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Diversified Cropping of Grains and *Atractylodes lancea* (Thunb.) DC. Enhances Ecological Benefits of Agroecosystems

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Abstract: Crop diversification is pivotal in sustainable agriculture, influencing soil microbial communities and soil nutrient cycling functions. Yet, the impacts of incorporating medicinal plants into crop diversification strategies on the functional characteristics of these microbial communities remain understudied. This research elucidates the benefits of diversified cropping systems by assessing soil nutrient content, diversity and composition of soil microorganisms, the abundance of functional genes involved in carbon (C), nitrogen (N) and phosphorus (P) cycling, and overall agricultural productivity; collectively referred to as ecological benefits. The experimental design included four treatment groups: (1) continuous maize (*Zea mays* L.) cultivation (MC); (2) maize–*A. lancea* (*Atractylodes lancea* Thunb.) intercropping (MA); (3) maize–sorghum (*Sorghum bicolor* L.) rotation (MS); and (4) maize–*A. lancea* intercropping combined with sorghum rotation (MSA). Findings indicate that diversified cropping treatments significantly enhance the alpha diversity of soil bacterial communities over fungal communities. NH_4^+ and NO_3^- predominantly influence the composition of soil bacterial communities, with a notable increase in the relative abundance of Acidobacteriota, Gemmatimonadota, and Chloroflexi. Compared to MC treatment, the MA and MSA treatments significantly increased the abundance of C (121.44%, 294.26%), N (206.57%, 294.26%), and P (112.02%, 225.84%) cycling genes. The inverse variance weighting evaluation demonstrates that, compared to the MC treatment, the MS (5.34) and MSA (8.15) treatments significantly boost soil ecological benefits. Overall, diversifying the cultivation of *A. lancea* with grains can enhance the ecological benefits of the soil. This study offers new perspectives on diversified planting, particularly in terms of species selection and practical combinations on farmland.

Keywords: diversified cropping; medicinal plants; soil microorganisms; metagenomics; nutrient cycling

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1. Introduction

In the twenty-first century, global agricultural intensification has accelerated as nations strive to meet the escalating food demands driven by population growth [1]. However, such intensification often precipitates ecological challenges including biodiversity loss, soil nutrient imbalances, and soil degradation [2,3]. Enhancing plant diversity is increasingly recognized as a viable strategy to amplify the ecological benefits of farmland, bolstering ecosystem functions like biodiversity, soil nutrient enhancement, and productivity [4–6].

Intercropping and crop rotation are the main methods that enhance the diversification of agricultural crops [7]. They both enhance plant resource utilization through niche differentiation and complementarity [8]. Intercropping enhances agro-ecosystem benefits through interspecies interactions and synergies. For instance, intercropping white lupin (*Lupinus albus* L.) with barley (*Hordeum vulgare* L.) elevates soil iron and manganese levels via root interactions, while maize (*Zea mays* L.) intercropped with alfalfa (*Medicago sativa* L.) increases phosphorus availability through root exudates [9,10]. And crop rotation focuses on optimizing the residual effects of preceding crops on soil nutrients [11]. Therefore, the reasonable combination of plant species is a core issue in the diversification of agricultural species.

Incorporating food crops with medicinal plants is a promising approach, given the significant economic value and demand for medicinal plants [12]. It is worth noting that medicinal plants produce various secondary metabolites like phenolics, terpenoids, and nitrogen- and sulfur-containing compounds [13]. At low concentrations, the compounds can enhance nutrient cycling and plant growth [14,15]. However, at high concentrations, these compounds may inhibit microbial activity and be toxic to plants [16]. For example, 1,2-benzenediol at 0.2 $\mu mol \cdot L^{-1}$ significantly boosts crop growth and nitrogen utilization, but concentrations at 2.0 $\mu mol \cdot L^{-1}$ and above exhibit inhibitory effects [17]. Diversified cropping helps mitigate the buildup of these metabolites in the soil thus reducing their potential harmful effects [18,19]. Therefore, diversified cropping systems not only alleviate the harm caused by continuous cropping but also contribute to enhancing the overall benefits of the ecosystem.

Soil microorganisms are crucial for maintaining various agricultural ecosystem functions, particularly in regulating soil nutrient cycling [20]. Diversified cropping can significantly alter the composition and functions of microbial communities in the soil [21,22]. For instance, in sugarcane (*Saccharum officinarum*) and soybean (*Glycine max*) intercropping systems, the prevalence of thick-walled fungi and ascomycetes increases, enhancing soil organic matter decomposition [23]. In maize–legume intercropping systems, the abundance of ammonia-oxidizing bacteria rises, facilitating nitrification [24].

The overarching goal of diversified cropping is to elucidate how increases in aboveground plant species can regulate belowground microbial structures and functions, affect soil nutrient dynamics, and create feedback mechanisms that maximize ecosystem benefits [25]. At present, it remains unclear how medicinal plants affect the nutrient cycling functions of soil microbial communities during the diversification process, or if their perennial traits can be leveraged to expand diversification configurations and enhance ecological advantages. Therefore, we investigated the impact by comparing four cropping systems with and without the inclusion of *Atractylodes lancea* (Thunb.) DC (https://www.gbif.org/species/3122433, accessed on 13 October 2024) over two years of field trials. We hypothesize that: (1) incorporating medicinal plants into intercropping systems with food crops enhances the abundance of soil carbon (C), nitrogen (N), and phosphorus (P) cycling functional genes by influencing the diversity and composition of soil microbial communities, which in turn augments soil nutrient cycling functions; (2) such intercropping configurations not only boost soil productivity but also improve overall ecological benefits of agroecosystems; and (3) the integration of crop rotation strategies with these intercropping systems will further amplify these effects. Moreover, we will assess the soil ecological benefits of diversified cropping systems involving medicinal plants across four dimensions: (1) biodiversity, specifically the diversity and composition of microbial communities; (2) soil nutrient content, focusing on the levels of soil C, N, and P; (3) soil nutrient cycling functions, as indicated by the abundance of soil microbial genes related to C, N, and P cycling; and (4) soil productivity, evaluated through crop yield and the resultant economic benefits.

2. Materials and Methods

2.1. Experimental Location and Design

The study area is located within the Changchun Experimental Station of the Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, in Changchun, Jilin Province, Northeastern China (43°59′ N, 125°23′ E). The region experiences a temperate continental semi-humid monsoon climate. The average temperature from May to October is 18.08 °C, with an average precipitation of 636.8 mm, of which 86.94% occurs during the growing season. According to the United States Department of Agriculture's soil taxonomy (1993), the predominant soil type is Typic Hapludoll, characterized by its black color and clay loam texture [26]. The selected experimental plots were continuously cultivated with maize for eight years, from 2014 to 2021. The initial soil nutrient content, designated as CK, is detailed in Appendix A Table A1.

This study began in May 2022 and concluded in October 2023, spanning two years. The experiment selected maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) as the primary cultivated food crops in the northeastern region, and Atractylodes (*Atractylodes lancea* Thunb.), a root and stem economic crop, locally used as a medicinal plant. The diversified design is based on the niche complementarity theory, ensuring that the selected species meet the prerequisites of equitable distribution of light resources, have no known mutual toxicity, and promote economic benefits [18,27,28]. As shown in Figure 1a, the experiment included four cropping systems: (1) single crop system (continuous maize cultivation, MC); (2) double crop intercropping system (maize–*A. lancea* intercropping, MA); (3) double crop rotation system (maize–sorghum rotation, MS); and (4) triple crop intercropping system (maize–*A. lancea* intercropping in 2022, maize–sorghum rotation in 2023, MSA). The aforementioned four cropping systems comprise their respective treatments, each repeated 5 times with a 1 m isolation area between each system to prevent cross-interference. The tested maize variety was "Liangyu 99", purchased from Dandong Denghai Liangyu Seed Industry Co., Ltd. (Dandong, China). The Atractylodes variety was "*Atractylodes Chinensis* (DC.) *Koidz*.", purchased from Tonghua County Jimei Agricultural Development Co., Ltd. (Tonghua, China). [29]. And the sorghum variety was "Jiliang No. 6", provided by Jilin Agricultural University. All experiments utilized a double-row cropping configuration, with a row spacing of 0.4 m for maize/sorghum, a plant spacing of 0.15 m for maize, and 0.1 m for sorghum, with a strip spacing of 1.6 m for all crops. The *A. lancea* strips were located between the corn or sorghum crops strips, at a distance of 0.6 m from the food crops strips, with a plant spacing of 0.4 m for *A. lancea*. Each experimental plot covers an area of about 300 square meters. Since *A. lancea* is a perennial herbaceous plant, and to avoid excessive soil disturbance affecting the experimental results, all treatments in the subsequent year were conducted using no-tillage methods. In 2023, food crops were sown adjacent to the 2022 planting sites. To minimize experimental error, consistent field and fertilization management practices were applied across all cropping treatments. Specifically, 300 kg·ha^{-1} of nitrogen fertilizer (CH_4N_2O, Sinochem Fertilizer Co., Ltd. (Beijing, China), Sinochem-Tianji brand urea, standard urea {46-0-0}) was administered annually in May.

2.2. Soil Sample Collection and Experiment Index Setting

In this study, the experimental design includes rotation treatments thus setting the growth cycle of each cropping system to two years. Soil samples were collected on 25 September 2023. At this time, maize and sorghum were in the harvesting period, while *A. lancea* was in the growth or harvesting period. Sampling points were uniformly established at a distance of 0.3 m from the food crop strips to ensure consistent soil property impacts across all treatments. Soil samples were collected from the top 20 cm of the soil profile using an auger (5 cm in diameter and 20 cm long), positioned at the center of the point and 0.3 m from the edge of the food crop strips. Five soil cores were randomly extracted along a straight line to compose a single soil sample. After removing roots, stones, litter, and debris, each bulk sample was divided into two subsamples. One subsample was immediately stored at −80 °C for DNA extraction, while the other was preserved at 4 °C for soil property analysis.

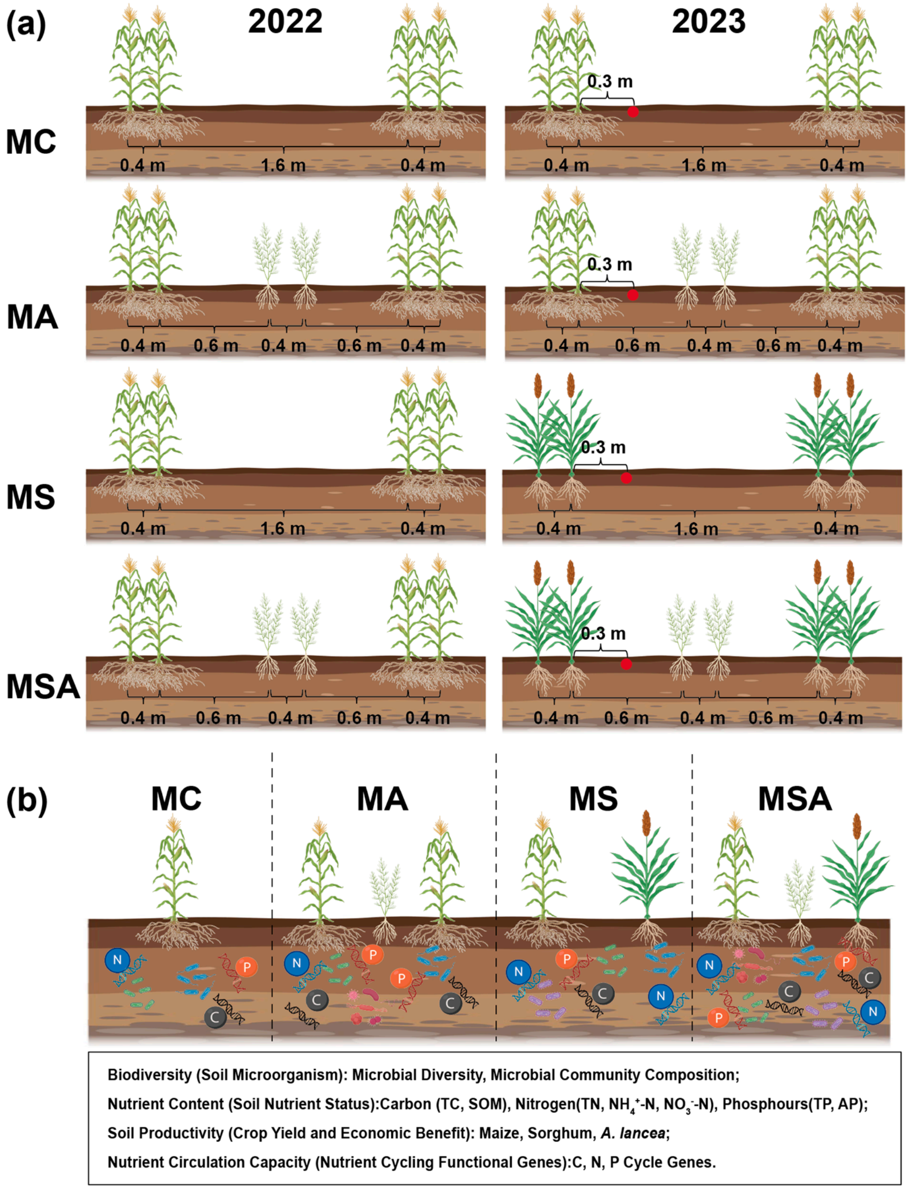

Figure 1. The field experiment arrangement and selection of the experimental indicators. (**a**) In 2022 and 2023, the planting conditions for the aboveground crops were classified as follows: continuous maize cultivation (MC), maize–*A. lancea* intercropping (MA), maize–sorghum rotation (MS), and maize–*A. lancea* intercropping in 2022, with a maize–sorghum rotation in 2023 (MSA). The red dots indicate the sampling locations. (**b**) The appropriate indicators were selected to evaluate the impact of the diversification of medicinal plants and food crops on soil nutrient cycling functions. These included microbial community diversity and composition, which serve as indicators of soil biodiversity; soil chemical properties, which reflect soil nutrient content; the abundance of functional genes related to carbon, nitrogen, and phosphorus cycling, which indicate soil nutrient cycling capacity; and crop yield and economic benefits, as indicators of soil productivity.

To clarify the impact of different diversified cropping systems on soil microbial communities, nutrient cycling capacity, and productivity, we quantified four functions related to these aspects based on the definitions in soil ecosystem functions [30]: (1) biodiversity (soil microbial diversity {alpha diversity} and composition {beta diversity}); (2) soil nutrient content, including total carbon (TC), soil organic matter (SOM), total nitrogen (TN), ammonium nitrogen ($NH_4{}^+$-N), nitrate nitrogen ($NO_3{}^-$-N), total phosphorus (TP), and available phosphorus (AP); (3) soil nutrient cycling capacity (the abundance of functional genes for carbon, nitrogen, and phosphorus cycles); and (4) soil productivity (crop yield and economic benefits), totaling 14 indicators (Figure 1b).

2.3. Determination of Soil Chemical Properties and Productivity Indicators

After the collected soil samples were air-dried at room temperature, they were divided into two parts. One portion of the air-dried soil samples was ground and sieved through a 2 mm sieve mesh. Soil pH and electrical conductivity (EC) were measured in a 1:5 (w/v) soil–water suspension using a pH meter (PHSJ-4F, Leici, Shanghai, China) and a conductivity meter (DDSJ-308F, Leici, China), respectively [31,32]. Grind another portion of the air-dried sample finely and pass it through a 0.15 mm sieve for the determination of other soil parameters [33]. SOM was quantified using the $K_2Cr_2O_7$ oxidation method [34]. TC and TN were determined using an Elemental Analyzer System (Vario Macro Cube, Hanau, Germany) using the combustion method. $NH_4{}^+$-N and $NO_3{}^-$-N were extracted with 2 M KCl [35]. TP was digested using HF-$HClO_4$, and AP was extracted with 0.5 M $NaHCO_3$ [36].

After the crops mature, collect the fruits of all plants in each treatment and record the yield per unit area. Subsequently, the yield conversion is carried out based on the ratio between the actual area of the experimental plot (300 m^2) and a hectare (10,000 m^2). The economic benefit is derived from the product of the crop price and yield. The crop prices are based on the local market prices in Jilin Province for 2023: maize at 0.29 $CNY \cdot kg^{-1}$, sorghum at 0.57 $CNY \cdot kg^{-1}$, and *A. lancea* at 2.29 $CNY \cdot kg^{-1}$.

2.4. Soil DNA Extraction, High-Throughput Sequencing, and Data Processing

Total DNA was extracted from 0.5 g of the soil using the Fast DNA SPIN Kit (MP Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was dissolved in sterile distilled water, and its quantity and quality were assessed using a NanoDrop 2000 (Thermo Scientific, Schwerte, Germany) and a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification of the 16S rRNA gene within the V3-V4 region of bacteria was performed using the primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACACHVGGGTWTCTAAT), while the ITS1 region of the fungi was amplified using the primers ITS1F (CTTGGTCATTTAGAG-GAGAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC). The PCR amplification of the 16S rRNA gene involved an initial denaturation step at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 40 s, and elongation at 72 °C for 30 s. This was completed with a final extension at 72 °C for 10 min. The PCR amplification of the ITS gene involved an initial denaturation step at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 40 s. This was completed with a final extension at 72 °C for 7 min. Following PCR amplification, the products were purified, quantified, and normalized to create sequencing libraries (Illumina Inc., San Diego, CA, USA). Sequencing was conducted by Biomarker Technologies Company (Beijing, China) on the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA). Raw reads obtained from sequencing were filtered using Trimmomatic (v0.33), and primer sequences were identified and removed using Cutadapt (v1.9.1), resulting in clean reads. Denoising was performed using the DADA2 method in QIIME2 (2020.6) [37,38]. The sequence ends were concatenated, and chimeric sequences were removed to obtain the final valid data (non-chimeric reads). The final sequences were clustered into operational taxonomic units (OTUs) based on a 97% similarity threshold, and the taxonomic annotation was conducted using Mothur (v1.40.45) against the SILVA (release 138) and FUNGuild (v1.1) databases [39,40].

2.5. Shotgun Metagenomic Sequencing and Data Processing

To elucidate the role of soil bacterial communities in nutrient cycling across various diversified cropping systems, we conducted a quantitative analysis of functional genes associated with carbon (C) cycling (including carbon fixation and degradation), nitrogen (N) cycling (encompassing nitrification, denitrification, and assimilatory reduction), and phosphorus (P) cycling (covering organic phosphorus mineralization, inorganic phosphorus dissolution, and phosphorus transport) under different diversified cropping treatments. Referring to the research methods of Ji et al., the extracted DNA strands were subjected to segmental purification [41]. Sequencing libraries were then created based on the purified DNA samples, and quality checks were performed before generating 2×150 bp paired-end reads on the NovaSeq 6000 Illumina platform. Subsequently, quality control of the raw data was performed using Fastp to obtain clean, high-quality data. Host gene sequences were aligned using Bowtie2 software (version 2.5.4) to remove contaminating human host sequences. The mixed samples ($n = 5$) were assembled using MEGAHIT (version 1.1.2) software, and contigs smaller than 200 bp were filtered out. The assembly results were evaluated using QUAST (version 2.3). To obtain non-redundant reference gene sequences, CD-HIT (version 4.6.6) software was used to remove redundancy, with similarity and coverage thresholds set at 95% and 90%, respectively. The EggNOG-mapper software (version 2.1.4) was used for the annotation of reference genomes against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Finally, Salmon was used to calculate the Transcripts Per Million (TPM) values for all samples, representing gene expression levels.

2.6. Statistical Analysis

All data were organized using Microsoft Office Excel 2019, with each variable presented as the mean ± standard deviation (SD). Alpha diversity of the microbial communities (ACE, Chao1, Simpson and Shannon index) was analyzed using the "vegan" and "picante" packages in R v4.3.3 based on OTU abundance data [13]. Significant differences between experimental treatment groups were analyzed using one-way ANOVA followed by Tukey's HSD test, conducted in SPSS v20.0 (IBM, Chicago, IL, USA). A significance level of $p < 0.05$ was considered statistically significant [42]. Principal Coordinate Analysis (PCoA) was conducted on microbial community structure and physicochemical structure based on the Bray–Curtis distance matrix. Additionally, permutation multivariate analysis of variance (PERMANOVA) was employed using the "vegan" package in R to assess significant differences in the distance matrix [43]. Data merging of the OTUs was conducted at the phylum level, and the proportion of the abundance of related microorganisms at the phylum level to the total OTUs abundance was calculated for subsequent analysis. Mantel's analysis was used to examine the correlation between microbial diversity and composition and soil nutrient content, accomplished through the "mantel.partial" function in the "vegan" package in R [44]. We employed ordinary least squares (OLS) regression to evaluate the linear relationship between key bacterial phyla levels and soil nutrient content. Additionally, the abundance of functional genes and metabolic pathways in the bacteria was categorized through comparison with the KEGG database. Data visualization was conducted using the OmicStudio tool (available at https://www.omicstudio.cn/tool/99, accessed on 15 July 2024) and the Chiplot tool (available at https://www.chiplot.online/bar_plot_errorBar.html, accessed on 15 July 2024).

Principal component analysis (PCA) was used to reduce collinearity among the selected indicators and normalize the data. Consequently, the 10 retained indicators were utilized to assess the overall ecological benefits of various diversified cropping systems. These 10 indicators include microbial diversity (Chao1 and Shannon indices), microbial community composition (bacterial phylum-level abundance), nutrient cycling capability (total abundance of soil C, N, and P cycling functional genes), soil nutrient content (soil C, N, and P content), and soil productivity (crop yield and economic benefits). For each indicator, we then calculated the effect size and variance between the variable groups (MA, MS, and MSA) and the control group (MC) using the random effects model (ROM) in R. The variance between variables (τ^2) was calculated using the restricted maximum likelihood method (REML), and the total variance of each variable was composed of the sum of the variance of each variable and τ^2. The weight of each variable was represented by the reciprocal of the total variance [45]. Then, using the inverse variance weighting method, the product of weights and effect sizes was calculated to indicate the impact of each variable on the soil microbial community function [46,47]. Finally, the cumulative sum of the product of weights and effect sizes was obtained to evaluate the results of the different diversified cropping systems. All the aforementioned analyses were conducted using the "Rma.mv" function from the "metafor" package in R version 4.3.3.

3. Results

3.1. The Impact of Different Diversified Cropping Systems on Soil Nutrient Content, Microbial Diversity, and Composition

The PCoA results for soil nutrients revealed that all diversified treatments significantly altered the composition of soil nutrients (Figure 2a, Supplementary Table S1). Compared to the MC treatment, both the MA and MSA treatments significantly increased the levels of NO_3^--N, TP, and AP while concurrently reducing the levels of TN, NH_4^+-N, and EC (Appendix A Table A1). Additionally, the MSA treatment significantly boosted the SOM content (Appendix A Table A1). Conversely, apart from increases in NO_3^--N and pH, all other indicators for the MS treatment were found to be lower than those of the MC treatment (Appendix A Table A1).

High-throughput sequencing technology was employed, yielding a total of 16 samples across 4 groups (n = 4). This produced 2,609,705 bacterial clean reads, with each sample generating at least 149,084 clean reads and an average of 163,107 clean reads. For fungi, 2,378,153 clean reads were produced, with each sample contributing at least 112,373 clean reads and an average of 148,635 clean reads. Following quality filtering and DADA2 denoising, a total of 2270 bacterial operational taxonomic units (OTUs) and 20,214 fungal OTUs were identified. The rarefaction curves for all samples reached saturation, indicating that the sequencing data adequately captured the present species diversity (Appendix A Figure A1).

Alpha diversity analysis indicated significant increases in the Chao1 index (Figure 2b, Tukey's HSD test, Supplementary Table S2) and Shannon index (Figure 2c, Tukey's HSD test, Supplementary Table S2) of the soil bacterial communities across all the diversified treatments. Specifically, the Chao1 index for the MSA treatment was significantly higher than those for the MA and MS treatments. In contrast, the diversified treatments did not significantly alter the Chao1 index of the fungal communities (Figure 2d, Tukey's HSD test, Supplementary Table S2). However, the Shannon index for the fungal communities was significantly higher under the MA and MS treatments compared to the MC and MSA treatments (Figure 2e, Tukey's HSD test, Supplementary Table S2). PCoA results demonstrated significant differences in the community composition of both bacteria (Figure 2f) and fungi (Figure 2g) among the different diversified cropping systems and the control (MC). Post-diversification, the relative abundance of the major bacterial groups (>1%, taxa abundance divided by the total sum of taxa) showed decreases in Proteobacteria and increases in Acidobacteriota, Gemmatimonadota, Myxococcota, Methylomirabilota, and Chloroflexi (Figure 2h, Supplementary Table S3, ANOVA, Tukey's HSD test). For fungi, the effects of the diversified cropping on the major groups (>1%, taxa abundance divided by the total sum of taxa) were less consistent. The relative abundances of Ascomycota significantly decreased in the MA and MSA treatments, while Chytridiomycota showed significant decreases in the MS and MSA treatments. Conversely, the MSA treatment significantly increased the relative abundance of Basidiomycota, and both the MA and MSA treatments significantly elevated the relative abundance of Mortierellomycota (Figure 2i, Supplementary Table S4, ANOVA, Tukey's HSD test).

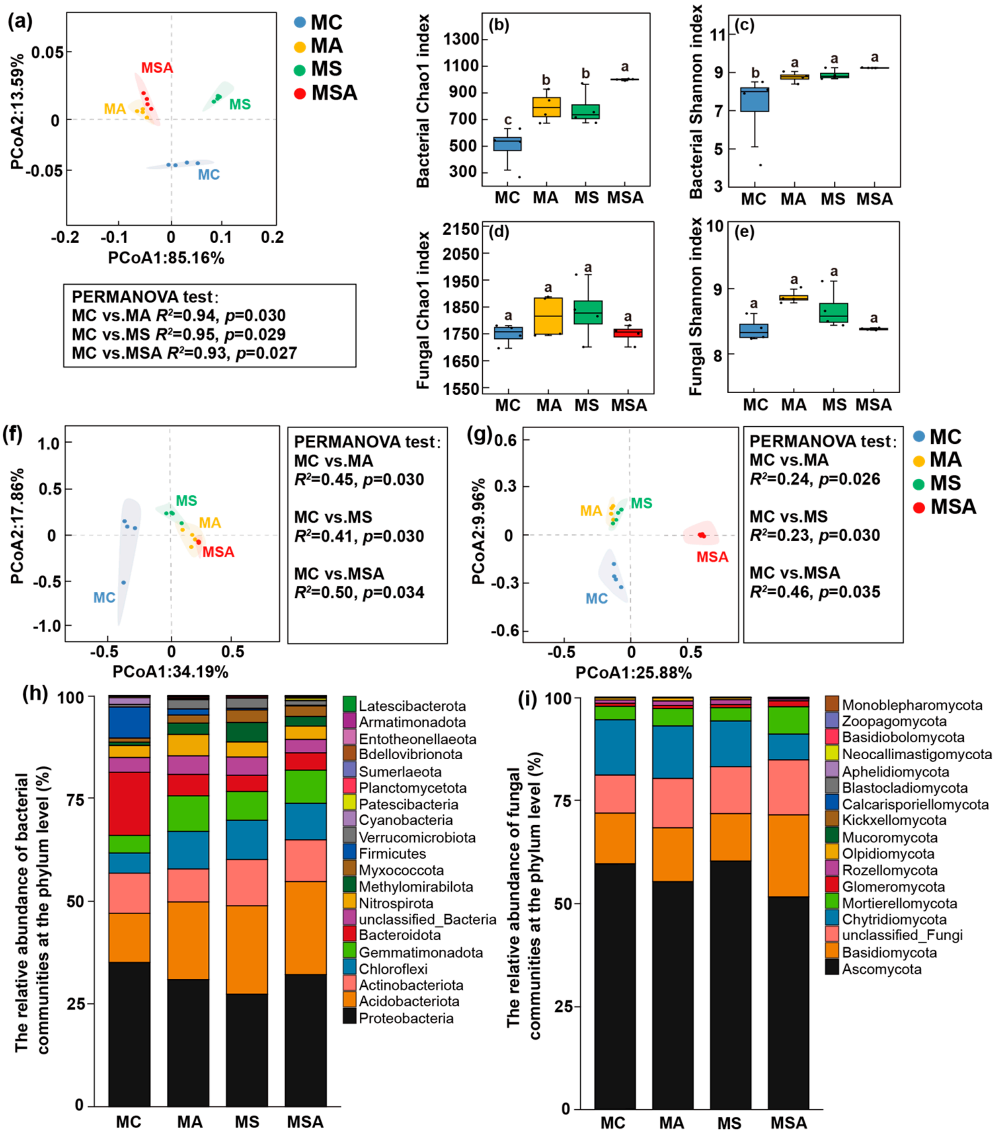

Figure 2. The impact of the diversification process on soil properties, microbial community diversity, and composition. (**a**) Principal Coordinate Analysis (PCoA) was performed on soil chemical properties (including pH, EC, TC, SOM, TN, NH_4^+-N, NO_3^--N, TP, and AP). Permutational multivariate analysis of variance (PERMANOVA) was employed to assess the statistical significance of the ordination plots. (**b**–**e**) The Chao1 index for bacterial communities with different treatments (**b**), the Shannon index for bacterial communities (**c**), the Chao1 index for fungal communities (**d**), and the Shannon index for fungal communities (**e**) (n = 16 samples). The symbols and error bars represent the mean value and standard deviation of four biological replicates, respectively. The distinct letters on top indicate significant differences across between sample groups (Tukey's honestly significant difference test, $p < 0.05$). (**f**,**g**) The PCoA of soil bacterial (**f**) and fungal (**g**) community compositions were based on the OTUs abundance from 16S rRNA and ITS sequencing. (**h**,**i**) Differences in relative abundance at the phylum level for bacterial (**h**) and fungal (**i**) communities under various diversification treatments were also assessed using 16S rRNA and ITS sequencing data.

3.2. Effects of Changes in Soil Microbial Community Diversity and Composition on Soil Nutrients in Diversified Cropping Systems

Under the MC treatment, microbial diversity (bacterial and fungal diversity) is mainly related to the content of TN and TP, and the bacterial community structure (relative abundance at the phylum level) is related to the content of NH_4^+-N ($p < 0.05$, Figure 3a). Under different diversified cropping systems, only the diversity and structural changes in bacterial communities show a significant correlation with soil nutrients, and this occurs only in diversified cropping systems with medicinal plants. In the MA treatment, bacterial diversity is significantly correlated with NO_3^--N and TP, and changes in the bacterial community structure are significantly correlated with NH_4^+-N content ($p < 0.05$, Figure 3b). In the MSA treatment, only changes in the bacterial community structure were significantly correlated with TC and NO_3^--N ($p < 0.05$, Figure 3c), while in the MS treatment, there is no significant correlation between changes in microbial community diversity and structure and soil nutrients ($p > 0.05$, Figure 3d).

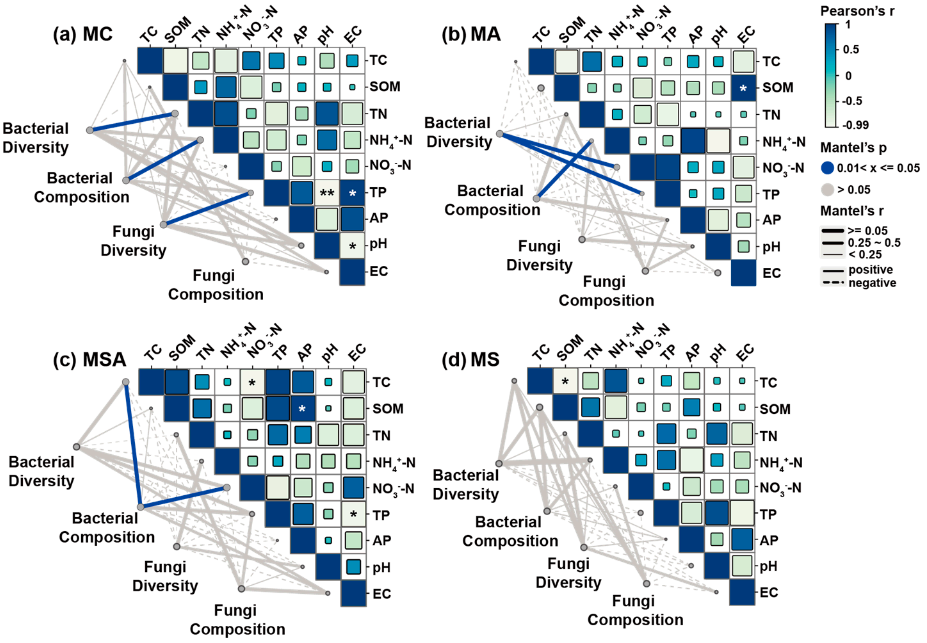

Figure 3. The influence of soil microbial diversity and composition on nutrient dynamics. (**a**–**d**) Mantel's analysis was utilized to evaluate the diversity and composition of soil microbial communities relative to soil environmental factors: (**a**) continuous maize cultivation (MC); (**b**) maize–*A. lancea* intercropping (MA); (**c**) maize–*A. lancea* intercropping in 2022, maize–sorghum rotation in 2023 (MSA); and (**d**) maize–sorghum rotation (MS). A heatmap on the right of each image set illustrates the Pearson correlations between the soil environmental factors, with the color gradient indicating the magnitude of the Pearson correlation coefficient. Asterisks signify significant differences, with "*" and "**" denoting $p < 0.05$ and $p < 0.01$, respectively. The diversity and composition of the bacterial and fungal communities are depicted on the left of each image set, represented by Chao1 and Shannon indices and determined by the abundance of OTUs at the phylum level (based on Bray–Curtis distances). The widths of the lines depict Mantel's r statistic, while the color indicates the significance of the p-value derived from 999 permutations. Significant correlations are highlighted in blue ($0.01 < p < 0.05$), and non-significant correlations are shown in gray ($p > 0.05$).

3.3. *Effects of Different Diversified Cropping Systems on Soil Microbial Communities and Soil Nutrient Cycling Functions*

The PCoA results of soil bacterial functional gene abundance showed that the diversified cropping altered the abundance of functional genes in soil microbial communities (Figure 4a). The MA treatment significantly increased the abundance of functional genes related to C cycling by 121.44%, N cycling by 206.57%, and P cycling by 112.02% in the soil microbial communities (Figure 4b). In contrast, the MS treatment did not improve the abundance of these functional genes and, in fact, led to significant decreases in genes related to C cycling by 35.70% and P cycling by 39.11% (Figure 4c). The MSA treatment further enhanced the abundance of these functional genes beyond the increases observed with the MA treatment (Figure 4d).

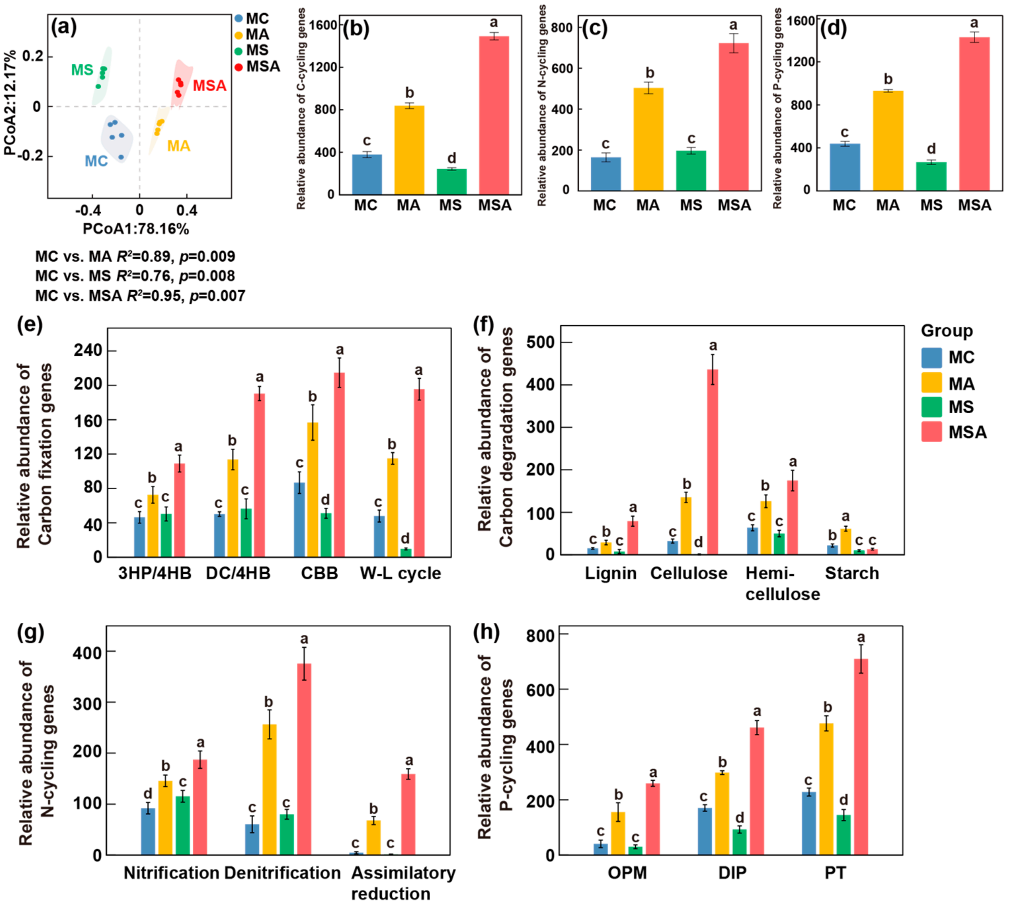

Figure 4. The impact of diversification processes on the nutrient cycling function of soil microbial communities. (**a**) Principal Coordinate Analysis (PCoA) assessed the nutrient cycling functions of soil microbial communities based on metagenomic sequencing (KO gene abundance using the transcripts per million (TPM) metric, n = 20 samples). PERMANOVA was employed to assess the statistical significance of the ordination plots. (**b**–**d**) There were notable differences in the overall abundance of carbon (**b**), nitrogen (**c**), and phosphorus (**d**) cycling functional genes in the soil microbial communities across the different diversification treatments. (**e**–**h**) Gene abundance changes in specific functional pathways of the soil microbial communities under the different diversities included carbon fixation (**e**), carbon degradation (**f**), nitrogen cycling (**g**), and phosphorus cycling (**h**). The symbols and error bars represent the mean value and standard deviation of five biological replicates, respectively. The distinct letters on top indicate significant differences across the sample groups (Tukey's honestly significant difference test, $p < 0.05$). 3HP/4HB: 3-hydroxypropionic acid/4-hydroxybutyric acid cycle. W-L cycle: acetyl coenzyme a pathway. CBB: Calvin–Benson–Bassham cycle. DC/4HB: dicarboxylate/4-hydroxybutyric acid cycle. OPM: organic phosphorus mineralization. DIP: dissolved inorganic phosphorus. PT: phosphorus transport.

For carbon fixation pathways, the MA and MSA treatments significantly increased the abundance of functional genes associated with four key pathways. Specifically, the abundance of genes including *accA*, *acsA*, *acs*, *rpiA*, *PGK*, and *ppc* escalated with the incorporation of increasing numbers of medicinal plant species in the farmland (Figure 4e, Supplementary Table S5). However, concerning carbon degradation pathways, only three pathways showed an increasing trend under the MA and MSA treatments: lignin decomposition (*lig*), cellulose decomposition (*bglX*, *cdh*), and hemicellulose decomposition (*xylA*, *xylB*). Contrarily, the MSA treatment did not enhance, and instead reduced by 39.75%; the abundance of functional genes was associated with starch polysaccharide degradation (Figure 4f, Supplementary Table S5). Additionally, the abundance of functional genes involved in nitrification (*narH*), denitrification (*narB*), and assimilation reduction (*nasA*) within the N cycle, as well as inorganic phosphorus mineralization (*phoD*, *phoN*), organic phosphorus solubilization (*gcd*, *ppa*, *ppx*), and phosphorus transport (*pstB*, *pstC*, *pstS*, *phnD*, *ugpA*, *ugpB*, *ugpC*) within the P cycle, showed a rising trend with the increase in the number of plant species incorporated into the farmland (Figure 4g,h, Supplementary Table S5).

3.4. Yield and Economic Benefits of Different Diversified Cropping Systems

Table 1 shows that in 2022, the maize yield in the MA and MSA treatments decreased by 6.84% compared to the MC treatment. In 2023, the maize yield in the MA treatment decreased by 6.63% compared to the MC treatment, and the sorghum yield in the MSA treatment decreased by 5.23% compared to the MS treatment. After the full crop harvest in 2023, the total aboveground crop yield over two years under the MA and MSA treatments was not significantly different from the MC treatment, but the yield under the MS treatment (37,493.25 ± 756.00 kg·ha^{-1}) was significantly lower than the MA treatment (39,275.70 ± 980.70 kg·ha^{-1}). In addition, the diversified cropping systems significantly improved the economic benefits per unit of farmland area. The economic benefits for the MA and MS treatments increased by 52.42% and 53.24%, respectively, compared to the MC treatment. The MSA treatment showed the greatest improvement, with an increase of 101.85% compared to the MC treatment.

Table 1. Crop yields and economic benefits under different planting treatments.

Treatment	2022	2023			Total Yield (kg·ha^{-1})
	Maize Yield (kg·ha^{-1})	**Maize Yield (kg·ha^{-1})**	**Sorghum Yield (kg·ha^{-1})**	***A. lancea* Yield (kg·ha^{-1})**	
MC	17,908.95 ± 360.75 a	21,108.15 ± 218.25	-	-	39,017.10 ± 559.65 ab
MA	16,683.75 ± 304.50 b	19,707.00 ± 925.05	-	2885.10 ± 155.85	39,275.70 ± 980.70 a
MS	18,104.85 ± 308.55 a	-	19,388.40 ± 1045.80	-	37,493.25 ± 756.00 b
MSA	16,682.70 ± 202.65 b	-	18,373.65 ± 1444.95	2821.80 ± 73.50	37,878.00 ± 1277.85 ab
	Maize Output Value (CNY·ha^{-1})	**Maize Output Value (CNY·ha^{-1})**	**Sorghum Output Value (CNY·ha^{-1})**	***A. lancea* Output Value (CNY·ha^{-1})**	**Total Output Value (CNY·ha^{-1})**
MC	5193.60 ± 104.61 a	6121.36 ± 63.29	-	-	11,314.96 ± 162.32 c
MA	4838.29 ± 88.30 b	5715.02 ± 268.24	-	6606.75 ± 356.15	17,160.05 ± 402.81 b
MS	5250.42 ± 89.46 a	-	11,051.36 ± 596.09	-	16,301.78 ± 511.16 b
MSA	4837.98 ± 58.76 b	-	10,472.94 ± 823.63	6461.79 ± 168.14	21,772.72 ± 737.38 a

Note: MC: continuous maize planting; MA: maize–*A. lancea* intercropping; MS: maize–sorghum rotation; MSA: maize–*A. lancea* intercropping with sorghum rotation. The data in the table are presented as mean ± standard deviation ($n = 4$). The different lowercase letters within the same column indicate significant differences between the sample groups (ANOVA, Tukey's HSD, $p < 0.05$). The blank area indicates that the crop was not planted this year. The economic benefits were calculated based on the actual market prices of crops at the end of 2022, multiplied by their yields. The unit price for maize was 0.29 CNY·kg^{-1}, for sorghum it was 0.57 CNY·kg^{-1}, and for *A. lancea* it was 2.29 CNY·kg^{-1}.

3.5. Ealuation of Soil Bacterial Community Function and Ecological Benefits in Different Diversified Cropping Systems

The evaluation of the ecological benefits under the different diversified cropping treatments using the inverse variance weighting method revealed significant differences. Compared to the MC treatment, both the MA treatment (5.34) and the MSA treatment (8.15) significantly increased the ecological benefits of the diversified planting system ($p < 0.01$). Conversely, the MS treatment (−4.52) significantly reduced the ecological benefits (Figure 5, Supplementary Table S6).

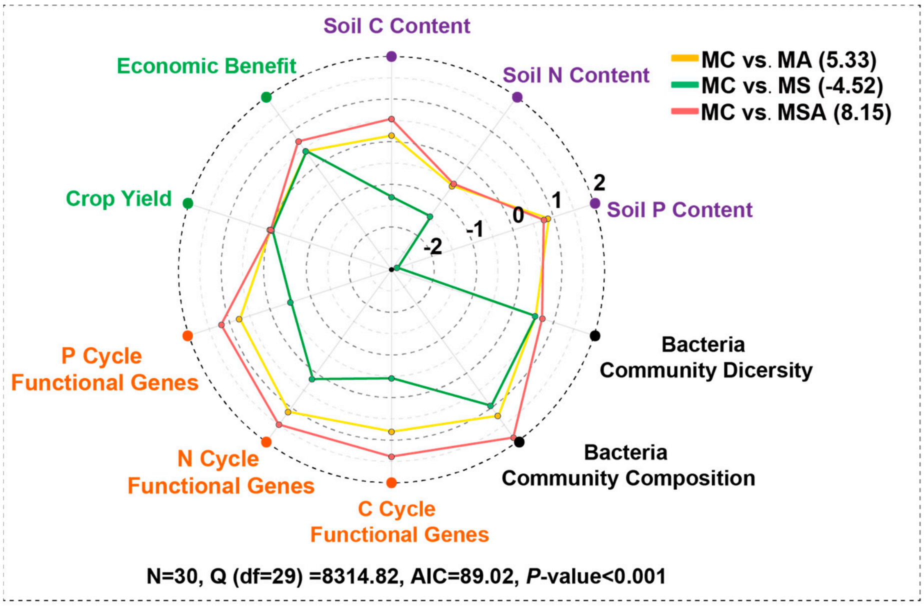

Figure 5. A comprehensive evaluation of soil nutrient cycling functions under the various diversified cropping treatments, depicting variables for soil nutrient content, soil nutrient cycling functions, soil productivity, and biodiversity with purple, orange, green, and black borders, respectively. The yellow, green, and red areas visualize the cumulative impacts of the MA, MS, and MSA treatments compared to MC on soil ecosystem functions.

4. Discussion

4.1. A. lancea Primarily Influence the Soil Bacterial Community During Crop Diversification, More than the Fungal Community

Our findings show that species diversification in farmland, particularly through the incorporation of *A. lancea*, favorably influences the overall microbial community, which aligns with observations by Cappelli [48]. Specifically, the intercropping of *A. lancea* and food crops (MA treatment) significantly augmented the alpha diversity of the soil microbial communities, particularly affecting bacterial more than fungal communities (Figure 2b–e). This outcome supports our initial hypothesis. Interestingly, similar increases in microbial diversity were observed in the crop rotation system without *A. lancea* (MS treatment), suggesting that both intercropping and rotation may similarly enhance microbial diversity in the short term [49]. Further, incorporating crop rotation (MSA treatment) into intercropping appears to boost the richness of soil bacterial communities (Chao1 index) but simultaneously decreases fungal richness (Figure 2e). This differential response likely stems from the higher sensitivity of bacterial communities to environmental changes compared to fungal communities, which may be less adaptable to the disturbances caused by crop diversification [50,51]. Despite the increase in microbial diversity, there remains no consensus on whether this directly translates to enhanced soil ecosystem functions [52,53]. Functionality might be more dependent on the composition of the microbial communities than merely their diversity [54].

Generally, the composition of soil microbial communities is closely linked to changes in soil physicochemical properties [55,56]. Although *A. lancea* do not notably enhance overall soil microbial diversity, our study found that they significantly strengthen the relationship between soil bacterial communities and soil physicochemical factors (Figure 3). In particular, the composition of the soil bacterial communities in the MA and MSA treatments was primarily influenced by the availability of nitrogen in the form of ammonium nitrogen and nitrate nitrogen. The diversification of *A. lancea* and food crops led to a significant increase in the relative abundance of the phyla Acidobacteriota, Gemmatimonadota, and Chloroflexi (Figure 2h, Supplementary Table S3). This finding aligns with Wang et al., who noted that bacterial communities are more responsive to soil nitrogen fluctuations compared to fungi [52]. The observed decrease in total nitrogen and ammonium nitrogen content is likely the key factor driving the increased abundance of Acidobacteriota, Gemmatimonadota, and Chloroflexi. Numerous studies have demonstrated that these three phyla are oligotrophic, meaning they can thrive in nutrient-limited environments by utilizing minimal resources. Additionally, they contribute to soil nutrient cycling by secreting organic acids that facilitate the transformation of C, N, and P [57–60].

In summary, we found that in diversified cultivation systems involving *A. lancea*, the increase in aboveground species diversity enhances the alpha diversity of soil bacterial communities and raises the relative abundance of oligotrophic bacterial groups. However, this process had less of an effect on the soil fungal community. The primary factors influencing these results may be nutrient competition and niche differentiation between bacteria and fungi [61]. Under crop diversification, different plant species secrete various secondary metabolites, leading to a more complex soil nutrient structure [62,63]. Bacteria, with their ability to metabolize a wider range of compounds and reproduce rapidly, can quickly occupy ecological niches that might otherwise support fungi during the early stages of nutrient competition. This can result in the decline of some rare fungal species [64–66]. Additionally, diversified cropping (MA and MSA) increases the nutrient load demand per unit of soil, as aboveground plants compete for limited resources, reducing the nutrients available for microbial growth (Appendix A Table A1). In response, bacterial communities may enhance their diversity by competing for different resources and evolving various metabolic pathways to adapt to these conditions [67]. In contrast, fungal communities tend to be more stable in nutrient-poor conditions, as they can decompose and utilize complex nutrient structures [68]. Furthermore, the medicinal plant *A. lancea*, used in this study's diversified cropping systems, secretes a large number of secondary metabolites and has high nutrient requirements, potentially intensifying this nutrient competition and affecting microbial dynamics [69,70].

4.2. During Crop Diversification, A. lancea Significantly Enhance the Abundance of Functional Genes Associated with C, N, and P Cycling in Soil Microbial Communities

Both intercropping and crop rotation, alongside diversified cropping strategies, enhance the ability of soil microorganisms to convert soil nutrients effectively [71,72]. This study found that the inclusion of *A. lancea* in these systems significantly increased the content of TP, AP, and NO_3^--N in the soil, while reducing TN and NH_4^+-N levels (Appendix A Table A1). The MA treatment notably increased the abundance of functional genes related to C, N, and P cycling within the soil microbial community, aligning with our hypotheses (Figure 4). However, these positive effects were not observed in the MS treatment, which even showed a reduction in the abundance of functional genes for C and P cycling. This suggests that different methods of crop diversification distinctly influence microbial function and soil nutrient dynamics. Intercropping directly impacts underground microbial functions through root exudates and signaling molecules. For example, in a maize/faba bean (*Vicia faba*) intercropping system, maize secretes flavonoids that enhance rhizobial activity around the faba bean rhizosphere, promoting nitrogen fixation by faba beans, which reduces soil pH and increases phosphorus availability to support maize growth [73,74]. In contrast, crop rotation regulates microbial function indirectly by utilizing the residual effects of the previous crop on soil nutrients [75]. For instance, during the soybean (*Glycine max* L.)/maize rotation, nitrogen-fixing bacteria in the soybean's root nodules can regulate key enzymes in the nitrogen mineralization pathway, creating legacy effects that impact the abundance of nitrogen cycling functional genes during the subsequent maize growing period [76].

Medicinal plants such as *A. lancea* secrete a wide array of organic compounds, including sesquiterpenoids, triterpenoids, polyacetylenes, coumarins, phenylpropanoids, flavonoids and flavonoid glycosides, steroids, benzoquinones, and polysaccharides [77], which potentially enhance the abundance of functional genes related to C, N, and P cycling [78,79]. However, the crops chosen for rotation, sorghum and corn, which are both from the Poaceae family, do not vary significantly in nutrient utilization or types of metabolites, limiting the benefits of nutrient legacy. This is a likely reason why the MS treatment did not enhance the abundance of functional genes related to C, N, and P cycling in the soil microbial community [80,81].

Furthermore, the MSA treatment was found to further enhance the abundance of functional genes related to C, N, and P cycles in soil microbial communities (Figure 4e–h). Interestingly, under the MS treatment, the pathways for cellulose degradation and assimilatory nitrogen reduction saw the greatest decreases. However, in the MSA treatment, these pathways actually showed the greatest increases in functional gene abundance. This suggests that including sorghum rotation in the maize/*A. lancea* intercropping system might trigger special interspecies interaction mechanisms, amplifying the benefits of intercropping, potentially through the mutual stimulation of certain metabolic products. Future studies will include metabolomics data to explore these interactions further [7].

4.3. A. lancea May Not Increase Yield During Crop Diversification, but Can Improve Economic Productivity and Enhance Ecological Benefits of Farmland

Crop yield is a direct indicator of farmland productivity, and while intercropping medicinal plants with cereal crops is often associated with enhanced soil productivity [82], our study observed a significant decrease in the yields of food crops in both the MA and MSA treatments (Table 1). This finding contradicts our hypothesis that intercropping *A. lancea* with food crops would enhance soil productivity. Nonetheless, *A. lancea* possess a higher economic value, and despite the lower yields during intercropping, the economic benefits significantly surpassed those from monoculture corn planting (Table 1). Diversified cropping is generally reported to boost the ecological benefits of soil [83], a finding that aligns with our observations where the ecological benefits of the soil under the MA (5.36) and MSA (8.15) treatments were significantly greater than those under the MC treatment. Contrarily, the ecological benefit assessment for the MS treatment (−4.52) was lower than that of the MC treatment. Upon analyzing the composition of the evaluation factors, we identified that the primary limiting factor for the ecological benefits in the MS treatment was the soil nitrogen content (Supplementary Table S6). This limitation is not only present in the MS treatment; the assessment results of soil nitrogen content in both the MA and MSA treatments were also negative (Supplementary Table S6). *A. lancea*, used in our intercropping system, is a high nitrogen-demanding crop, and the cultivation of sorghum, which requires more nitrogen fertilizer than corn, exacerbates this demand [69,84]. Given that only 300 $\text{kg}\cdot\text{ha}^{-1}$ of urea was applied annually, the nitrogen supply was insufficient, constraining further improvements in soil productivity and ecological benefits [84,85]. Therefore, in diversified cropping systems, implementing scientific fertilizer management and ensuring timely nitrogen supplementation are crucial for optimizing productivity and ecological outcomes.

4.4. Research Limitations

This study assesses the influence of *A. lancea* on soil microbial communities when intercropped with food crops, particularly focusing on how these plants affect the abundance of functional genes associated with C, N, and P cycling in soil microbial communities through metagenomic analysis. Despite these insights, the absence of data on the differences in metabolic products between various species combinations limits our understanding of the precise mechanisms through which *A. lancea* influence these functional genes. Future research will aim to fill this gap. Moreover, as *A. lancea* are perennial, the two-year scope of this study does not encompass their full growth cycle. It remains unclear whether additional changes occur over a longer period. Investigating the impact of incorporating *A. lancea* into agricultural ecosystems on insect communities is also of great importance. Therefore, ongoing monitoring and extended observation will be crucial to uncover further dynamics within these intercropping systems.

5. Conclusions

In summary, this study demonstrates that *A. lancea* contribute positively to diversified cropping systems by enhancing the alpha diversity of microbial communities and increasing the abundance of functional genes associated with carbon (C), nitrogen (N), and phosphorus (P) cycling. While *A. lancea* alone may not improve soil productivity, their intercropping with food crops leads to increased economic and ecological benefits. Implementing appropriate rotational treatments that combine *A. lancea* with food crops can further amplify these effects. This research offers new insights into diversified planting, especially concerning the selection and practical combinations of species in agricultural settings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14122327/s1, Supplementary Table S1: Differences in soil chemical properties under different diversified cropping systems. Supplementary Table S2: Comparison of microbial diversity among different planting treatments and post hoc test *p*-values. Supplementary Table S3: Differences in relative abundance of soil bacterial phyla under different planting treatments. Supplementary Table S4: Differences in relative abundance of soil fungal phyla under different planting treatments. Supplementary Table S5: Differences in functional gene abundance of soil bacterial communities under different planting treatments. Supplementary Table S6: Effect size and weight of nutrient cycling functions in soil bacterial communities under various diversified cropping systems based on inverse variance weighting method.

Author Contributions: C.T. and E.W. conceptualized and designed the study. M.L., L.Y., X.Y., Z.Y. and E.W. performed the experiments. M.L. and E.W. analyzed the data and prepared the figures and tables. Y.S. and E.W. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw sequences were submitted to the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) under study accession numbers PRJNA1123888, PRJNA1123895, PRJNA1124387.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A

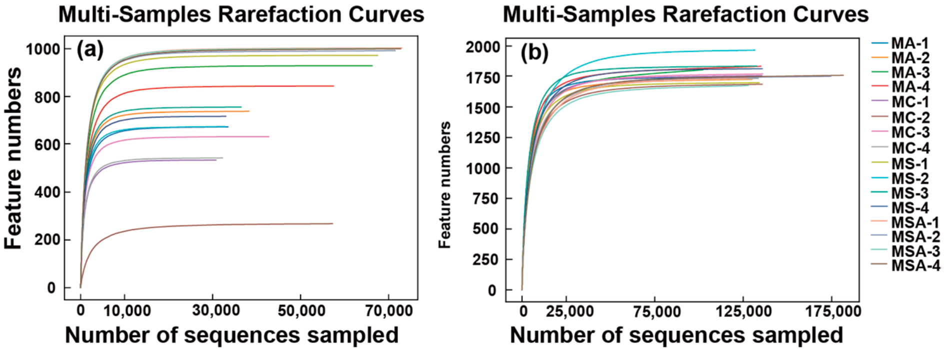

Figure A1. The rarefaction curves of the bacterial and fungal communities. A depiction of the rarefaction curves for (**a**) bacterial and (**b**) fungal communities, respectively. The horizontal axis denotes the number of sequencing reads sampled randomly from the data, while the vertical axis represents the observed number of features (OTUs).

Table A1. Differences in soil chemical properties under different diversified cropping systems.

Index	TC (g·kg^{-1})	OM (%)	TN (g·kg^{-1})	NH_4^+-N (mg·kg^{-1})	NO_3^--N (mg·kg^{-1})	TP (mg·kg^{-1})	AP (mg·kg^{-1})	pH	EC (mS·cm^{-1})
CK	16.42 ± 0.17 b	3.09 ± 0.28 b	1.54 ± 0.06 c	5.19 ± 0.38 b	37.20 ± 2.42 a	544.65 ± 8.33 b	45.14 ± 1.57 d	6.43 ± 0.06 d	96.26 ± 2.91 a
MC	18.90 ± 1.64 a	3.22 ± 0.07 b	1.98 ± 0.07 a	15.04 ± 0.35 a	4.56 ± 0.22 e	530.18 ± 19.12 b	55.38 ± 1.55 c	6.94 ± 0.03 c	62.68 ± 1.54 b
MA	19.28 ± 0.49 a	3.29 ± 0.10 b	1.66 ± 0.05 b	2.50 ± 0.17 c	18.49 ± 0.35 c	599.30 ± 12.58 a	83.65 ± 0.50 a	6.99 ± 0.07 c	52.29 ± 1.22 c
MS	16.84 ± 0.20 b	2.79 ± 0.27 c	1.48 ± 0.03 c	2.13 ± 0.07 c	6.42 ± 0.24 d	455.90 ± 6.38 c	42.89 ± 1.04 e	7.23 ± 0.02 a	38.34 ± 1.94 e
MSA	19.21 ± 1.00 a	3.86 ± 0.17 a	1.50 ± 0.01 c	5.27 ± 0.32 b	26.69 ± 0.57 b	581.50 ± 9.41 a	81.30 ± 1.66 b	7.13 ± 0.03 b	43.67 ± 2.55 d

Note: CK: soil chemical properties in May 2022 before planting crops. MC: continuous maize cultivation; MA: maize–*A. lancea* intercropping; MS: maize–sorghum rotation; MSA: maize–*A. lancea* intercropping in 2022, maize–sorghum rotation in 2023. TC: total carbon; SOM: soil organic matter; TN: total nitrogen; NH_4^+-N: ammonium nitrogen; NO_3^--N: nitrate nitrogen; TP: total phosphorus; AP: available phosphorus; EC: electrical conductivity. Table data shown as mean ± standard deviation (*n* = 4). Different lowercase letters in same column indicate significant differences between sample groups (ANOVA, Tukey's HSD test).

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