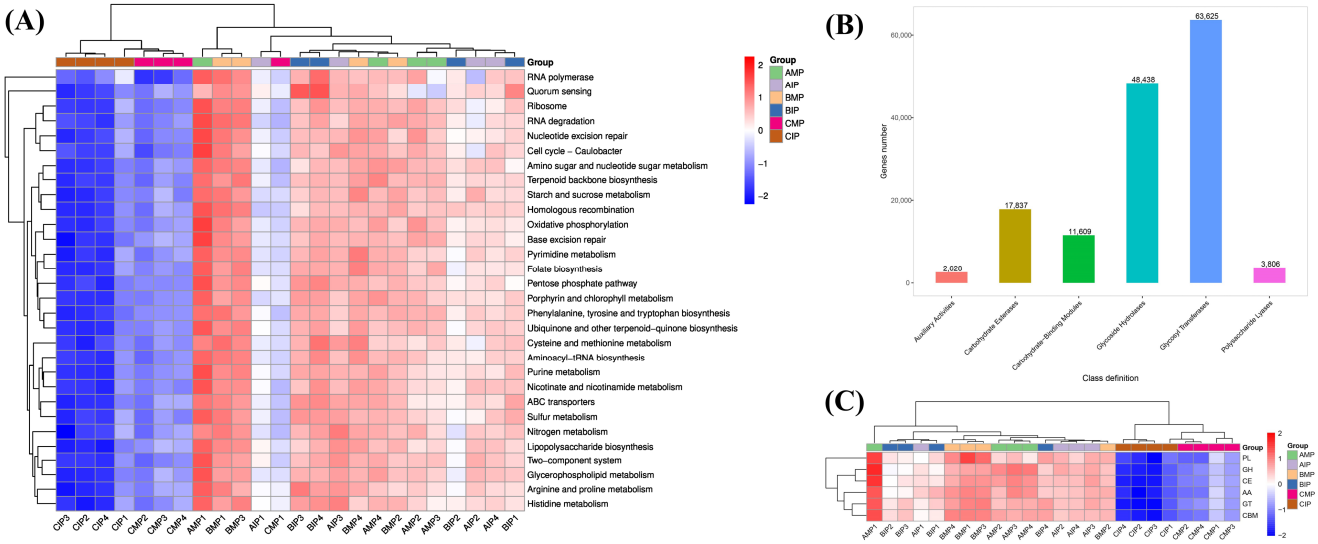

Figure 4. Functional pathways associated with microbes in the peanut rhizosphere soil. (**A**) KEGG functional pathways were annotated in all samples; (**B**,**C**) The annotation and abundance information of samples in CAZy database; AMP, monocropping peanuts with low nitrogen levels; BMP, monocropping peanuts with intermediate nitrogen levels; CMP, monocropping peanuts with high nitrogen levels; AIP, intercropping peanuts with low nitrogen levels; BIP, intercropping peanuts with intermediate nitrogen levels; CIP, intercropping peanuts with high nitrogen levels.

CAZy database annotations revealed that 63,625 genes were annotated as glycosyl transferases (GTs), 48,438 as glycoside hydrolases (GHs), 17,837 as carbohydrate esterases (cEs), 11,609 as carbohydrate-binding modules (CBMs), 3806 as polysaccharide lyases (PLs), and 2628 as auxiliary activities (aAs). The relative abundance of six functional classes of CE genes in rhizosphere soil microbes from the monocropping and intercropping groups decreased significantly with rising levels of nitrogen fertilizer application. At a given nitrogen level, the relative abundance of these six functional classes of CE genes was higher in rhizosphere soil microbes from the monocropping group relative to the intercropping group (Figure 4B,C).

3.5. The Impact of Environmental Factors on Rhizosphere Soil Microbial Community Structure and Function

In order to identify environmental factors with an impact on soil microbial community structure and function, RDA and Spearman correlation analyses were conducted. RDA analyses indicated that soil AN (p = 0.002), OM (p = 0.006), pH (p = 0.007), and AK (p = 0.012) significantly affected the rhizosphere soil microbial community structure (Figure 5A and Table S11). Spearman analyses of the associations between environmental factors and KEGG level 2 functional pathways revealed a positive correlation between soil TK and the abundance of cell motility, carbohydrate metabolism, and glycan biosynthesis

and metabolism pathways (Figure 5B and Table S12). In addition, soil AP was positively correlated with the abundance of the translation, cellular, and energy metabolism pathways, while soil AN was negatively correlated with the abundance of the metabolism of other amino acids, carbohydrate metabolism, and glycan biosynthesis and metabolism pathways. In addition, a negative correlation was detected between soil pH and the enrichment of the environmental adaptation pathway.

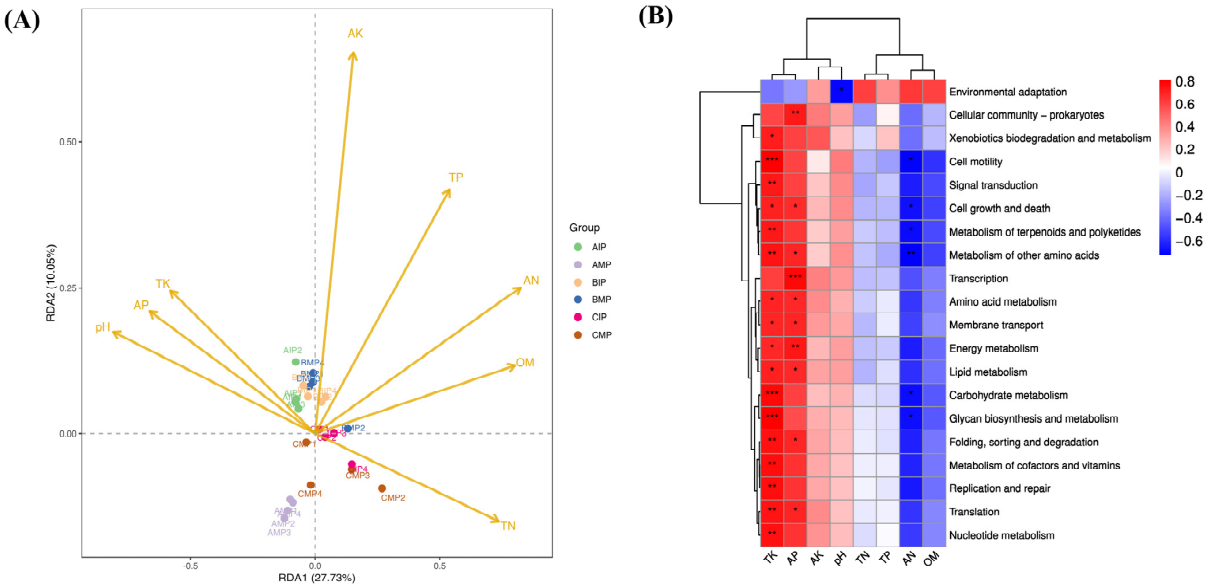

Figure 5. Analysis of the relationships between soil characteristics and rhizosphere microbial community structure and function. (**A**) Redundancy analysis plots corresponding to the associations between microbial communities and the environmental characteristics of soil. (**B**) Spearman correlation analyses of correlations between KEGG level 2 functional pathways and the environmental characteristics of soil. * mean $p < 0.05$, ** means $p < 0.01$, *** mean $p < 0.001$.

3.6. Analyses of the Nitrogen Cycle in Peanut Rhizosphere Soil Microbes

Next, metagenomic sequencing results were used to identify nitrogen cycle-related genes, followed by the KEGG annotation and analysis of those genes (Figure 6). Through the creation of gene sets and analyses of nitrogen cycling-related genes, the abundance of *niFH*, *niFK*, and *niFD* genes associated with nitrogen fixation in the rhizosphere soil from peanuts under intercropping conditions, as well as the nitrification-related *pmoA-amoA*, *pmoB-amoB*, and *pmoC-amoC* genes, was more abundant than under monocropping conditions at low levels of nitrogen application, whereas the opposite trend was evident under high levels of nitrogen fertilization. The denitrification-related *norC* and *nosZ* genes showed a reduced abundance in the rhizosphere soil from the intercropping conditions relative to monocropping conditions under high levels of nitrogen amendment. The abundance of the dissimilating nitrate reduction-related *napA* gene was elevated in the rhizosphere soil under intercropping conditions relative to monocropping conditions for all three levels of nitrogen amendment, with *napA* abundance rising with increasing nitrogen levels in both groups. In summary, most nitrogen cycling-related genes were present in greater abundance in peanut rhizosphere soil under intercropping conditions relative to monocropping conditions at low levels of nitrogen application, suggesting the greater activity of the nitrogen conversion cycle in intercropping peanut rhizosphere soil when lower nitrogen levels were present. In contrast, high nitrogen levels were associated with a lower abundance of these genes in the intercropping group relative to the monocropping group, suggesting that the peanut rhizosphere soil nitrogen transformation cycle is more active under high nitrogen levels for monocropping systems (Figure 6 and Tables S13 and S14).

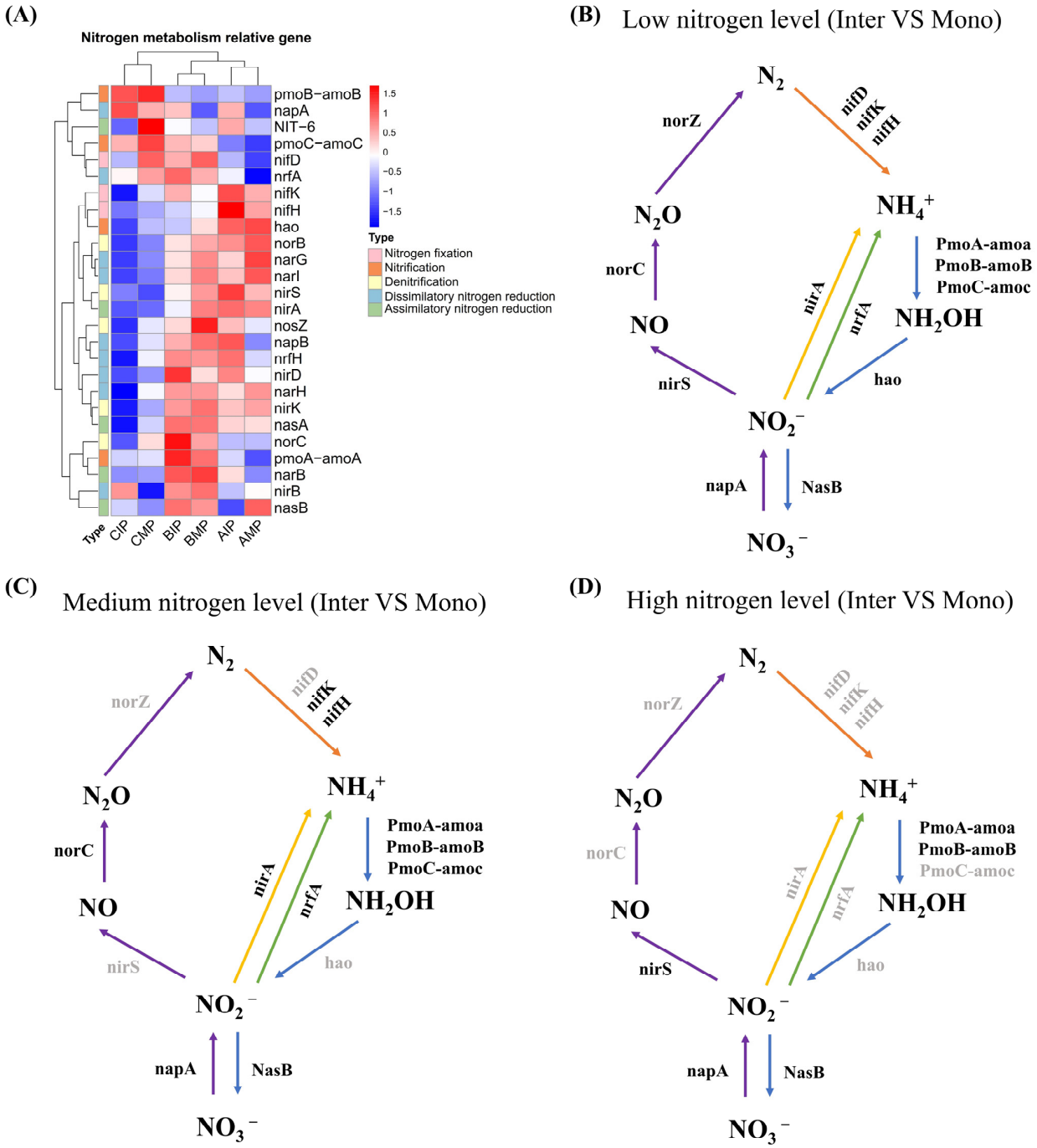

Figure 6. Schematic overview of the primary nitrogen transforming functions for monocropped and intercropped peanut plants under low, intermediate, and high levels of nitrogen application based on KEGG pathway annotation and enrichment results. (**A**) A heatmap presenting the relative abundance of nitrogen metabolism genes. (**B**–**D**), nitrogen cycling at low (**B**), intermediate (**C**), and high (**D**) levels of nitrogen amendment under intercropping conditions relative to monocropping conditions. Orange arrows correspond to nitrogen fixation, blue arrows indicate nitrification, purple arrows indicate denitrification, yellow arrows indicate assimilatory nitrogen reduction, and green arrows indicate dissimilatory nitrogen reduction. Higher gene abundance in intercropping vs. monocropping, the gene color will be black, and vice versa, it will be gray.

4. Discussion

Intercropping cultivation strategies are sustainable agricultural production strategies [43], with sugarcane/peanut intercropping having emerged as a model that is increasingly popular in Guangxi, China [32]. Prior studies have demonstrated that intercropping can facilitate the full utilization of natural resources while reducing fertilizer use, improving crop yields, and decreasing the incidence of disease and pest-related damage [43–45]. The present study explored the effects of different levels of nitrogen fertilizer application on sugarcane/peanut intercropping systems, with a particular focus on how peanut rhizosphere soil microbial communities respond to intercropping and different levels of nitrogen fertilizer application. These results provide valuable insights into peanut rhizosphere soil microecology and the factors that affect these microbial communities.

Intercropping can allow for the more efficient use of soil nutrients, and the intercropping of grasses and legumes can result in increases in both soil pH and AN levels [46]. Here, sugarcane/peanut intercropping was found to increase peanut rhizosphere soil pH and AN levels, consistent with prior results [47]. These data, together with other past results, suggest that these soil changes resulting from peanut and sugarcane intercropping may be associated with nitrogen fixation mediated by peanut nodules and plant root exudates [48]. Sugarcane competitive nutrient absorption and utilization when intercropped with peanuts can drive enhanced peanut nitrogen fixation, thereby leading to greater AN levels within the peanut rhizosphere soil. Relative to peanut monocropping, root exudates under sugarcane/peanut intercropping conditions were more abundant, further resulting in the observed change in rhizosphere soil pH.

Peanut intercropping results in enhanced soil microbial richness and diversity [27,31,36]. Here, metagenomic sequencing of rhizosphere soil samples collected under peanut mono/intercropping conditions with low, intermediate, and high levels of nitrogen amendment was conducted, leading to the identification of microbes that were ultimately classified into 197 phyla, 172 classes, 369 orders, 862 families, 4013 genera, and 36,360 species. At the phylum level, greater *Gemmatimonadetes* abundance was observed in rhizosphere soil samples from intercropped peanuts relative to monocropped peanuts at all tested nitrogen levels, while the opposite trend was evident for *Chloroflexi* abundance. *Gemmatimonadetes* is a phylum of Gram-negative aerobic heterotrophic bacteria with fully functional type 2 (pheophytin-quinone) photosynthetic reaction centers [49]. The intercropping of sugarcane and peanuts results in enhanced photosynthetic efficiency, with light contributing to improved *Gemmatimonadetes* growth rates. Thus, *Gemmatimonadetes* richness under intercropping conditions is higher than that under monocropping conditions [50]. This study additionally found that as nitrogen fertilizer usage increased, relative *Gemmatimonadetes* abundance levels rose, in line with past reports [51]. *Chloroflexi* can facilitate inorganic carbon conversion into organic material while also enabling the aerobic oxidation of nitrite [52]. Wu et al. [53] found that as levels of nitrogen fertilizer application rose, *Chloroflexi* abundance declined, whereas Cao et al. [54] used soil amendment to increase *Chloroflexi* abundance. As nitrogen fertilizer amendment levels rose in the present study, *Chloroflexi* abundance first increased and then declined, with higher abundance levels under monocropping conditions relative to intercropping conditions. These differences in *Chloroflexi* abundance may be a consequence of the observed shifts in soil properties. At the genus level, rising nitrogen fertilizer levels were associated with gradual increases in *Enterococcus* abundance under monocropping and intercropping conditions, whereas *Bradyrhizobium* and *Variovorax* abundance gradually declined. *Nitrospira* abundance also rose gradually in the monocropping group, while it exhibited the opposite trend under intercropping conditions. Tang et al. [32] found that the intercropping of peanut and cassava plants resulted in increased *Nitrospira* abundance, whereas *Bradyrhizobium* abundance levels rose in the context of corn and peanut intercropping [26]. Thus, the present study revealed some differences relative to prior reports with respect to the characteristics of monocropping and intercropping systems, while also providing new details regarding the effects of different levels of nitrogen application under these conditions. *Bradyrhizobium*

is capable of utilizing plant-derived carbohydrates to exchange fixed nitrogen, whereas *Nitrospira* bacteria with nitrifying activity are capable of converting soil ammonia nitrogen into nitrate nitrogen to maintain soil nitrogen cycling [55,56]. As nitrogen fertilizer levels rose, *Bradyrhizobium* abundance under monocropping conditions declined, while *Nitrospira* abundance increased and decreased in the monocropping and intercropping systems, respectively. This suggests that under low nitrogen levels, stronger nitrogen cycling activity was evident in the sugarcane/peanut intercropping system as compared to the monocropping system, while the opposite was true under high levels of nitrogen amendment. Metagenomic sequencing was also used to identify nitrogen cycle-related genes, followed by the KEGG annotation and analysis of these genes. Through the establishment of gene sets and specific analyses of genes associated with nitrogen cycling, the nitrogen fixation-related *nifH*, *nifK*, and *nifD* genes, as well as the nitrification-related *pmoA-amoA*, *pmoB-amoB*, and *pmoC-amoC* genes, were found to be more abundant in the rhizosphere soil of intercropped peanuts relative to monocropped peanuts at low levels of nitrogen application, whereas the opposite was true when high levels of nitrogen were applied. The denitrification-related *norC* and *nosZ* genes were less abundant for intercropped peanuts relative to monocropped peanuts under high nitrogen levels. The observed changes in soil microbe abundance and nitrogen cycling-related gene levels provided further validation regarding these nitrogen cycling results (Figure 6). Intercropping was associated with the enrichment of nitrogen-fixing bacteria and greater efficiency of soil nitrogen utilization [57]. Ruibao et al. [58] determined that as levels of applied nitrogen fertilizer rose, nitrogen-fixing bacterial abundance decreased. The present study simultaneously tested three levels of nitrogen fertilizer amendment, peanut monocropping, and sugarcane/peanut intercropping systems to gain more detailed insights into nitrogen cycling patterns under these conditions, offering a theoretical foundation for future studies focused on nitrogen cycling under mono/intercropping with different modes of fertilization.

KEGG and CAZy pathways can offer insight into the characteristics of functional genes and the functional traits of microbes present within peanut rhizosphere soil samples. KEGG pathway analyses indicated that gradual decreases in the relative abundance of the cell cycle *Caulobacter*, amino sugar, nucleotide sugar metabolism, nucleotide excision repair, oxidative phosphorylation, two-component system, and glycerol phosphate metabolism pathways were evident for both monocropping and intercropping systems with rising levels of the nitrogen fertilizer application. Soil microbes survive primarily through the absorption of nutrients, amino acids, and other sources of energy, consistent with the observed enriched functional genes and pathways in this study [59]. The amino sugar and nucleotide metabolism pathway, as well as the starch and sucrose metabolism pathway, may serve as important sources of nutrients and energy for microbes in the soil. In contrast to prior reports, however, increases in the abundance of the genes associated with these two pathways were evident under monocropping conditions relative to intercropping conditions [27,32,60]. In the context of continuous sugarcane cropping, amino acid biosynthesis abundance gradually increases over the course of 0, 10, and 30 years [61]. CAZy database analyses revealed gradual decreases in the relative abundance of six functional classes of carbohydrate enzyme genes in rhizosphere soil microbes under intercropping and monocropping conditions as nitrogen fertilizer levels increased. At a given nitrogen level, the relative abundance of these six functional classes of carbohydrate enzyme genes was higher in rhizosphere soil microbes from monocropped peanuts relative to intercropped peanuts. Previous studies have demonstrated that soil microorganisms associated with walnut/hairy vetch intercropping offer great carbohydrate metabolism potential [62], while metagenomic analyses have demonstrated the dominant role of carbohydrate metabolism pathways in the context of sugarcane/peanut intercropping [36]. These results are partially inconsistent with the present findings, underscoring the need for further research to resolve these differences.

Changes in intercropping strategy and nitrogen application levels result in significant changes in the physicochemical properties of soil, including soil pH, nutrient content, and

microbial biomass levels. Here, RDA analyses indicated that rhizosphere soil AN ($p = 0.002$), OM ($p = 0.006$), PH ($p = 0.007$), and AK ($p = 0.012$) significantly affected the microbial community structure. This aligns well with prior studies [36,61,63,64]. Pang et al. and Lewis et al. [61,65] reported negative correlations between soil pH and replication and repair, polysaccharide biosynthesis, and metabolic pathways, whereas Tang et al. [66] conducted Spearman correlation analyses of different metabolites in sugarcane rhizosphere soil and the physicochemical characteristics of soil in the context of sugarcane/peanut intercropping, revealing positive correlations between the levels of adenine, D-proline, and adenosine, as well as properties such as AP, AK, and pH. Similarly, Spearman correlation analyses of the relationships between KEGG level 2 functional pathways and soil characteristics revealed a positive correlation between soil TK and cell motility, carbohydrate metabolism, and glycan biosynthesis and metabolism pathways. Soil AP was also positively correlated with the abundance of the translation, cellular community-prokaryotes, and energy metabolism pathways, while soil AN was negatively correlated with the abundance of the metabolism of other amino acids, carbohydrate metabolism, and glycan biosynthesis and metabolism pathways. In addition, a negative correlation was detected between soil pH and the enrichment of the environmental adaptation pathway. At present, there is growing interest in examining the correlations between KEGG level 2 functional pathways and soil environmental parameters through soil microbial metagenomic sequencing analyses. Thus, the present results can serve as a reference and foundation for further efforts to explore the associations between the physicochemical properties and functions of soil.

5. Conclusions

Here, increases in nitrogen amendment were found to be associated with an upward trend in rhizosphere soil TN, AN, and OM content for both monocropped and intercropped peanuts, whereas the soil pH decreased. At a given level of nitrogen fertilizer amendment, TN, TP, and TK levels in rhizosphere soil samples from monocropped peanuts were generally higher than those for intercropped peanuts, while AN content and pH levels were significantly less than those for the intercropped peanuts. Macrogenomic sequencing-based analyses of soil microbial communities revealed that the diversity of microbial communities in the rhizosphere soil of intercropped peanuts was significantly higher than that of monocropped peanuts under high levels of nitrogen fertilizer application. The abundance of *Gemmatimonadetes* in rhizosphere soil for the intercropping group was higher than that for the monocropping group under all three tested levels of nitrogen application, whereas the opposite trend was observed for *Chloroflexi* abundance. *Nitrospira* abundance in the monocropping group gradually rose, whereas the opposite was evident in the intercropping treatment group. The rhizosphere soil microbial community structure was significantly impacted by soil characteristics, including AN, OM, pH, and AK. Analyses of nitrogen cycling-related genes further revealed the greater activity of the nitrogen conversion cycle in intercropping peanut rhizosphere soil when lower nitrogen levels were present, but the peanut rhizosphere soil nitrogen transformation cycle is more active under high nitrogen levels for monocropping systems. These findings suggest that while there are advantages to intercropping systems, excessive nitrogen application can have additional effects on the soil environment, underscoring the need for rational fertilization.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14030635/s1. Figure S1: Analyses of microbial community alpha diversity in the rhizosphere soil of peanuts; Figure S2: Analyses of beta diversity for microbial communities at the phylum, genus, and species levels; Figure S3: Relative abundance of microorganisms in rhizosphere soil under different classification levels; Figure S4: Statistics on the number of annotated genes in the KEGG database; Table S1: Sequencing data preprocessing statistical results title; Table S2: Statistical analysis of gene prediction results for 24 samples; Table S3: Statistical summary of non-redundant gene annotation results; Table S4: Relative species abundance of 24 samples at the phylum level; Table S5: Relative species abundance of 24 samples at the class level; Table S6: Relative species abundance of 24 samples at the order level; Table S7: Relative species abundance of

24 samples at the family level; Table S8: Relative species abundance of 24 samples at the genus level; Table S9: Relative species abundance of 24 samples at the species level; Table S10: Level 3 functional pathways annotated by KEGG database in 24 samples; Table S11: RDA analysis of environmental factors on microbial community structure; Table S12: Spearman correlation analysis between the KEGG level 2 functional pathway and environmental factors; Table S13: KEGG database annotation genes related to nitrogen cycling; Table S14: The relative abundance of nitrogen cycling related genes in 24 samples.

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