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Effects of Differential Tobacco Straw Incorporation on Functional Gene Profiles and Functional Groups of Soil Microorganisms

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Abstract

Straw returning is a critical practice with profound strategic importance for sustainable agricultural development. However, within a comprehensive soil health evaluation framework, research analyzing the impact of tobacco straw returning on soil ecosystem health from the perspectives of microbial taxa and functional genes remains insufficient. To investigate the effects of tobacco straw returning on virulence factor genes (VFGs), methane-cycling genes (MCGs), nitrogen-cycling genes (NCGs), carbohydrate-active enzyme genes (CAZyGs), antibiotic resistance genes (ARGs), and their host microorganisms in soil, this study collected soil samples from a long-term tobacco-rice rotation field with continuous tobacco straw incorporation in Shaowu City, Fujian Province. Metagenomic high-throughput sequencing was performed on the samples. The results demonstrated that long-term tobacco straw returning influenced the diversity of soil VFGs, MCGs, NCGs, CAZyGs, ARGs, and their host microorganisms, with richness significantly increasing compared to the CK treatment ($p < 0.05$). In the microbially mediated methane cycle, long-term tobacco straw returning resulted in a significant decrease in the abundance of the key methanogenesis gene *mttB* and the methanogenic archaeon Methanosarcina, along with a reduced *mtaB*/*pmoA* functional gene abundance ratio compared to CK. This suggests enhanced CH_4 oxidation in the tobacco-rice rotation field under straw returning. Notably, the abundance of plant pathogens increased significantly under tobacco straw returning. Furthermore, a significantly higher *norB*/*nosZ* functional gene abundance ratio was observed, indicating a reduced capacity of soil microorganisms to convert N_2O in the tobacco-rice rotation field under straw amendment. Based on the observation that the full-rate tobacco straw returning treatment (JT2) resulted in the lowest abundances of functional genes *prkC*, *stkP*, *mttB*, and the highest abundances of *nirK*, *norB*, *malZ*, and *bglX*, it can be concluded that shifts in soil physicochemical properties and energy substrates drove a transition in microbial metabolic strategies. This transition is characterized by a decreased pathogenic potential of soil bacteria, alongside an enhanced potential for microbial denitrification and cellulose degradation. Non-parametric analysis of matrix correlations revealed that soil organic carbon, dissolved organic carbon, alkaline-hydrolyzable nitrogen, available phosphorus, and available potassium were significantly correlated with the composition of soil functional groups ($p < 0.05$). In conclusion, long-term tobacco straw returning may increase the risk of soil-borne diseases in tobacco-rice rotation systems while potentially elevating N_2O and reducing CH_4 greenhouse gas emission rates. Analysis of functional gene abundance changes identified the full-rate tobacco straw returning treatment as the most effective among all treatments.

Keywords: tobacco stalk returning; metagenomics; functional gene; function group; plant pathogen

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1. Introduction

China is a leading tobacco-producing nation, consistently ranking first globally in both tobacco leaf and cigarette production. The tobacco industry serves as a pivotal sector supporting the national economy and has made significant contributions to improving living standards [1]. Fujian Province, with its comprehensive ecological conditions ideally suited to the "temperature, light, water, and soil" requirements of tobacco growth, stands as one of China's three major premium tobacco-growing regions. It cultivates approximately 200,000 hectares of tobacco [2]. Flue-cured tobacco, being a crop sensitive to continuous cropping, benefits from rotation systems which enhance root vitality and disease resistance in subsequent crops. Since the 1980s, the region has predominantly adopted a flue-cured tobacco–rice rotation system. The soil microbial communities (including bacteria, fungi, archaea, and protozoa) act as a core driver in agroecosystems, with their community structure, diversity, and functional activity serving as key indicators of soil biological fertility. Recent advances in microbial ecology have increasingly demonstrated that soil microflora is closely linked to crop health and productivity through multiple direct and indirect pathways. On one hand, microorganisms drive the biogeochemical cycles of essential elements such as carbon, nitrogen, and phosphorus, directly determining nutrient supply efficiency via processes including organic matter decomposition, biological nitrogen fixation, and nutrient activation. On the other hand, they form a biological defense line against soil-borne pathogens by competing for ecological niches, secreting antagonistic substances, and inducing systemic resistance in plants. Furthermore, microbial secretion of extracellular polysaccharides promotes the formation of soil aggregates, improving soil physical structure, while phytohormones produced by microbes directly regulate root development and plant growth. Through long-term agricultural practice and scientific research, straw returning has been recognized as an effective management measure for maintaining soil fertility. Rich in organic matter and macro-nutrients, straw serves as an important external input of organic carbon, not only providing energy and material foundations for microbial growth but also driving the evolution of microbial community structure and function by altering the soil micro-environment. Studies have shown that the decomposition of straw significantly enhances the diversity of soil bacteria and fungi, activates key functional microbial groups involved in carbon and nitrogen cycling (e.g., cellulose-decomposing bacteria, nitrogen-fixing bacteria, and arbuscular mycorrhizal fungi), and promotes soil enzyme activities, thereby accelerating organic matter mineralization and nutrient release [3]. This microbe-driven nutrient transformation mechanism effectively improves the bioavailability of soil nitrogen, phosphorus, and potassium [4], while the formation of beneficial microbial networks suppresses the proliferation of soil-borne pathogens. Concurrently, improved soil physicochemical properties and a stable nutrient supply create favorable conditions for root development and plant physiological metabolism, ultimately leading to optimized plant biomass accumulation and yield formation. The tobacco-rice rotation system generates a substantial amount of straw. It is estimated that Fujian Province produces between 100,000 and 200,000 tons of tobacco straw annually [2]. Returning tobacco straw to the field can reduce the costs associated with environmental remediation and straw disposal caused by traditional burning or landfilling. Moreover, after microbial decomposition, the straw is converted into organic matter, which can effectively substitute for partial chemical fertilizer input, particularly enhancing potassium utilization efficiency and reducing potassium fertilizer application. Studies indicate that tobacco straw is rich in nutrients such as sugars, proteins, lipids, and nitrogen-free extracts [5,6]. Its application improves soil fertility and nutrient supply status and shows notable effects in controlling rice sheath blight [7]. Treated tobacco straw can alter soil microbial community structure, increase the bacteria-to-fungi ratio, enhance soil microbial diversity, and contribute to the

suppression of tobacco bacterial wilt [8]. An appropriate amount of tobacco straw returning can increase the content of soil available nitrogen, phosphorus, and potassium, positively influencing tobacco yield and quality [9–11].

Tobacco straw incorporation drives the evolution of soil ecological functions through multiple molecular mechanisms. During the paddy flooding phase, its rapid decomposition leads to a sharp decrease in redox potential. This anaerobic environment directly determines the net methane flux by regulating the relative abundance of the *mcrA*/*mttB* genes in methanogenic archaea versus the *pmoA* gene in aerobic methanotrophs [12]. In terms of the nitrogen cycle, straw acts as an electron donor, activating the denitrification cascade. The expression ratios of functional genes such as *nirK*/*nirS* (nitrite reduction), *norB* (nitric oxide reduction), and *nosZ* (nitrous oxide reduction) determine whether nitrogen is released as the greenhouse gas N_2O or the inert N_2 [13]. Simultaneously, lignocellulose decomposition requires the microbial community to coordinately express specific CAZyme families: *GH5*/*GH9* families act on cellulose backbone deconstruction, *GH10*/*GH11* families target hemicellulose hydrolysis, while auxiliary activity families such as *AA9* disrupt the lignin sheath structure through oxidative reactions [14]. Residual alkaloids and heavy metals in the straw may promote the co-transfer of antibiotic resistance genes (ARGs) and virulence factor genes (VFGs) via co-selection pressure. This risk is further amplified under alternating wet and dry conditions-the synergy between microbial stress responses triggered by moisture fluctuations and increased activity of mobile genetic elements (e.g., plasmids, transposons) ultimately accelerates the dissemination of the resistome and virulome within the soil microbial network [15]. Currently, research on the ecological effects of straw return in rice-based systems has formed a substantial body of knowledge. Multiple meta-analyses indicate that straw return has a significant positive effect on enhancing soil organic carbon (SOC) sequestration [16], but its impact on greenhouse gas emissions is complex, often promoting CH_4 emissions while exerting variable effects on N_2O flux [17]. Furthermore, emerging research perspectives are focusing on straw input as an environmental selective pressure on the soil micro-ecology, revealing that it can influence the abundance and dissemination of antibiotic resistance genes (ARGs) and indirectly modulate the potential pressure of soil-borne pathogens by altering the microbial community structure [18]. However, although the comprehensive effects of straw return have been preliminarily elucidated in conventional rice systems, a significant knowledge gap remains regarding the specific tobacco-rice rotation system. Tobacco straw, as a biomass containing specific secondary metabolites (e.g., nicotine), may produce unique effects distinct from those of conventional straws like rice or wheat after incorporation. Currently, there is a lack of systematic analysis, particularly utilizing modern biological techniques such as metagenomics. Therefore, this study aims to fill this gap by thoroughly investigating the specific microbial ecological effects induced by differential tobacco straw incorporation in the tobacco-rice rotation system.

At the metagenomic level, this study employs shotgun sequencing to comprehensively analyze the functional potential of the soil microbial community. Unlike marker gene sequencing (e.g., 16S rRNA gene sequencing), which only reflects taxonomic structure, shotgun metagenomics involves random fragmentation and sequencing of total microbial DNA from the environment. This approach not only accurately identifies species composition but also fully captures the functional gene repertoire of the microbial community (including metabolic pathways, antibiotic resistance genes, and virulence factors), thereby overcoming the limitations of marker gene sequencing in functional prediction accuracy and resolution [19]. Combined with bioinformatics workflows (including sequence assembly, gene prediction, functional annotation, and host tracking), this method enables direct linkage of microbial community structure to functional mechanisms, providing

key technical support for in-depth exploration of how tobacco straw returning regulates soil ecosystem functions [20]. While numerous studies have reported the effects of straw returning on soil physicochemical properties, microbial communities, greenhouse gas emissions, and pathogen dynamics, detailed analyses of the comprehensive impact of long-term tobacco straw returning on the soil ecological environment are still lacking. Based on the above background, this study proposes the following core hypotheses: different application rates of tobacco straw will alter soil microbial community structure, particularly affecting the abundance and composition of microbial taxa with specific metabolic functions (e.g., carbohydrate metabolism, nitrogen cycling); increased straw application will significantly influence the abundance of functional genes related to biogeochemical cycles (e.g., nitrogen cycle genes, methane cycle genes), and these changes will be closely associated with shifts in the abundance of specific host microorganisms; while modifying beneficial functional genes, tobacco straw returning may also increase the potential dissemination risk of antibiotic resistance genes and virulence factor genes by affecting the distribution of host microorganisms; the interconnected changes in microbial community structure and function will ultimately impact the health and stability of the soil ecosystem, with threshold effects dependent on the application rate. To test these hypotheses, this study investigates soils under long-term tobacco straw returning in a tobacco–rice rotation system. Using metagenomics, we analyze the correspondence between functional genes and their hosts, thereby linking soil microbial community composition with its functional attributes. We aim to examine the effects of different tobacco straw application rates on virulence factor genes, methane cycling genes, nitrogen cycling genes, carbohydrate metabolism genes, antibiotic resistance genes, and their host microorganisms. The findings are expected to provide a reference for a comprehensive analysis and objective evaluation of the impact of tobacco straw returning on integrated soil health.

2. Materials and Methods

2.1. Overview of Test Site

The experiment employed *Cuibi No. 1*, (Fujian branch of China national tobacco corporation, Fuzhou, China) a major flue-cured tobacco cultivar in Fujian Province, and the rice cultivar *Nongliangyou* (*China national rice research institute*, *Hangzhou*, *China*) for the late season. A five-year continuous monitoring study was conducted from October 2019 to October 2024 in Fangqian Village, Xiaojiafang Town, Shaowu City (117°26′67″ E, 27°14′36″ N). The experimental region is characterized by a mid-subtropical maritime monsoon climate. The soil is classified as a hydragric paddy soil with a silt loam texture. The fundamental physicochemical properties of the soil at the initiation of the experiment were as follows: pH 5.42, organic matter 37.29 g/kg, total nitrogen 2.14 g/kg, total phosphorus 0.9 g/kg, total potassium 19 g/kg, available nitrogen 197.18 mg/kg, available phosphorus 50.61 mg/kg, and available potassium 195.12 mg/kg, particle size distribution: clay (<0.002 mm) 14.3%; silt (0.02–0.002 mm) 32.2%; fine sand (0.2–0.02 mm) 47.8%; coarse sand (2–0.2 mm) 5.7%, cation exchange capacity (CEC) 6.18 cmol(+) kg^{-1}, exchangeable acidity 3.78 cmol(+) kg^{-1} [21].

2.2. Experimental Design

The field experiment comprised four treatments (Table 1). The planting densities were 1.15 m × 0.5 m for tobacco and 0.2 m × 0.2 m for late-season rice. Conducted from 2019 to 2024, the trial was repeated annually over five consecutive cropping years. Each year, a full straw return practice was maintained, with average application rates of 3750 kg ha^{-1} (dry weight basis) for rice straw and 2500 kg ha^{-1} (dry weight basis) for tobacco straw. In the tobacco-rice rotation system, irrigation and fertilization for both crops followed local

conventional practices (see Table S2 and Figure S1 in the Supplementary Materials), with consistent application rates and management measures across all treatments.

Table 1. Design of field plot experiments.

Experimental Design	Treatment			
	CK	**JT1**	**JT2**	**JT3**
Plot area (m^2)	40	40	40	40
Number of plots	3	3	3	3
Straw return amount	Full rice straw + No tobacco straw	Full rice straw + Half tobacco straw	Full rice straw + Full tobacco straw	Full rice straw + doubled tobacco straw

2.3. Test Method

2.3.1. Experimental Soil

Soil samples were collected once during the peak growth stage of tobacco in 2025. An S-shaped five-point sampling method was employed to form one composite sample per plot, with three replicates established. During sampling, the surface litter was first removed, and samples were taken from the 0–20 cm depth. From each composite sample, 50 g of soil was stored at −80 °C for soil microbial community structure and functional analysis, while 200 g was air-dried indoors for determining soil physicochemical properties.

2.3.2. Determination of Soil Physical and Chemical Properties

Soil pH was measured potentiometrically with a pH meter using a 1:2.5 (w/v) soil-to-water suspension in distilled water. Total nitrogen (TN) content was determined by the semi-micro Kjeldahl method. Total phosphorus (TP) content was measured using the acid dissolution-molybdenum antimony anti-colorimetric method. Soil organic matter (SOM) content was quantified by the $K_2Cr_2O_7$-H_2SO_4 external heating method (Walkley-Black method). Available phosphorus (AP) was extracted with $NaHCO_3$ and determined by the molybdenum antimony anti-colorimetric method. Available potassium (AK) was extracted with 1 mol/L NH_4OAc solution and measured by flame photometry. Alkali-hydrolyzable nitrogen (AN) was determined by the alkali hydrolysis diffusion method. Dissolved organic carbon (DOC) was extracted with ultrapure water and analyzed using TOC analyzer. The mechanical composition (particle size distribution) was analyzed by the hydrometer method. The cation exchange capacity of the soil was determined using the ammonium acetate exchange method [22–24].

2.3.3. Soil DNA Extraction and Metagenomic Sequencing

There were four treatments, with three replicates per treatment, resulting in a total of 12 sequencing samples. Each sample used 0.3 g of soil. Genomic DNA was extracted from the soil samples using the Mag-Bind® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The concentration and purity of the extracted DNA were quantified, and its integrity was verified by 1% agarose gel electrophoresis. The DNA was then fragmented using an M220 ultrasonicator (Covaris, Woburn, MA, USA) to an average size of approximately 350 bp. These fragments were used to construct paired-end libraries with the NEXTFLEX® Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, USA). All libraries were subjected to metagenomic sequencing on an Illumina NovaSeq™ X Plus platform (Illumina Inc., San Diego, CA, USA) by Shanghai Majorbio Bio-pharm Technology Co., Ltd. Paired-end 150. The raw sequencing data have been deposited in the NCBI Sequence Read Archive under the BioProject accession number PRJNA1301848.

The data were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com, 9 June 2025). Briefly, the paired-end Illumina reads were trimmed of

adaptors. And low-quality reads (length < 50 bp or with a quality value < 20) were removed by fastp (https://github.com/OpenGene/fastp, version 0.23.0, 9 June 2025). Metagenomics data were assembled using MEGAHIT (https://github.com/voutcn/megahit, version 1.1.2, 9 June 2025), which makes use of succinct de Bruijn graphs. Contigs with a length $\geq$ 500 bp were selected as the final assembling result, and then the contigs were used for further gene prediction and annotation. A non-redundant gene catalog was constructed. Using CD-HIT (http://www.bioinformatics.org/cd-hit/, version 4.6.1, 9 June 2025) with 90% sequence identity and 90% coverage. High-quality reads were aligned to the non-redundant gene catalogs to calculate gene abundance with 95% identity using SOAPaligner (https://github.com/ShujiaHuang/SOAPaligner, version 2.21, 9 June 2025). Using SOAPaligner (https://github.com/ShujiaHuang/SOAPaligner; version 2.21, 9 June 2025), the high-quality reads from each sample were mapped to the non-redundant gene set with a sequence identity threshold of 95%. The abundance of each gene in the corresponding sample was then calculated.

2.3.4. Soil Microbial Function Annotation

Sequences were aligned against the KEGG 20241007 (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/, 19 June 2025), VFDB 20240301 (Virulent Factor Database, http://www.mgc.ac.cn/VFs/, 19 June 2025), CAZy v12 (Carbohydrate-active enzymes, http://www.cazy.org/, 19 June 2025) and CARD v3.2.9 (Comprehensive Antibiotic Resistance Database, https://card.mcmaster.ca/, 19 June 2025) databases to construct specialized gene sets, including virulence factor genes (VFGs), methane-cycling genes (MCGs), nitrogen-cycling genes (NCGs), carbohydrate-active enzyme genes (CAZyGs), and antibiotic resistance genes (ARGs). The amino acid sequences of the non-redundant gene catalog were subjected to BLASTP analysis (BLAST Version 2.2.28+; http://blast.ncbi.nlm.nih.gov/Blast.cgi, 9 June 2025) against the KEGG, VFDB, and CARD databases with an E-value cutoff of 1×10^{-5} [25]. This step provided functional annotations, associating genes with KEGG orthology, virulence factors, and antibiotic resistance functions. The abundance of each functional category (e.g., KO, Pathway, EC number, Module) was calculated as the sum of the abundances of all genes annotated to that category. Sequences that did not yield significant hits in the initial BLASTP run against the VFDB were realigned against the VFDB prediction dataset. The abundance of each antibiotic resistance function was calculated as the cumulative abundance of all genes annotated to that specific function. For annotation against the CAZy database, the amino acid sequences were analyzed using the hmmscan tool [14] with a significance E-value threshold of 1×10^{-5} to assign carbohydrate-active enzyme annotations. The abundance of each CAZy family was subsequently calculated as the sum of the abundances of all genes assigned to it [26]. Finally, the abundance information for all functional genes (MCGs, NCGs, VFGs, CAZyGs, ARGs) and their corresponding host taxonomic groups was consolidated for downstream analysis.

2.3.5. Data Analysis

Principal coordinate analysis (PCoA), community bar plots, and redundancy analysis (RDA) were performed using R language (version 3.3.1). Normality and homogeneity of variances were tested prior to conducting analysis of variance (ANOVA). One-way ANOVA followed by Least Significant Difference (LSD) post hoc tests (α = 0.05) for multiple comparisons was carried out using SPSS 24.0. Graphs were generated with GraphPad Prism 8. The effects of different treatments on bacterial community Alpha-diversity and predicted functional gene abundances were assessed using mothur v.1.30.2 (https://mothur.org/wiki/calculators/, 9 June 2025). Significant differences were

determined by the Kruskal–Wallis rank sum test. Beta diversity, representing the similarity or dissimilarity of bacterial community structures among treatments, was analyzed via Principal Co-ordinates Analysis (PCoA) based on a Bray–Curtis dissimilarity matrix. Permutational multivariate analysis of variance (Adonis) was employed to test for significant differences in microbial community/functional structure between groups. The relationship between functional gene hosts and environmental factors was examined using the Mantel test, a non-parametric method for assessing correlation between two matrices.

3. Results and Analysis

3.1. Effects of Different Tobacco Straw Returning Modes on Soil Microbial Functional Genes and Functional Group Diversity

Bioinformatic analysis of soil samples subjected to different tobacco straw returning treatments revealed changes in the diversity of VFGs, MCGs, NCGs, CAZyGs, ARGs and their host microorganisms. As shown in Table 2, tobacco straw returning resulted in a significant increase ($p < 0.05$) in the diversity of these functional genes and their respective host communities. Figure 1 illustrates that tobacco straw returning altered the structure of functional microbial communities in the tobacco-rice rotation soil. The composition of the total microbial community (bacteria, archaea, fungi total species taxa), methane cycle microbial community (MCGs taxa), nitrogen cycle microbial community (NCGs taxa), potential pathogenic microbial community (VFGs taxa), carbohydrate metabolism related microbial community (CAZyGs taxa), and antibiotic resistance microbial community (CARGs taxa) of the full-rate tobacco straw returning treatment JT2 and CK treatment were located on the left and right sides of the first principal component PCoA1, respectively. This distribution indicates a significant difference in β diversity compared to the control. In contrast, the JT1 and JT2 treatments exhibited overlapping clusters, suggesting a relatively similar species composition of soil microbial communities between these two treatments.

Table 2. Changes in soil microbial functional genes and functional group diversity under different tobacco straw returning treatments.

Treatment	VFGs		MCGs		NCGs	
	Ace Index	Shannon Index	Ace Index	Shannon Index	Ace Index	Shannon Index
CK	2395 ± 382.3 a	6.374 ± 0.189 a	51.67 ± 3.667 a	2.965 ± 0.178 a	38.33 ± 0.33 a	2.755 ± 0.128 a
JT1	2777 ± 387.7 b	6.563 ± 0.232 b	55.33 ± 0.333 a	2.787 ± 0.329 b	38.67 ± 2.00 a	2.628 ± 0.135 b
JT2	2782 ± 352.7 b	6.606 ± 0.202 b	54.67 ± 0.667 a	2.636 ± 0.180 c	40.33 ± 1.67 a	2.621 ± 0.088 bc
JT3	2747 ± 29.67 b	6.576 ± 0.044 b	54.00 ± 1.667 a	2.785 ± 0.151 b	40.00 ± 1.33 a	2.668 ± 0.048 c
Treatment	**CAZyGs**		**CARGs**			
	Ace Index	**Shannon Index**	**Ace Index**	**Shannon Index**		
CK	890.7 ± 167.0 a	5.479 ± 0.139 a	1033 ± 158.0 a	5.472 ± 0.162 a		
JT1	1058 ± 190.0 bd	5.618 ± 0.123 b	1191 ± 161.7 b	5.634 ± 0.183 b		
JT2	1081 ± 150.7 b	5.602 ± 0.125 b	1195 ± 142.7 b	5.656 ± 0.161 b		
JT3	1041 ± 23.0 cd	5.604 ± 0.016 b	1176 ± 19.0 b	5.633 ± 0.022 b		
Treatment	**VFGs Taxa**		**MCGs Taxa**		**NCGs Taxa**	
	Ace Index	**Shannon Index**	**Ace Index**	**Shannon Index**	**Ace Index**	**Shannon Index**
CK	6097 ± 184.30 a	5.21 ± 0.805 a	728.3 ± 239.7 a	4.840 ± 0.487 a	477.7 ± 118.3 a	4.505 ± 0.346 a

Table 2. *Cont.*

Treatment	VFGs Taxa		MCGs Taxa		NCGs Taxa	
	Ace Index	Shannon Index	Ace Index	Shannon Index	Ace Index	Shannon Index
JT1	9039 ± 334.08 b	6.02 ± 0.294 b	968.0 ± 173.7 bd	5.326 ± 0.454 b	596.0 ± 89.3 b	4.851 ± 0.017 b
JT2	8952 ± 487.00 b	5.51 ± 0.601 b	902.0 ± 216.3 c	5.294 ± 0.365 b	567.0 ± 110.3 c	4.489 ± 0.323 a
JT3	8855 ± 297.33 b	5.81 ± 0.510 b	944.7 ± 42.7 cd	5.205 ± 0.032 c	588.0 ± 29.0 bc	4.828 ± 0.362 b
Treatment	**CAZyGs Taxa**		**CARGs Taxa**			
	Ace Index	**Shannon Index**	**Ace Index**	**Shannon Index**		
CK	3689 ± 85.12 a	5.073 ± 0.853 a	3470 ± 169.33 a	5.059 ± 0.775 a		
JT1	5609 ± 38.00 bd	5.926 ± 0.109 b	5241 ± 73.67 b	5.833 ± 0.296 b		
JT2	5647 ± 187.70 b	5.182 ± 0.741 b	5172 ± 281.11 b	5.354 ± 0.610 c		
JT3	5421 ± 225.70 cd	5.814 ± 0.744 b	5168 ± 502.00 b	5.669 ± 0.479 b		

Note: CK, no application of tobacco straw; JT1, half-rate tobacco straw incorporation; JT2, full-rate tobacco straw incorporation; JT3, double-rate tobacco straw incorporation. Different lowercase letters indicated significant differences between different treatments ($p < 0.05$). The data in the table are mean ± standard deviation ($n = 3$).

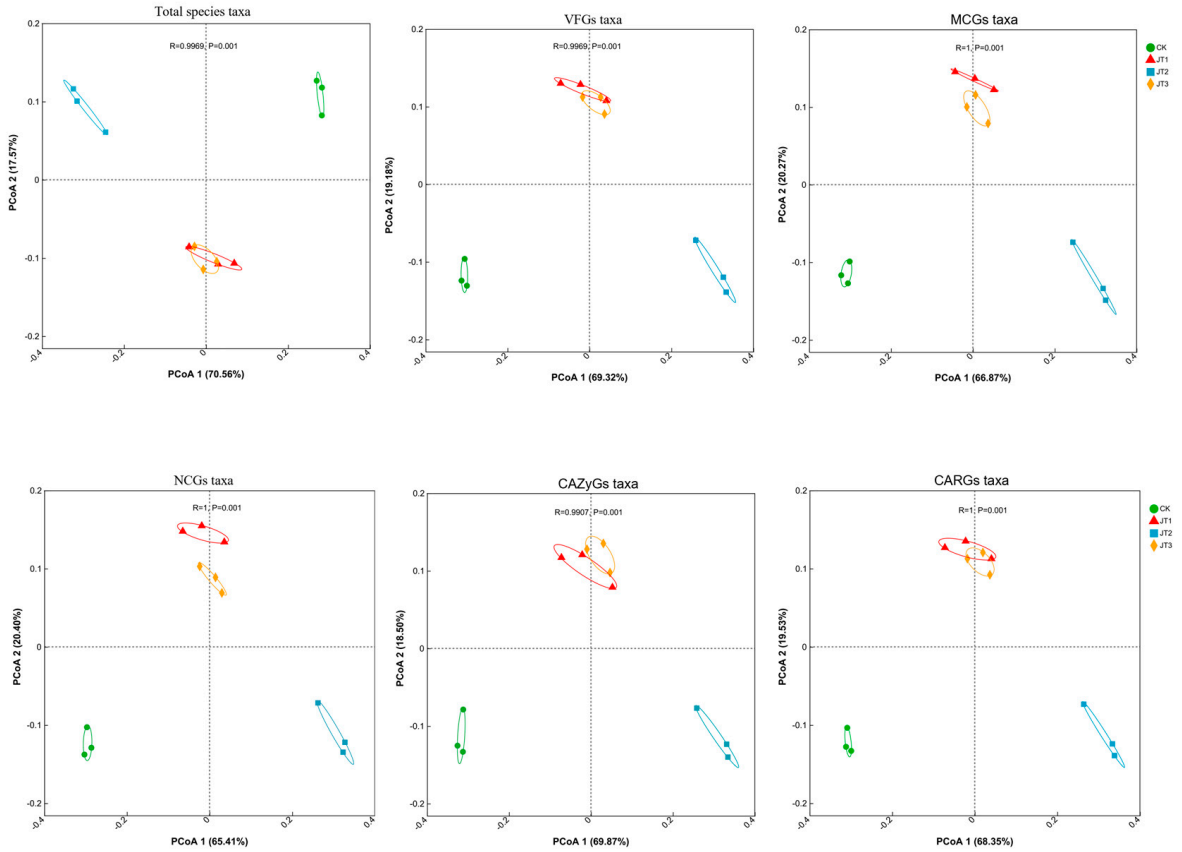


Figure 1. Changes of β diversity of soil microbial functional groups in different treatments. CK, no application of tobacco straw; JT1, half-rate tobacco straw incorporation; JT2, full-rate tobacco straw incorporation; JT3, double-rate tobacco straw incorporation ($n = 3$, $p < 0.05$).

3.2. Effects of Different Tobacco Straw Returning Modes on the Abundance of Soil Microbial Functional Groups

Figure 2 displays the relative abundance of soil microbial functional guilds under different treatments. In the JT1 and JT3 treatments, Pseudomonadota served as the dominant phylum within both the potentially pathogenic microorganisms and the carbohydrate-

active enzyme-associated microorganisms. In the JT2 treatment, Pseudomonadota and Actinomycetota jointly dominated these two functional guilds. Furthermore, Pseudomonadota and Actinomycetota were identified as the predominant phyla in the methane-cycling and nitrogen-cycling microbial communities across all treatments. To further identify microbial functional taxa whose abundance changed significantly following the application of different quantities of tobacco straw, a LEfSe analysis was performed to compare the four treatments. The results, shown in Figure 3, revealed significant differences ($p < 0.05$) at the phylum level. Specifically, the JT2 treatment showed a significant enrichment of Bathyarchaeota (a phylum with methane metabolic capabilities). The JT3 treatment exhibited significant differences in the abundances of Nanoarchaeota and Nitrososphaerota (the latter plays a crucial role in the nitrogen cycle). At the genus level, the JT1 treatment showed significant differences in the abundances of *Thalassiosira* (a genus implicated in the carbon cycle) and *Methanoregula*.

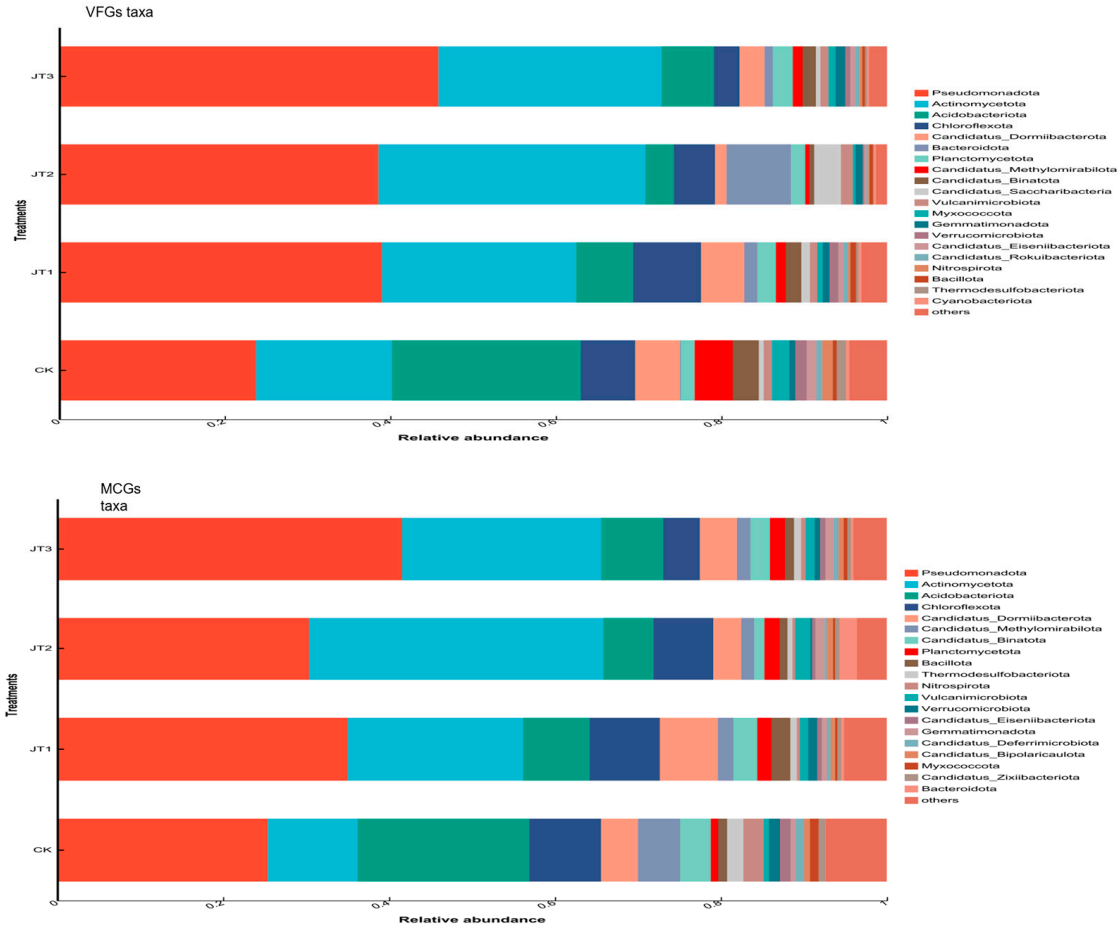


Figure 2. *Cont.*

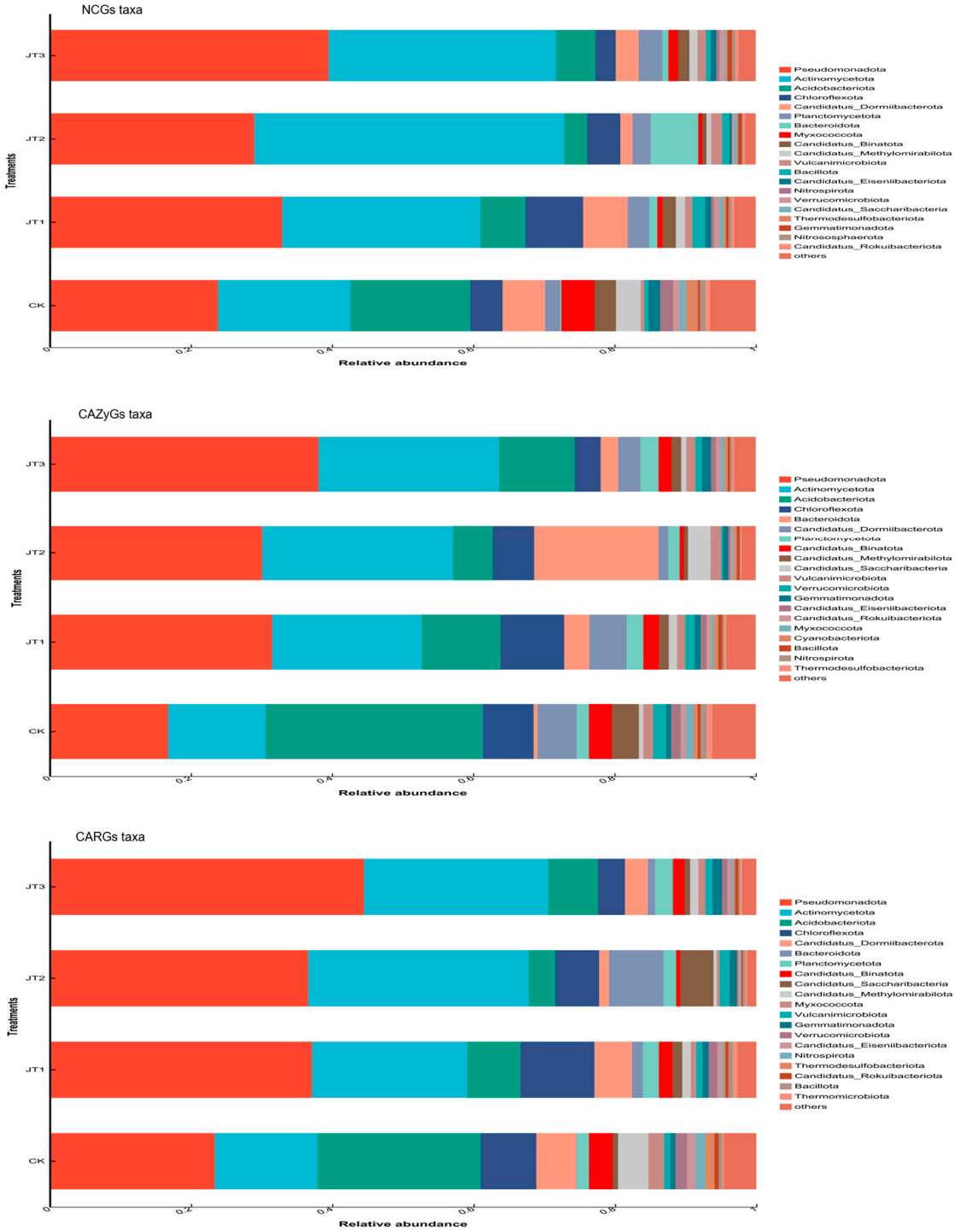


Figure 2. Abundance changes in soil microbial functional groups under different treatments. CK, no application of tobacco straw; JT1, half-rate tobacco straw incorporation; JT2, full-rate tobacco straw incorporation; JT3, double-rate tobacco straw incorporation.

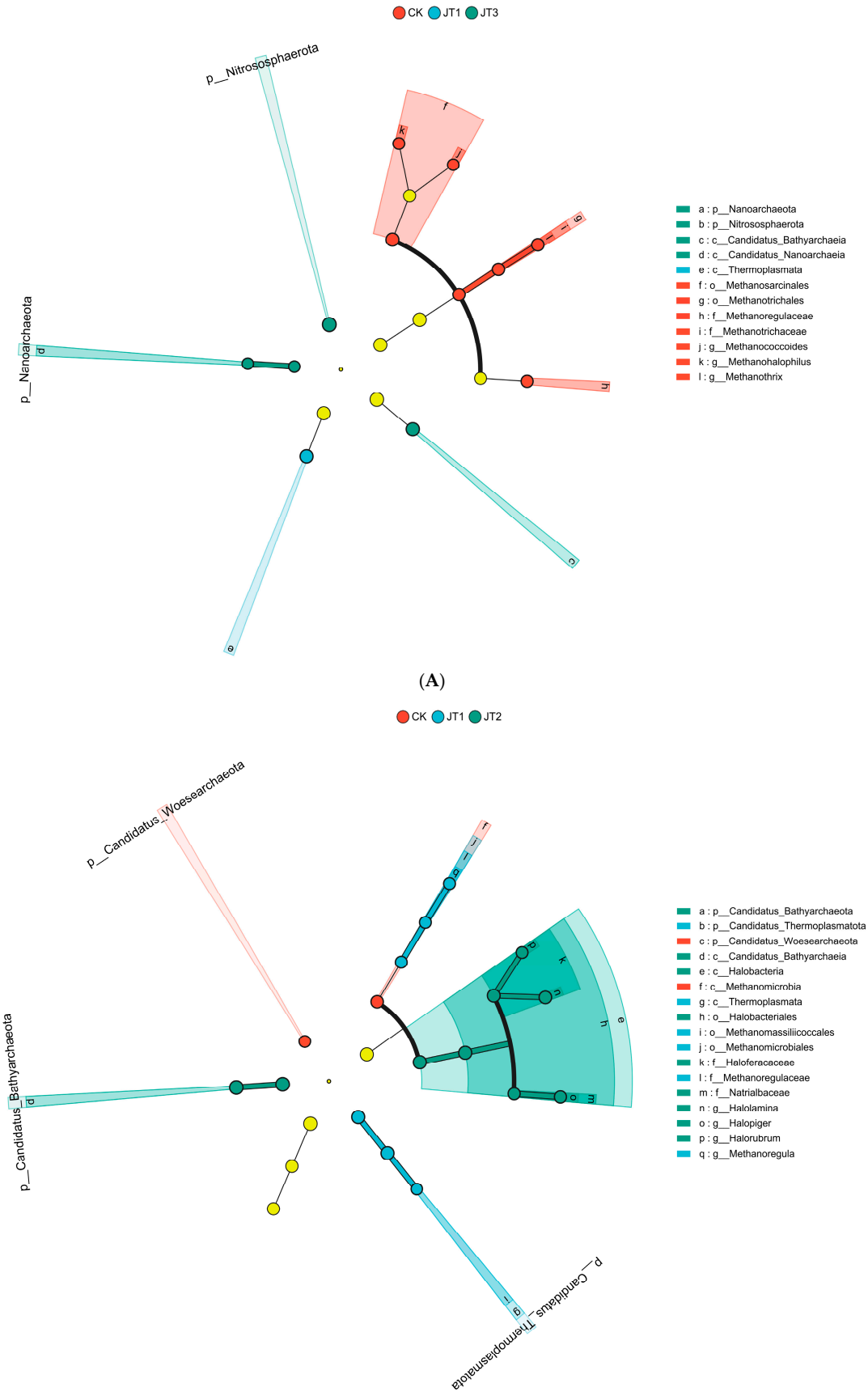
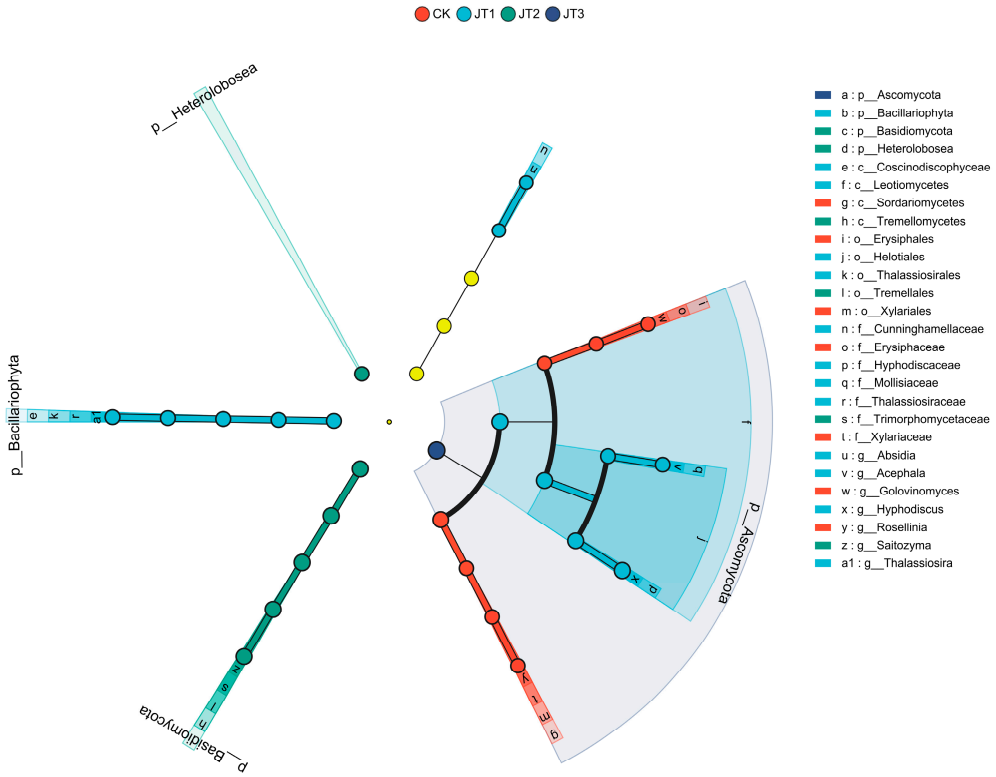
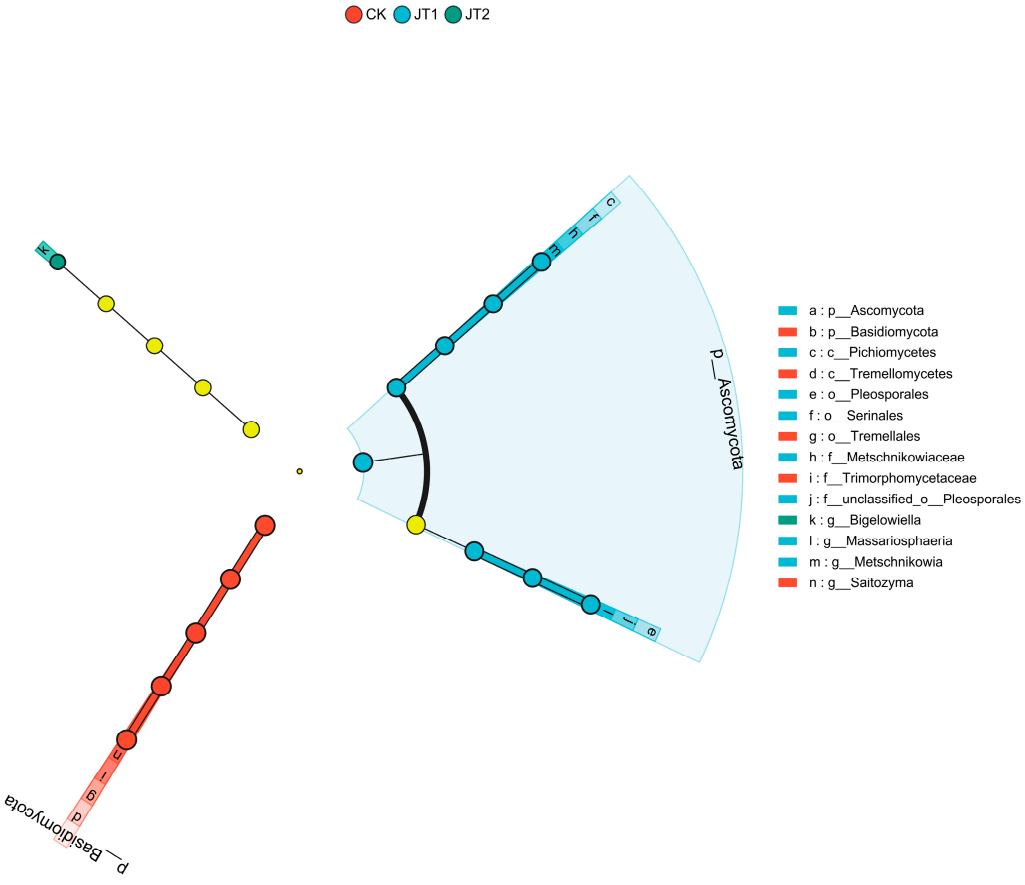


Figure 3. *Cont.*



(**C**)



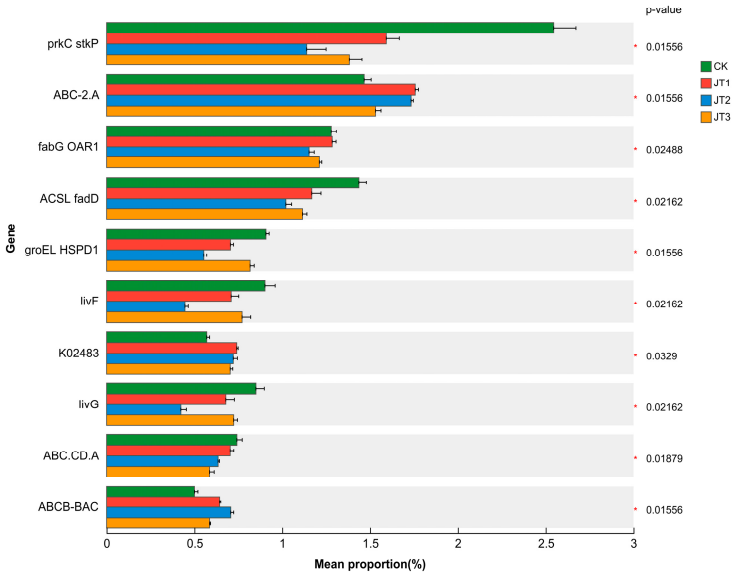
(**D**)

Figure 3. Changes of soil microbial functional groups under different treatments. CK, no application of tobacco straw; JT1, half-rate tobacco straw incorporation; JT2, full-rate tobacco straw incorporation;

JT3, double-rate tobacco straw incorporation. (**A**), MCGs taxa; (**B**), NCGs taxa; (**C**), CAZyGs taxa; (**D**), CARGs taxa. Nodes of different colors represent microbial taxa that are significantly enriched in the corresponding groups and have a significant impact on intergroup differences; pale yellow nodes represent microbial taxa that show no significant differences across groups or have no significant effect on intergroup variations.

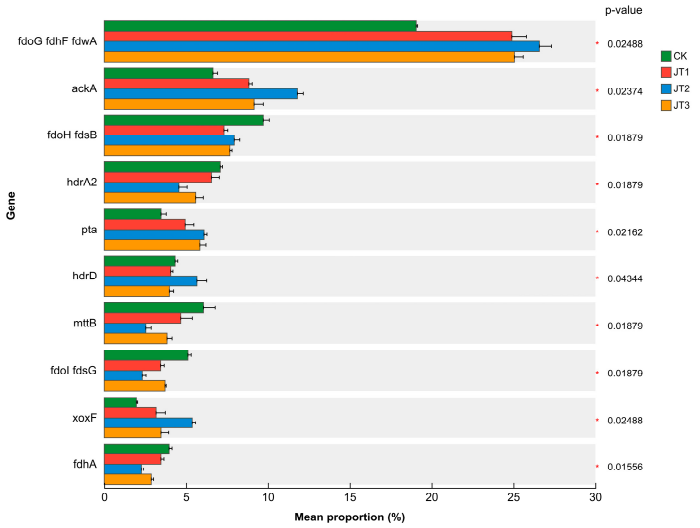
3.3. Effects of Different Tobacco Straw Returning Modes on the Abundance of Soil Microbial Functional Genes

Among the VFGs detected across the different treatments, the abundance of *prkC/stkP*, indirectly affecting bacterial virulence, decreased significantly following tobacco straw incorporation, with the JT2 treatment exhibiting the lowest abundance (Figure 4). Conversely, the abundance of *K02483*, a key virulence gene directly associated with bacterial pathogenicity, increased significantly after tobacco straw incorporation, with the JT1 treatment showing the highest abundance. The abundance of mttB, an important methanogenesis gene, was significantly reduced by tobacco straw incorporation, reaching its minimum in the JT2 treatment. Within the NCGs, the abundance of *gltD*, a key gene involved in microbial nitrogen assimilation, decreased significantly with tobacco straw incorporation and was lowest under the JT2 treatment. In contrast, the abundances of *nirK* and *norB*, crucial genes for microbial denitrification, increased significantly, with the JT2 treatment recording the highest levels. Regarding the CAZyGs, the abundances of *malZ* and *bglX* increased significantly after tobacco straw incorporation, peaking in the JT2 treatment. Analysis of the ARGs revealed that tobacco straw incorporation led to a reduction in the abundances of the *TC.HAE1*, *ABC.CD.A*, and *ABC.CD.P* functional genes. From the analysis results of functional gene abundance, it can be seen that the abundance of some bacterial virulence-related functional genes, the abundance of important genes related to microbial denitrification, and the abundance of functional genes related to carbohydrate metabolism in JT2 treatment were significantly different from other treatments, indicating that the full return of tobacco straw can reduce the pathogenic ability of soil bacteria and enhance the potential ability of soil microbial denitrification and cellulose degradation by changing the physical and chemical properties and energy substrates of soil.

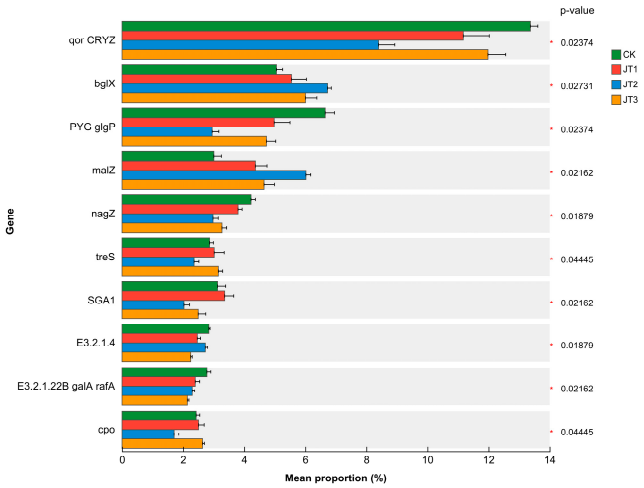


(**A**)

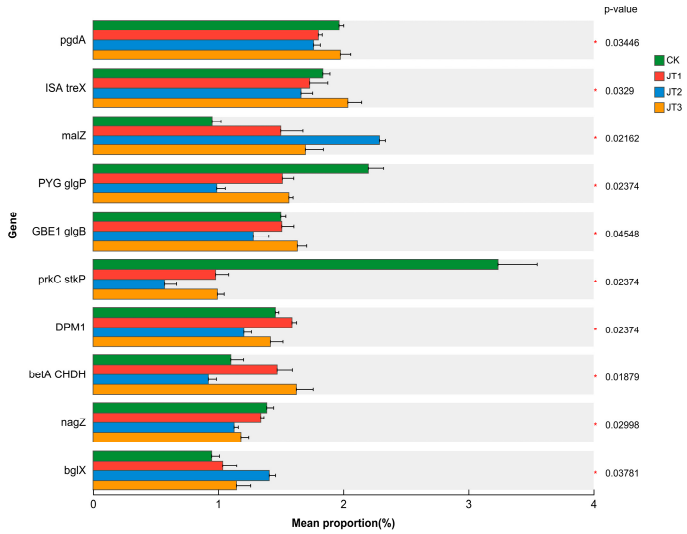
Figure 4. *Cont.*



(**B**)

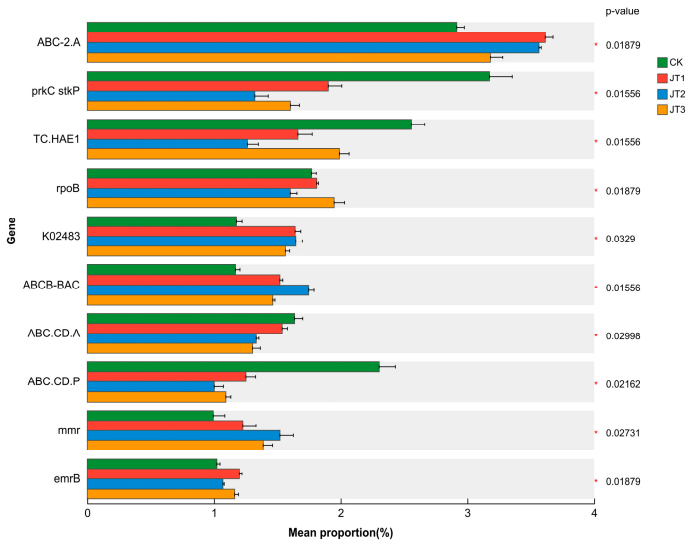


(**C**)



(**D**)

Figure 4. *Cont.*



(**E**)

Figure 4. Difference analysis of soil microbial gene abundance in different treatments. (**A**), VFGs; (**B**), MCGs; (**C**), NCGs; (**D**), CAZyGs; (**E**), CARGs. Single asterisk (*) indicates statistically significant differences ($n = 3$, $p < 0.05$).

3.4. The Potential Impact of Different Tobacco Straw Returning Modes on the Ecological Environment

To further elucidate the potential impacts of tobacco straw incorporation on soil-borne pathogens and greenhouse gas emissions, we integrated gene data from target species with their corresponding functional annotations by querying pathogen-host interaction databases. By leveraging the correspondence between functional genes and their microbial hosts, we further identified microbial taxa harboring the *mtaB* (methane production) and *pmoA* (methane oxidation) genes, as well as those carrying the *norB* (N_2O production) and *nosZ* (N_2O consumption) genes. Results from the differential analysis (Figure 5) indicated that tobacco straw incorporation increased the abundance of plant pathogens. Specifically, the abundances of the tobacco bacterial wilt pathogen *Ralstonia solanacearum*, the tobacco brown spot pathogen *Alternaria alternata*, the rice sheath blight pathogen *Rhizoctonia solani*, and the rice blast pathogen *Magnaporthe oryzae* were all significantly higher in the straw incorporation treatments (JT1, JT2, JT3) compared to the control (CK). Among these, the JT2 treatment showed the highest overall abundance of plant pathogens. In environments rich with certain lignin degradation products, methanogens containing the *mtaB* gene become particularly important. The *mtaB/pmoA* ratio represents the balance between methanogenic potential and methanotrophic capacity [27]. Under tobacco straw incorporation treatments (JT1, JT2, JT3), the abundance of the methanogenic archaeon *Methanosarcina* significantly decreased compared to the CK treatment. Concurrently, the ratio of *mtaB* to *pmoA* functional gene abundance was lower than in CK, indicating a decreased ratio of CH_4 production to CH_4 oxidation. This suggests that in tobacco-rice rotation fields amended with tobacco straw, CH_4 is more readily converted into other substances. Regarding the nitrogen cycle, the *norB/nosZ* ratio is widely used as a proxy for predicting the endpoint of denitrification and the potential for N_2O emissions [28]. A significantly increased ratio of *norB* to *nosZ* functional gene abundance was observed, indicating a reduced capacity of soil microorganisms to convert N_2O in the tobacco straw-amended tobacco-rice rotation fields.

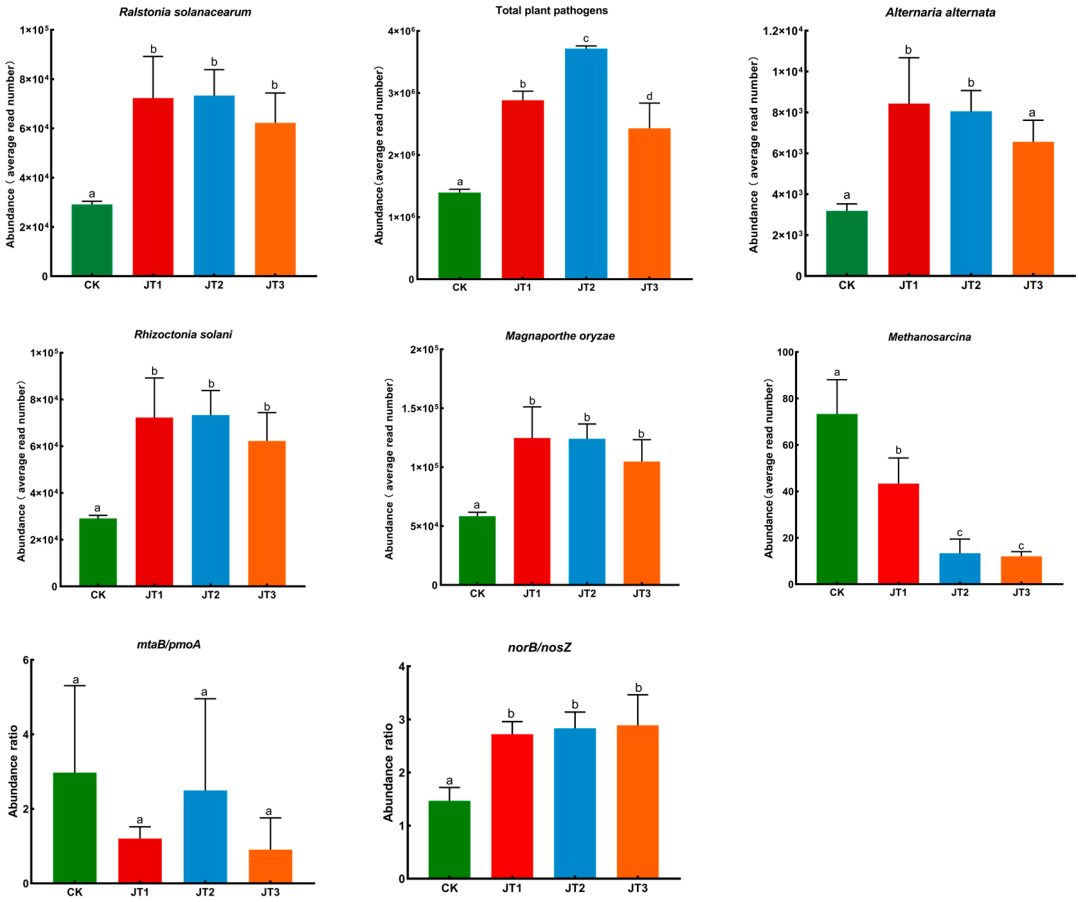


Figure 5. Abundance changes of typical pathogens, methanogens and key functional genes under different treatments. CK, no application of tobacco straw; JT1, half-rate tobacco straw incorporation; JT2, full-rate tobacco straw incorporation; JT3, double-rate tobacco straw incorporation. Different lowercase letters indicated significant differences between different treatments (n = 3, $p < 0.05$).

3.5. Effects of Environmental Factors on Soil Microbial Functional Groups

The nonparametric analysis of the correlation between the two matrices (Table 3) revealed that soil organic carbon, dissolved organic carbon, alkaline-hydrolyzable nitrogen, available phosphorus, and available potassium all exerted significant influences on the community composition of VFGs, MCGs, NCGs, CAZyGs, and CARGs carriers ($p < 0.05$). This indicates that the functional gene-carrying bacterial communities are sensitive to changes in environmental carbon, nitrogen, phosphorus, and potassium.

Table 3. The relative importance of environmental factors to soil microbial functional groups under different treatments.

Community	Correlation Coefficient					
	pH	Soil Organic Carbon	Dissolved Organic Carbon	Alkali-Hydrolyzed Nitrogen	Available Phosphorus	Available Potassium
VFGs	0.107	0.561 *	0.505 *	0.584 *	0.823 *	0.509 *
MCGs	0.128	0.531 *	0.497 *	0.589 *	0.777 *	0.499 *
NCGs	0.109	0.516 *	0.490 *	0.559 *	0.807 *	0.467 *
CAZyGs	0.108	0.601 *	0.526 *	0.625 *	0.818 *	0.555 *
CARGs	0.112	0.548 *	0.493 *	0.569 *	0.824 *	0.496 *

Note: Single asterisk (*) indicates significant correlations at the 0.05 probability level.

4. Discussion

Tobacco straw incorporation directly enhances soil organic matter content, providing a rich carbon source for soil microbial activity. This, in turn, increases the diversity, abundance, and activity of soil microorganisms and influences the activity of enzymes associated with carbon, nitrogen, and phosphorus cycling. As tobacco straw is rich in potassium, its incorporation significantly replenishes the soil potassium pool, potentially reducing the need for potassium fertilizers. It also contributes a certain proportion of nitrogen, phosphorus, and other medium and trace elements. However, concurrent with these benefits, straw incorporation can also lead to the enrichment of soil pathogens and an increased incidence of soil-borne diseases. As a vital carbon sequestration practice, tobacco straw incorporation represents an important component of agricultural strategies to mitigate climate change [29]. Therefore, a systematic analysis of the comprehensive ecological effects of long-term tobacco straw incorporation in tobacco-rice rotation systems is crucial. Such an analysis can provide a solid theoretical foundation and scientific guidance for optimizing straw management practices, maximizing its positive effects, and developing high-yield, efficient, healthy, and sustainable modern agricultural management systems. This study employed metagenomic technology to investigate the changes in virulence factor genes, methane cycling genes, nitrogen cycling genes, carbohydrate-active enzyme genes, antibiotic resistance genes, and their host microorganisms in a tobacco-rice rotation soil under long-term tobacco straw incorporation. The results demonstrate that tobacco straw incorporation significantly alters the structure of the soil microbial community, as well as the abundance and diversity of functional genes and functional guilds, which may subsequently influence plant disease occurrence and greenhouse gas emissions. Although rational crop rotation can disrupt the allelopathic effects associated with long-term monocropping, and has been shown to improve the soil environment for tobacco cultivation by increasing biodiversity, enhancing soil organic matter content, and reducing the incidence and transmission of soil-borne diseases [30,31], soil itself constitutes a vast reservoir of plant pathogens. Straw, being a rich carbon source, can stimulate the growth and reproduction of these pathogens when incorporated into the soil, leading to a rapid expansion of pathogen populations [32,33]. The findings of this study, which demonstrated a significant increase in the abundance of various virulence factor genes (VFGs) and an overall rise in the abundance of plant pathogens, including *Ralstonia solanacearum* (causing tobacco bacterial wilt), *Alternaria alternata* (causing tobacco brown spot), *Rhizoctonia solani* (causing rice sheath blight), and *Magnaporthe oryzae* (causing rice blast), support this conclusion. The dynamic changes in soil pathogen communities and virulence genes may profoundly impact long-term soil health and productivity through three primary mechanisms. First, the persistent enrichment of specific plant pathogens and their carried virulence genes directly increases the risk of soil-borne disease outbreaks, leading to crop root damage and reduced nutrient uptake efficiency, manifesting as intensified continuous cropping obstacles and suppressed biomass accumulation. Second, these pathogenic microorganisms secrete inhibitory substances such as antibiotics while competing with beneficial microbes for ecological niches, disrupting the original microbial interaction networks and reducing the diversity and stability of the soil microbial community, thereby weakening its natural buffering capacity against diseases. More critically, virulence genes may undergo horizontal transfer among microbial communities via mobile genetic elements, enabling originally non-pathogenic indigenous microbiota to acquire pathogenic potential and form more adaptive new pathogenic combinations. This degradation of ecological functions will ultimately transform soil from a metabolically active living system into a hotbed for disease transmission, not only increasing agricultural dependence on chemical pesticides but also directly constraining sustainable land productivity through crop yield

reduction and quality decline [34]. However, other research has indicated that long-term wheat straw incorporation can stimulate the growth of disease-suppressive fungi such as *Pseudogymnoascus* and *Schizothecium* [35]. Similarly, corn straw incorporation has been found to enhance soil fertility across black soils of high, medium, and low productivity, and to reduce the incidence and severity of corn stalk rot, with more pronounced effects in high-fertility soils [36]. This suggests that the impact of straw incorporation on plant pathogen dynamics varies depending on specific application conditions. Chen et al. [37] demonstrated that in rice-wheat rotation systems, incorporation of rice straw significantly increased the severity of wheat sheath blight when followed by inadequate management practices such as insufficient nitrogen fertilization, as the straw provided both primary inoculum and a nutritional substrate for the pathogen. Pre-treatment of straw before incorporation can mitigate the associated increase in soil-borne diseases [38]. Furthermore, the incorporation of decomposed rice and wheat straw has been shown to improve the yield, economic value, and sensory quality of flue-cured tobacco [39]. Additionally, the combined application of microbial inoculants with tobacco straw incorporation could be considered. For instance, research by He Yadeng [40] found that the combined application of fermentation products from the fungus *Trichoderma* THGY-01 and the bacterium *Bacillus* YB-1 significantly controlled tobacco soil-borne diseases such as bacterial wilt, *Fusarium* root rot, and *Rhizoctonia* blight.

In this study, long-term tobacco straw incorporation did not induce significant changes in the richness of methane-cycling functional genes. However, the abundance of the key methanogenesis gene *mttB* decreased significantly, and the ratio of *mtaB* (methane production) to *pmoA* (methane oxidation) gene abundances also declined compared to the CK control. This finding was corroborated by changes in key taxonomic abundances: while the overall diversity and richness of methane-cycling functional guilds increased significantly under long-term straw incorporation, the abundance of the methanogenic archaeon *Methanosarcina* decreased markedly. This suggests that in the tobacco-rice rotation system, a greater proportion of CH_4 is likely converted into other substances rather than being emitted. This phenomenon may be attributed to the fact that long-term straw incorporation increases soil organic matter content and promotes the formation of soil aggregates. This structure allows the soil to retain more pores even under flooded conditions, creating numerous aerobic-anaerobic microsites. This breaks up large-scale strictly anaerobic environments and creates favorable conditions for methane oxidation. CH_4 emissions are the net result of the activities of methanogens and methanotrophs, which are closely related to soil microbial community structure and abundance [41,42]. The long-term input of carbon sources selects for and enriches a large number of aerobic and facultative anaerobic bacteria and fungi that efficiently decompose cellulose. These microorganisms rapidly consume oxygen and decompose organic matter after straw incorporation, leading to the substantial consumption of methanogenic precursors under aerobic conditions [43]. Conversely, some studies have indicated that straw incorporation can increase CH_4 emissions, particularly in the context of global warming [44–46]. For instance, Hu et al. found that straw mulching and decomposition from various crops generally increased CH_4 emissions by 141.9% compared to non-incorporation practices [47]. Methane production is influenced by numerous factors, including environmental conditions, crop types, and duration of incorporation [48,49].

Tobacco straw incorporation significantly increased the diversity of microbial taxa involved in the nitrogen cycle. This finding aligns with previous studies reporting that rotary tillage with straw incorporation increased N_2O emissions by more than 15% compared to treatments without straw [50], and that both direct straw incorporation and fertilization can elevate N_2O emissions [51]. Tobacco straw contains nitrogenous heterocyclic compounds,

and its degradation process may release nitrogenous intermediates, stimulating specific microbial populations and indirectly influencing nitrogen cycling pathways, potentially leading to N_2O production. As the duration of incorporation increases, the total soil organic nitrogen accumulates. Following mineralization, this increases the overall soil nitrogen pool, providing a larger substrate base for nitrification and denitrification processes, thereby potentially enhancing the capacity for N_2O emissions [52]. From the perspective of nitrogen metabolic pathways, the increase in N_2O emissions can be attributed to the elevated abundance of genes involved in the denitrification pathway. In this study, the significantly increased ratio of *norB* (N_2O production) to *nosZ* (N_2O reduction) gene abundances indicates that long-term tobacco straw incorporation is likely to increase the net emission rate of N_2O. However, some studies have reported contrasting findings, suggesting that wheat straw addition can reduce N_2O flux [53], and that straw incorporation significantly decreased N_2O emissions in cotton fields [54].

The decomposition process following tobacco straw incorporation drives a shift in greenhouse gas emission patterns by regulating the expression of microbial functional genes. In the methane cycle, although the activity of the *mttB* gene in methanogenic archaea increases during the initial flooding period, the improved soil structure from straw amendment creates more aerobic-anaerobic interfaces, significantly activating the *pmoA* gene in methanotrophic bacteria. This enhances the efficient oxidation of methane to CO_2, resulting in net emission reduction. In the nitrogen cycle, however, the abundant readily decomposable organic carbon provided by straw decomposition strongly stimulates denitrifying microorganisms, leading to a surge in the expression of the *norB* gene encoding nitrite reductase, which accelerates the conversion of NO_2^- to N_2O. Meanwhile, the expression of the key gene *nosZ*, responsible for reducing N_2O to N_2, is relatively delayed, causing significant accumulation of N_2O at the terminal stage of denitrification. This study analyzed the abundance of functional genes across different treatments and found that the full incorporation of tobacco straw (JT2) significantly reduced the abundance of *prkC*, *stkP*, and *mttB* genes, while significantly increasing the abundance of *nirK*, *norB*, *malZ*, and *bglX* genes compared to other treatments. This indicates that tobacco straw incorporation alters soil physicochemical properties and energy substrates, leading to shifts in microbial metabolic strategies [55]. As a result, the pathogenic potential of soil bacteria decreases, while the potential for microbial denitrification and cellulose degradation increases. The full incorporation of tobacco straw (JT2) demonstrates superior performance compared to other treatments.

5. Conclusions

This study employed metagenomic techniques to investigate the characteristics of virulence factor genes (VFGs), methane-cycling genes (MCGs), nitrogen-cycling genes (NCGs), carbohydrate-active enzyme genes (CAZyGs), antibiotic resistance genes (ARGs), and their host microbial communities in soils under long-term tobacco straw incorporation. The results demonstrated that within microbially-mediated methane cycling, long-term tobacco straw incorporation significantly reduced the abundance of the key methanogenic gene *mttB* and the methanogenic archaeon *Methanosarcina* compared to the CK control. The functional gene abundance ratio of *mtaB*/*pmoA* also decreased, suggesting enhanced CH_4 conversion in the tobacco-rice rotation system under straw incorporation. The abundance of plant pathogens significantly increased under tobacco straw incorporation. Furthermore, a significantly increased functional gene abundance ratio of *norB*/*nosZ* was observed, indicating a reduced capacity of soil microorganisms to convert N_2O in the tobacco-rice rotation fields under straw incorporation. The full-rate tobacco straw plowing incorporation treatment (JT2) showed the lowest abundances of functional genes *prkC*, *stkP*, and

mttB, and the highest abundances of *nirK*, *norB*, *malZ*, and *bglX*. This suggests a shift in microbial metabolic strategies driven by altered soil physicochemical properties and energy substrates, leading to decreased potential soil bacterial pathogenicity and enhanced potential for microbial denitrification and cellulose degradation. Soil organic carbon, dissolved organic carbon, alkali-hydrolyzable nitrogen, available phosphorus, and available potassium were significantly correlated with the composition of functional microbial groups ($p < 0.05$). This study reveals that long-term tobacco straw incorporation may increase disease risk in tobacco-rice rotation systems, while potentially elevating N_2O emissions and reducing CH_4 emission rates. Analysis of functional gene abundance changes indicated that the full-rate straw plowing incorporation (JT2) was the most effective treatment among those tested. In conclusion, straw incorporation profoundly influences soil carbon and nitrogen transformation processes, and further validation considering different crop types, ecological regions, and soil conditions is warranted. Future research should integrate multi-omics approaches, including transcriptomics, proteomics, and metabolomics, to systematically investigate changes in soil microbial community structure and function under long-term tobacco straw incorporation, thereby providing further evidence for the underlying microbiological mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15222384/s1, Figure S1: The chronology of the tobacco-rice rotation system; Table S1: Sequencing depth; Table S2: Field management practices.

Author Contributions: H.Z.: Experimental design, Performed the experiment, Data curation and analysis, Writing—Original draft preparation. C.L. and Y.Y.: Sample collection, Sample pretreatment. L.C. and Y.F.: Sample pretreatment, DNA extraction. X.J.: Performed the experiment, Writing—Editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data and related metadata underlying the findings are already provided as part of the submitted article. The details of the data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare that no competing interests exist.

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