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Effects of Different Fertilizer Treatments on Rhizosphere Soil Microbiome Composition and Functions

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Abstract: Fertilization influences the soil microbiome. However, little is known about the effects of long-term fertilization on soil microbial metabolic pathways. In this study, we investigated the soil microbiome composition and function and microbial participation in the N cycle according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) functional annotation of different genes in a metagenomic analysis after long-term fertilization. Fertilizer application significantly changed the soil C/N ratio. Chemical fertilizer (NPK) treatment decreased soil pH, and chemical fertilizer combined with straw (NPK+S0.5) treatment increased ammonium nitrogen (NH_4^+-N) but decreased nitrate nitrogen (NO_3^--N). NPK, NPK+S0.5 and S0.5 applications did not change the soil microbiome composition or dominant phylum but changed the relative abundances of microbiome components. Moreover, fertilizer significantly influenced metabolic processes, cellular processes and single-organism processes. Compared with a no-fertilizer treatment (CK), the NPK treatment resulted in more differentially expressed gene (DEG) pathways than the NPK+S0.5 and S0.5 treatments, and these pathways significantly correlated with soil nitrate nitrogen (NO_3^--N), available phosphorus (AP) and the moisture content of soil (MC). KEGG analysis found that fertilizer application mainly affected the ribosome, photosynthesis and oxidative phosphorylation pathways. S0.5 and NPK+S0.5 increased microbial nitrogen fixation, and NPK and NPK+S0.5 decreased amoA and amoB and accelerated denitrification. Thus, organic fertilizer increased N fixation and nitrification, and inorganic N fertilizer accelerated denitrification. We found that the function of the soil microbiome under different fertilizer applications could be important for the rational application of fertilizer and for environmental and sustainable development.

Keywords: metagenomic analysis; rhizosphere; soil microbiome; microbiome function; fertilizer

1. Introduction

Fertilization is an important method of improving soil fertility and quality and increasing crop yield [1], although it also influences the level of soil pollution [2], leaching of nitrate in groundwater and

surface waters, enhanced emissions of nitrous oxide [3], soil hardness and organic matter mineralization. In recent years, to mitigate the environmental impacts of excessive rates of chemical fertilization in agroecosystems, the combined use of inorganic fertilizers with organic manures has attracted increasing interest in China [4]. Previous studies suggested that organic fertilizer application not only improves soil organic matter content, preserves moisture and fertility and reduces losses of soil nutrients such as N, P and K, but also helps maintain soil pH stability [5]. Straw application is an important way to increase soil organic fertilization, and crop residues have significant beneficial effects on soils' physical, chemical and biological processes. In addition, an increasing number of research studies have demonstrated that highly anthropogenic fertilizers with straw significantly enhance bacterial activity [6] and greenhouse gas emissions [7].

The soil rhizosphere microbiome has a critical role in plant growth, nutrition, health and breeding [8,9]. Kalivas et al. [2] concluded that the proportion of soil rhizosphere microorganisms (Proteobacteria and Acidobacteria) was higher in a no-fertilizer treatment. Fertilizers can exert profound effects on the biodiversity and structure and functions of soil microbial communities [10]. Soil microbial and fungal diversity was found to decrease significantly with fertilization [11]. Previous studies have found that dominant microbes are adapted to particular soil conditions and play significant roles in the preservation and regulation of key processes [12,13]. Ahmed et al. [14] reported that Proteobacteria, Actinobacteria, Gemmatimonadetes, Bacteroidetes, Firmicutes And Acidobacteria can tolerate salty soil. The dominant microbes differ in different types of soil and are altered by fertilizers [15].

The nitrogen (N) cycle is a collection of important biogeochemical pathways mediated by microbial communities [16]. Microbial transformations of nitrogen are often depicted as cycles consisting of nitrogen fixation, nitrification, denitrification, anammox, assimilation and ammonification [17]. Studies have shown that ammonia-oxidizing bacteria, nitrogen fixing bacteria, nitrifying bacteria, denitrifying bacteria and cellulose-decomposing bacteria have an important impact on the nitrogen cycle, and most ammonia-oxidizing bacteria belong to Proteobacteria [18,19]. At the taxonomic level, these are the largest and most diverse microbial species involved in soil organic N metabolism and nitrate reduction [20]. Previous studies have related soil N cycling to microbial genetic data that are mainly focused on a few gene families by PCR [21] or revealed changes in the soil bacterial community driven by soil organic C and N fertilizer and tillage crop residue management by 16S metagenomics [22]. Deng et al. [23] suggested that metagenomics can be used to reflect the structure and potential function of microbial communities.

However, the role of microbes in ecosystem functions and agricultural productivity in response to the different fertilizers is complicated and elusive. There is less understanding about how the taxonomic and functional compositions of soil microorganisms are involved in the whole N cycle. Moreover, little is known about the effect of long-term fertilization on soil microbial metabolic pathways. Therefore, shotgun metagenome sequencing was performed to characterize the taxonomic and functional composition involved in nitrogen cycling processes and provide insights into the influence of long-term fertilization on microbial metabolic pathways.

2. Materials and Methods

2.1. Soil Sampling

Soil samples were collected from a long-term experimental field belonging to Jilin Agricultural University in Changchun City, Jilin Province, Northeast China (43°47′42″ N, 123°20′45″ E). The area has a temperate continental monsoon climate, the average annual rainfall is 594.6 mm, and the accumulated temperature (≥10 °C) is 2850 °C. The average annual temperature is 7.8 °C; the lowest has been reported to be −24 °C and the highest 33 °C. During the growing season (April to October), the average monthly temperatures were 9.5 °C, 16 °C, 20 °C, 29.5 °C, 26.5 °C, 15.5 °C and 6 °C, respectively, in 2017. The soil type is black soil (Phaeozems), and the maize variety is Xianyu 335 (2014–2017).

The experimental plots were established in 1984, maize was sown in April, and all maize residues were removed from the plot after harvesting in October; there was no additional irrigation during this time. Soil samples were collected after the harvest period in 2017, each plot was sampled at five points, and the samples were mixed into one sample for a total of 12 test samples. The four fertilizer treatments were no-fertilization (CK), chemical fertilizer (NPK) (N-P_2O_5-K_2O: 0.15-0.075-0.075 kg/m^2), chemical fertilizer combined with straw (NPK+S0.5) (N-P_2O_5-K_2O: 0.15-0.075-0.075 kg/m^2; S0.5: straw 0.5 kg/m^2), and straw 0.5 kg/m^2 (S0.5). The area was 63 m^2 and each treatment was repeated three times, with random permutation; the chemical fertilizers were applied, and maize was sown annually from 1984 to 2017. We collected the rhizosphere soil (0–30 cm) in polyethylene bags by shaking the roots until the non-adhering soil fell off. The methods of soil sample preservation and pretreatment as well as the determination of the basic physical and chemical properties of the soil (pH, moisture content (MC), soil organic carbon content (SOC), soil total nitrogen (TN), ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N)) were described in our research team's previous papers [24].

2.2. DNA Extraction and Metagenomic Sequencing

We used the PowerSoil® DNA Isolation kit (MO BIO, USA) to separate and extract DNA from 0.50 g soil samples according to the manufacturer's instructions, and a total of 12 genomic DNA samples were obtained. Then, the sample genomic DNA was tested by 1% agarose gel electrophoresis. After that, the DNA was fragmented and end-repaired, an A was added at the 3′ end, and sequencing adapters were added at both ends of the fragments. After removal of self-ligated fragments by magnetic beads purification and fragment screening, library amplification was performed by PCR. Quality screening of the original data was carried out using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to obtain a high-quality data set (clean data: sequence length < 200 bp, no ambiguous bases, and mean quality score ≥ 20) for downstream macrogenomics analysis [25].

Then, the high-quality sequences of each sample were assembled to construct the metagenome contig and scaffold sequence set, and gene prediction was carried out to obtain the non-redundant protein sequence set. Subsequently, the protein sequence was functionally annotated with a variety of commonly used databases; the functional group abundance spectra of each grade were obtained, the differences were compared and analyzed, and the metabolic pathway enrichment analysis was carried out. We used Cluster Database software (version 2.1.0) at high identity and tolerance to obtain clean reads of each sample, and determined the abundance of genes in the corresponding sample.

2.3. Statistical Analysis

We used analysis of variance (ANOVA) to analyze the differences among groups, and the least significant difference (LSD) method was performed to compare groups [26]. The genes involved in the nitrogen cycle were clustered using heatmap software, and the genes with high and low abundance were distinguished by color gradient. We also determined the relationships between chemical properties and genes that participated in N cycle by redundancy analysis (RDA) using CANOCO software, Version 5.0 [27]. The representative gene set sequence (amino acid sequence) was annotated with the non-redundant (NR), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), SwissPro, and Gene Ontology (GO) databases with an e-value of 1×10^{-5} [28,29].

3. Results

3.1. Soil Chemical Properties

The soil chemical properties under different fertilization applications were significantly different. The pH decreased significantly with NPK and NPK+S0.5 compared to CK; the pH in the NPK treatment was 6.55, which was 0.48 lower than CK. However, fertilization application increased the soil C:N ratio; in particular, the ratio for NPK+S0.5 was 4.74 higher than that for CK. The content of NH_4^+-N decreased in the order NPK+S0.5 > S0.5 > CK > NPK, although the highest content of NO_3^--N was in the NPK

treatment. The soil moisture content (MC) also significantly differed with fertilization application, and the highest was in the CK treatment (Figure 1).

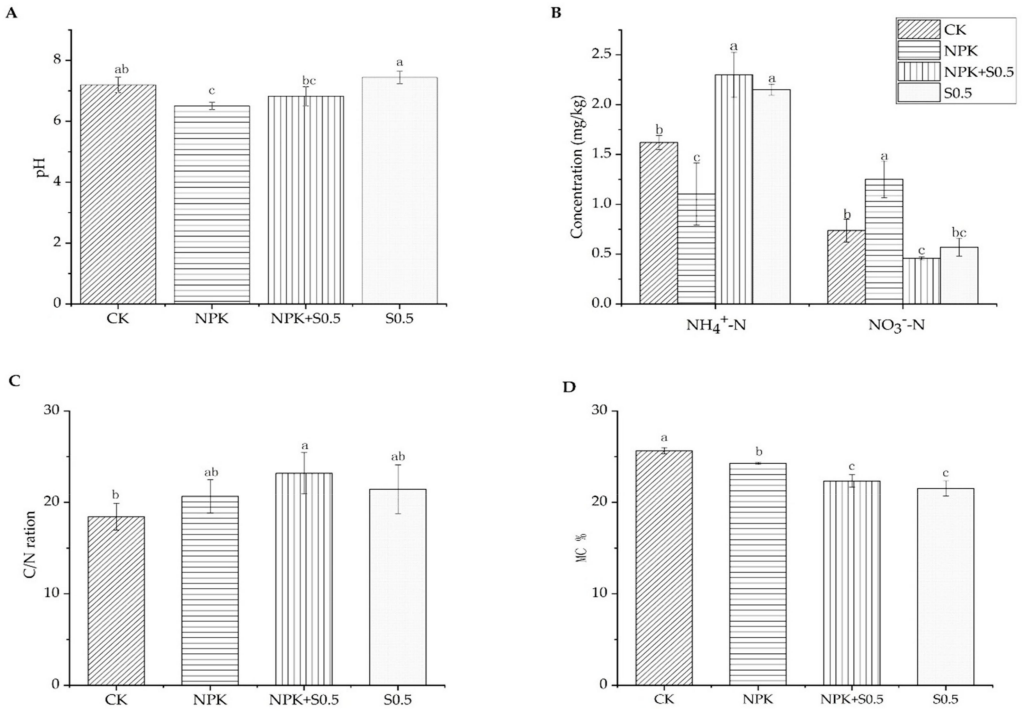


Figure 1. Soil chemical properties with different fertilizer applications. (**A**) pH changes with fertilizer application; (**B**) NH_4^+-N and NO_3^--N changes with fertilizer application; (**C**) C/N ratio changes with fertilizer application; (**D**) MC changes with fertilizer application. The lower case letters 'a', 'b' and 'c' indicate a significant difference ($p < 0.05$) among different samples for each treatment.

3.2. Soil Microbiome Community

To compare the influence of fertilizers on the microbiome we carried out soil metagenomic sequencing. The numerical data are summarized in Table A1. We obtained a total of 4.39×10^8 clean reads. The numerical data of the scaffolds after assembly are shown in Table 1. The greatest number of metagenome sequences were found for NPK+S0.5, with 238,996 DNA fragments for a total of 2.01×10^8 bp and a N50 sequence length of 779 bp. Taxonomic analysis showed that the genes were affiliated with bacteria, fungi, viruses and archaea; the predominant genes were in bacterial genes, which accounted for over 96% of the total genes, followed by fungal genes (1.63–2.44%), and the viral genes accounted for only 0.02–0.04% of the total (Figure 2). In addition, 36 phyla and 11 genera were found for bacteria, 10 phyla and 489 genera were found for fungi, four phyla and 104 genera were found for archaea, and 185 genera were found for viruses.

Table 1. Summary of numerical data for metagenomic sequencing.

Samples	Clean Reads (10^7)	Base Sum (10^{10})	Q20 (%)	Q30 (%)	GC (%)
CK	3.65 ± 0.02 a	1.09 ± 0.05 a	97.38 ± 0.04 a	93.15 ± 0.08 a	63.67 ± 0.08 a
NPK	3.35 ± 0.02 a	0.98 ± 0.04 a	97.42 ± 0.01 a	93.25 ± 0.02 a	63.69 ± 0.18 a
NPK+S0.5	3.81 ± 0.02 a	1.14 ± 0.06 a	97.39 ± 0.05 a	93.16 ± 0.12 a	64.14 ± 0.49 a
S0.5	3.40 ± 0.02 a	1.02 ± 0.05 a	97.43 ± 0.06 a	93.17 ± 0.05 a	65.13 ± 1.52 a

Note: Clean Reads: the number of pairs end sequences is counted in four rows and one unit after filtering; Base Sum: sequencing data volume; Q20: percentage of bases with mass value greater than or equal to 20; Q30: percentage of bases with mass value greater than or equal to 30 i; GC: percentage of G and C type bases. The lower case letter 'a' indicate a significant difference ($p < 0.05$) among different samples for each treatment.

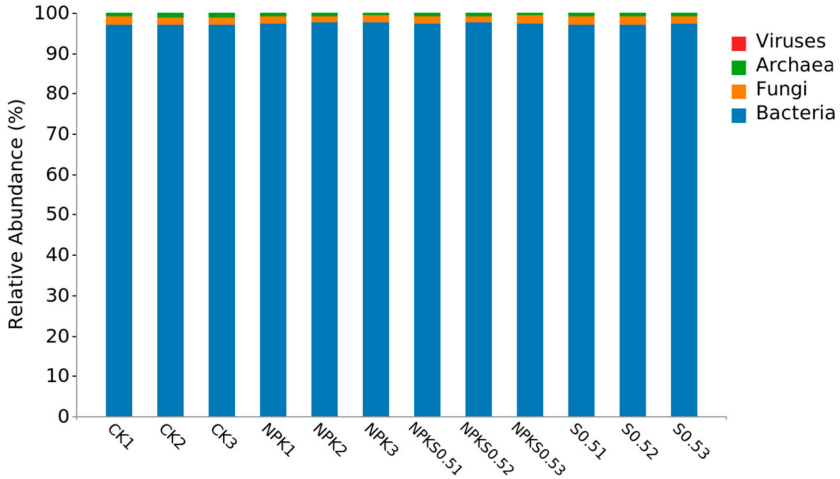


Figure 2. Relative abundance of soil microbiome under different fertilizer applications.

We also analyzed the dominant microbiome at the phylum and genus taxonomic levels, and the results showed that the microbial community was dominated by Proteobacteria (51.17–57.89%) at the phylum level, and that Acidobacteria (37.12–43.43%) and Firmicutes (1.13–1.24%) were next. At the genus level, the dominant microorganisms were Bradyrhizobium (56.75–10.75%), Streptomyces (6.80–7.861%) and Mycobacterium (3.78–4.96%). Proteobacteria and Acidobacteria changed significantly under different fertilizers, and NPK application alone decreased Proteobacteria but increased Acidobacteria. The dominant fungal phylum was Ascomycota (87.40–89.77%), followed by Basidiomycota (8.43–9.57%). *Sordaria*, *Fusarium* and *Paracoccidioides* (19.12% of the total) were the three most abundant genera in the fungal community. With NPK+S0.5 and S0.5 application, the relative abundance of Ascomycota increased significantly. In archaeal taxa, Crenarchaeota was the dominant phylum, accounting for 95.87–97.78% of archaea, and was significantly influenced by fertilizer application. Finally, we found that Alphabaculovirus (10.67–14.67%), *Jd18virus* (10.33–14.33%) and *Betabaculovirus* (1.0–2.0%) were the dominant viruses and that they had not significantly changed under fertilizer application (Table 2).

Table 2. Dominant microbiomes at the phylum and genus taxonomic levels.

		CK	NPK	NPK+S0.5	S0.5
		(%)	(%)	(%)	(%)
Bacteria	Proteobacteria	51.89 ± 0.47 b	57.32 ± 1.26 a	51.85 ± 3.65 b	51.71 ± 1.99 c
	Acidobacteria	42.53 ± 0.27 b	37.12 ± 1.36 c	43.43 ± 3.66 a	43.26 ± 1.78 a
	Firmicutes	1.16 ± 0.03 b	1.24 ± 0.08 a	1.22 ± 0.30 a	1.13 ± 0.11 b
Fungi	Ascomycota	87.40 ± 0.22 d	87.81 ± 1.04 c	89.77 ± 0.98 a	88.62 ± 0.55 b
	Basidiomycota	10.39 ± 0.43 b	10.57 ± 0.83 a	8.43 ± 0.93 d	9.71 ± 0.41 c
	Mucoromycota	0.94 ± 0.26 a	1.41 ± 0.22 a	1.20 ± 0.33 a	0.99 ± 0.21 a
Archaea	Crenarchaeota	95.87 ± 0.03 c	97.78 ± 0.05 a	97.39 ± 0.09 b	96.16 ± 0.22 bc
	Euryarchaeota	2.56 ± 0.20 a	1.08 ± 0.12 c	1.45 ± 0.28 c	2.17 ± 0.37 b
	Thaumarchaeota	1.46 ± 0.16 b	1.01 ± 0.12 c	1.05 ± 0.19 c	1.51 ± 0.16 a
Bacteria	*Bradyrhizobium*	7.66 ± 0.08 b	10.33 ± 0.10 a	10.75 ± 0.11 a	6.75 ± 0.07 b
	Streptomyces	7.34 ± 0.07 a	6.8 ± 0.07 a	7.86 ± 0.08 a	7.64 ± 0.08 a
	Mycobacterium	3.78 ± 0.04 b	4.13 ± 0.04 b	4.96 ± 0.05 a	3.84 ± 0.04 b
Fungi	*Sordaria*	5.83 ± 0.06 b	11.02 ± 0.11 a	7.03 ± 0.07 b	5.63 ± 0.06 b
	Fusarium	2.41 ± 0.02 a	2.64 ± 0.03 a	2.58 ± 0.03 a	3.15 ± 0.03 a
	Paracoccidioides	2.8 ± 0.03 a	1.95 ± 0.02 b	1.85 ± 0.02 b	2.2 ± 0.02 b
Archaea	*Halorubrum*	13.84 ± 0.14 a	14.36 ± 0.14 a	14.47 ± 0.12 a	13.92 ± 0.09 a
	Halococcus	9.48 ± 0.09 ab	9.01 ± 0.09 b	9.62 ± 0.10 a	9.27 ± 0.09 ab
	Haloferax	6.85 ± 0.07 a	7.1 ± 0.07 a	6.88 ± 0.05 a	7.04 ± 0.07 a
Viruses	*Alphabaculovirus*	10.67 ± 0.11 a	13.33 ± 0.13 a	14.67 ± 0.15 a	13 ± 0.13 a
	Jd18virus	11.00 ± 0.11 a	14.33 ± 0.14 a	10.33 ± 0.10 a	14.33 ± 0.14 a
	Betabaculovirus	1.00 ± 0.01 a	2.00 ± 0.02 a	1.33 ± 0.01 a	1.33 ± 0.01 a

Note: The lower case letters 'a', 'b' and 'c' indicate a significant difference ($p < 0.05$) among different samples for each treatment.

3.3. Soil Microbiome Functions

Compared with the CK treatment, NPK fertilizer application had a greater number of different genes. There were 1137 differentially expressed genes (DEGs) in CK vs. NPK, including 582 genes with high abundances and 555 genes with low abundances. Moreover, we analyzed the DEGs between organic fertilizer and inorganic fertilizer (NPK vs. S0.5). We found a total of 1581 DEGs, and most of the DEGs were in the NR and Pfam databases, indicating that the main DEGs were proteins (Table A2).

To explore the functions of metagenomes after treatment, we used the KEGG and GO databases to annotate metagenomes [26]. The DEGs were primarily enriched in biological processes, mainly metabolic processes, cellular processes and single-organism processes. The second most enriched category was molecular function, which mainly included binding, catalytic activity and structural molecule activity. In the cellular components, the DEGs were mainly enriched in cell part, macromolecular and organelle. Compared with the CK treatment, NPK treatment had more DEGs, and the NPK+S0.5 and S0.5 treatments had no DEGs in nucleic acid binding transcription factor activity, protein binding transcription factor activity, antioxidant activity, enzyme regulator activity, molecular transducer activity (molecular function), nucleoid, and extracellular region part (cellular component). Furthermore, there were some DEGs between the NPK and S0.5 treatments, including genes involved in catalytic activity, structural molecule activity, organelle, cell part and macromolecular complex (Figure 3).

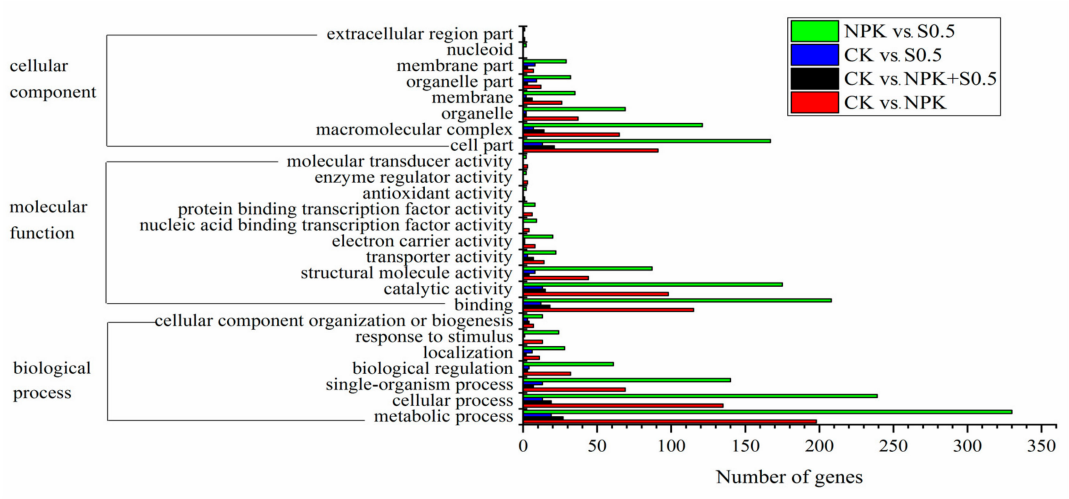
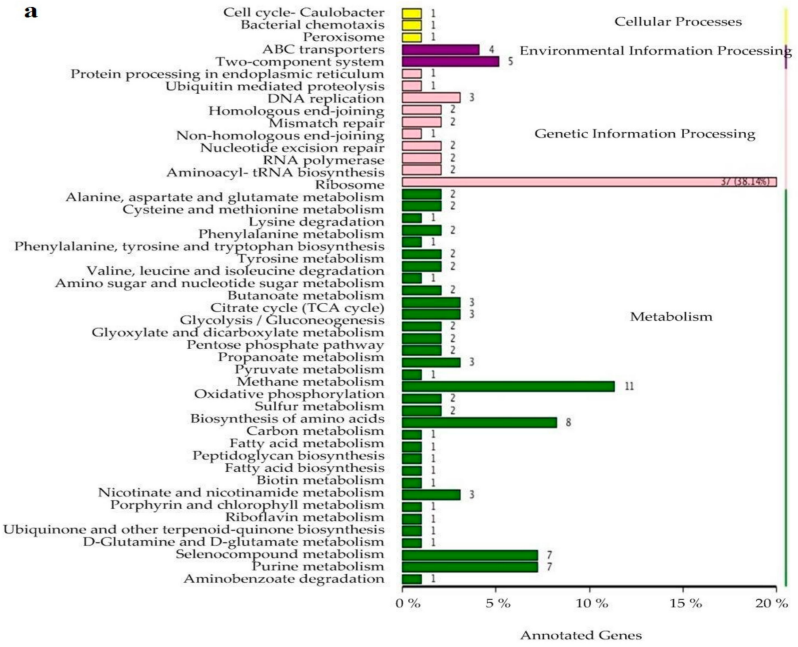


Figure 3. Statistical classification of gene ontology (GO) annotation for differentially expressed genes (DEGs).

KEGG annotation was used to analyze the pathways of DEGs to further determine their functions. The results showed that the pathway with the largest number of DEGs was ribosome; its average percentage of the total number of annotated genes was 30.21%, and the number of DEGs for NPK vs. S0.5 was 73. Forty-eight pathways were enriched by DEGs with NPK fertilizer application compared with the CK treatment, and 33 pathways belonging to metabolism, including oxidative phosphorylation, carbon metabolism and pyrimidine metabolism, had increased DEGs. There were 11 and seven pathways enriched by differential genes in CK vs. NPK+S0.5 and CK vs. S0.5, respectively, which were fewer pathways than in CK vs. NPK. Then, we found 62 pathways enriched by differential genes in NPK vs. S0.5; oxidative phosphorylation and biosynthesis of amino acids had 23 and nine differential genes, respectively, and ATP-binding cassette (ABC) transporters, purine metabolism and pyrimidine metabolism all had eight different DEGs (Figure 4).



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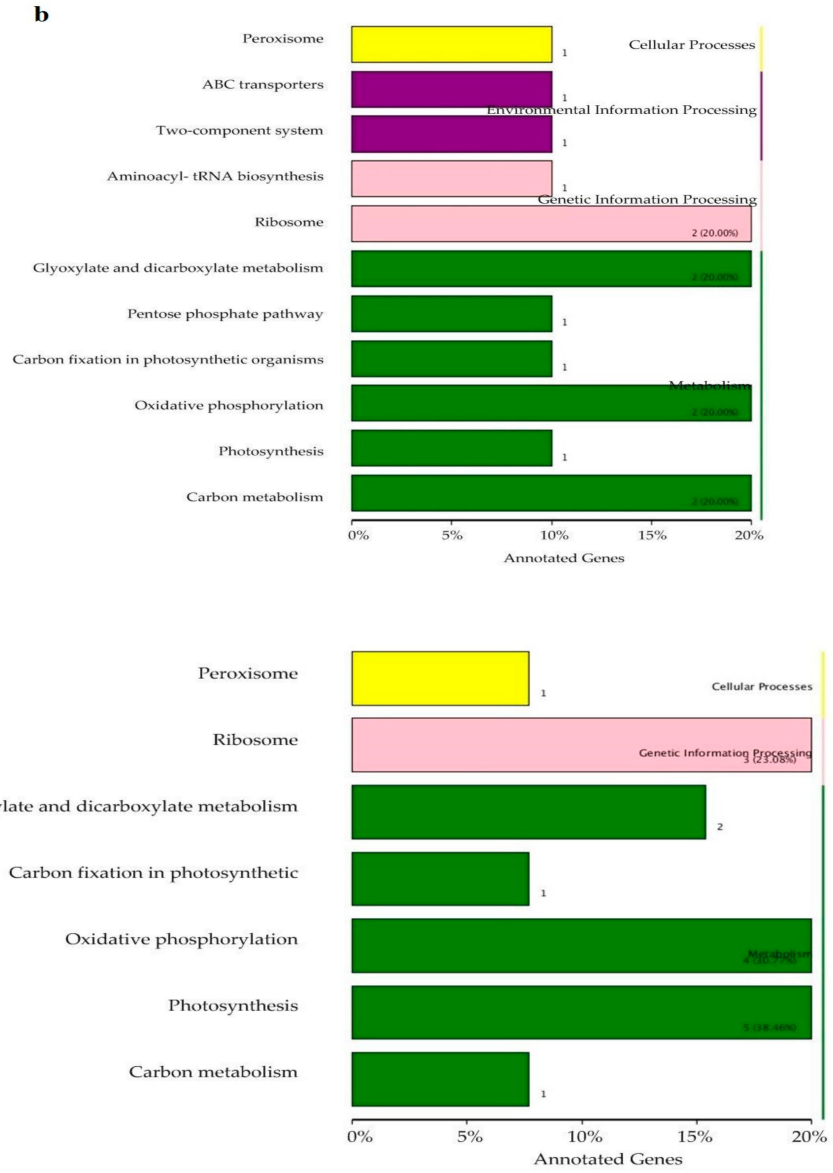

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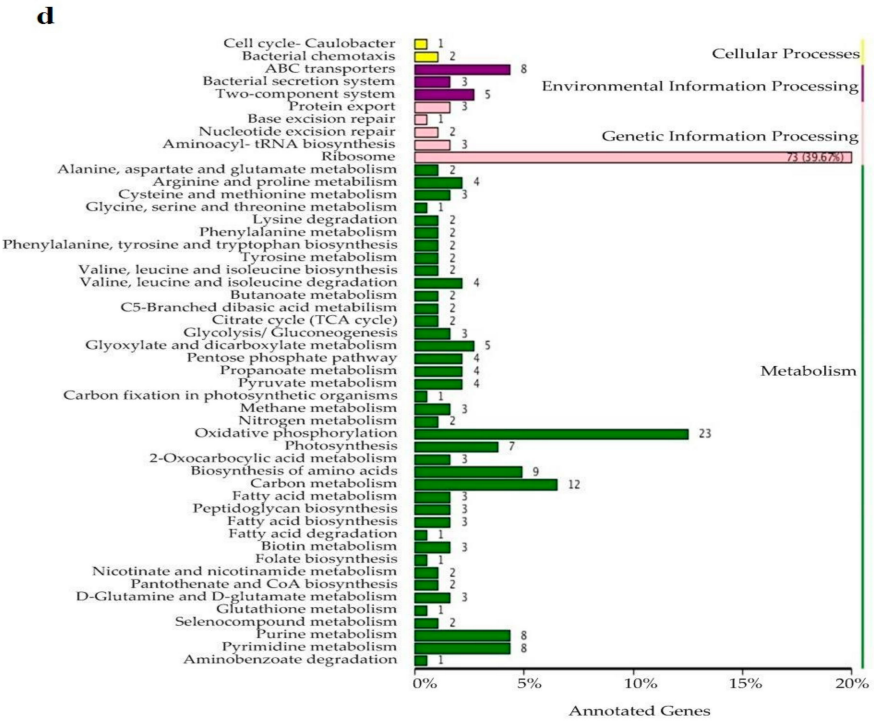

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG)classification map of DEGs. The *Y*-axis indicates the name of metabolic pathway. The *X*-axis indicates the number of genes annotated to the pathway and its proportion to the total number of genes annotated. (**a**) DEGs between CK and NPK; (**b**) DEGs between CK and NPK+S0.5; (**c**) DEGs between CK and S0.5; (**d**) DEGs between NPK and S0.5.

3.4. The Soil Microbiome Participating in N Cycling

There were seven pathways in the maize soil rhizosphere, including nitrogen fixation (nifH and nifD), nitrification (amoA, amoB and hao), and denitrification (narG nirK, nosZ, norZ and norB). The genes that participated in nitrogen fixation included nifH and nifD, which had increased abundances with organic fertilizers (NPK+S0.5 and S0.5 treatments). Genes that participated in nitrogen nitrification (amoA and amoB) had more abundant gene families in CK and S0.5 than in other treatments, although the hao gene showed the opposite pattern. The denitrification genes (narG, nirK, nosZ, norZ and norB) differed with fertilization application, and their abundance in the NPK and NPK+S0.5 treatments was higher than those in the CK and S0.5 treatments (Figure 5).

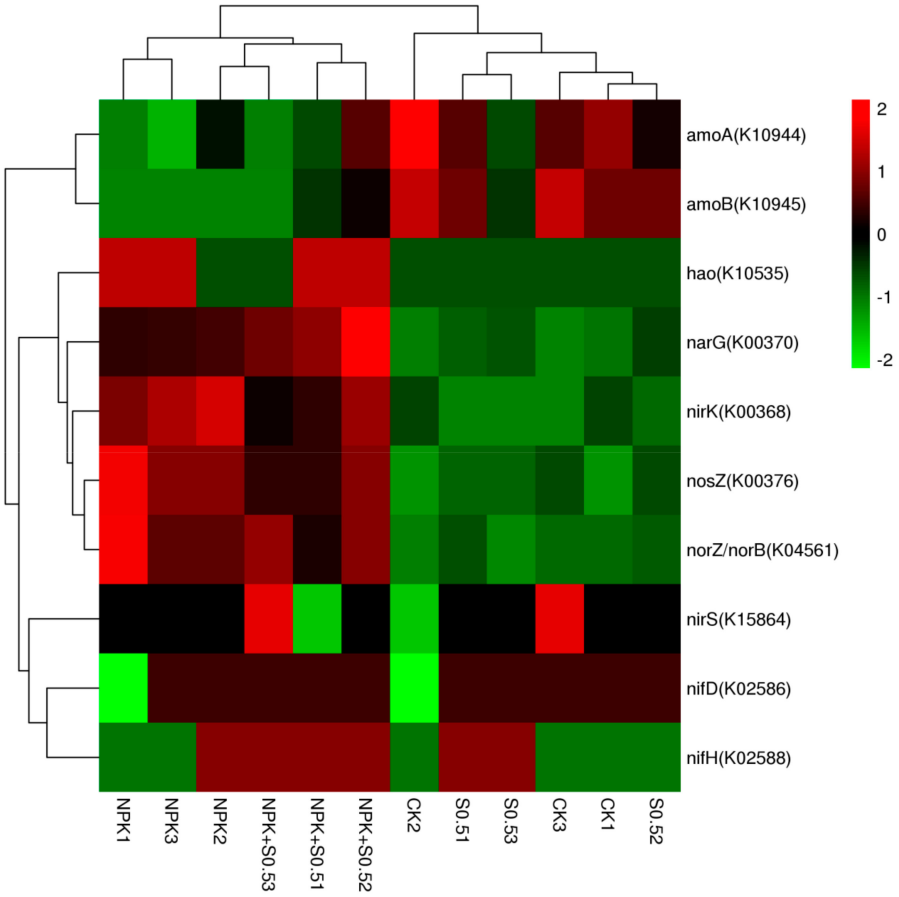

Figure 5. Heatmap of soil nitrogen cycle microbiome abundance genes with different fertilizer applications.

To analyze the function of genes participating in the N cycle, we used GO annotation. Figure 6 shows 33 gene annotations, and the dominant annotation was hydrolase activity acting on carbon–nitrogen (but not peptide) bonds (20.15–20.73%), followed by nitrogen compound metabolic processes (18.67–19.22%) and carbon–nitrogen ligase activity with glutamine as an amido-N donor (9.40–10.40%). Fertilizer application increased carbon–nitrogen ligase activity, and NPK treatment increased oxidoreductase activity, hydrolase activity, nitrogen utilization, phosphotransferase activity, cellular nitrogen compound catabolic processes and carbonyl sulfide nitrogenase activity but decreased the regulation of nitrogen utilization and the negative regulation of nitrogen compound metabolic process. The NPK+S0.5 treatment had an increased relative abundance for nitrogen fixation and organonitrogen compound metabolic processes. To further analyze the effects of fertilization on microorganisms participating in the N cycle, we analyzed the relationship between soil physical and chemical properties and N cycle genes. Interestingly, most of the metabolic pathways were significantly related to NO_3^--N (Figure 7). The application of organic fertilizer can significantly increase the content of soil nitrate nitrogen, which indicates that straw application has a significant impact on the N cycle.

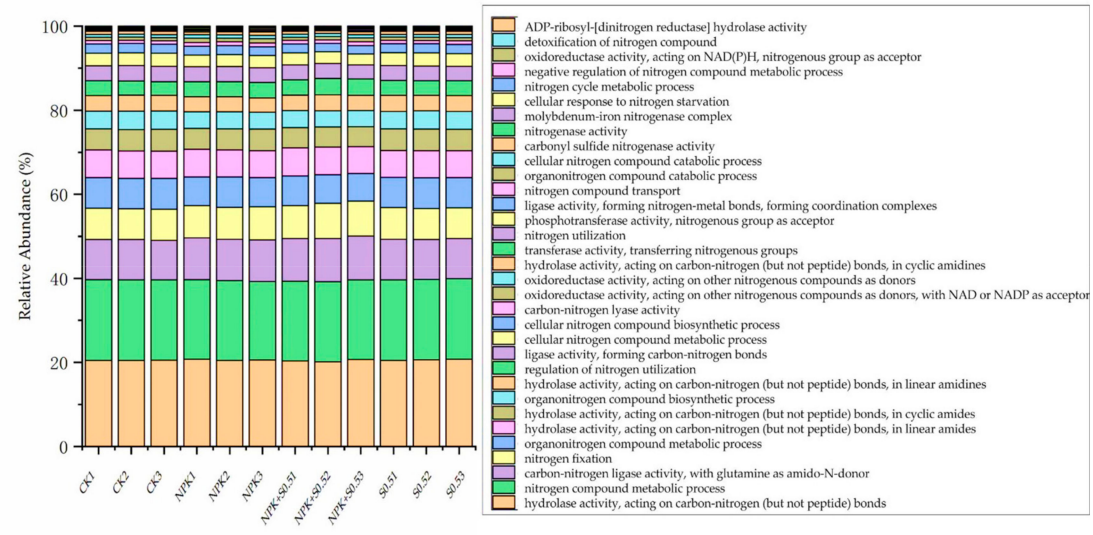

Figure 6. The relative abundance of the function of genes participating in the N cycle shown with GO annotation.

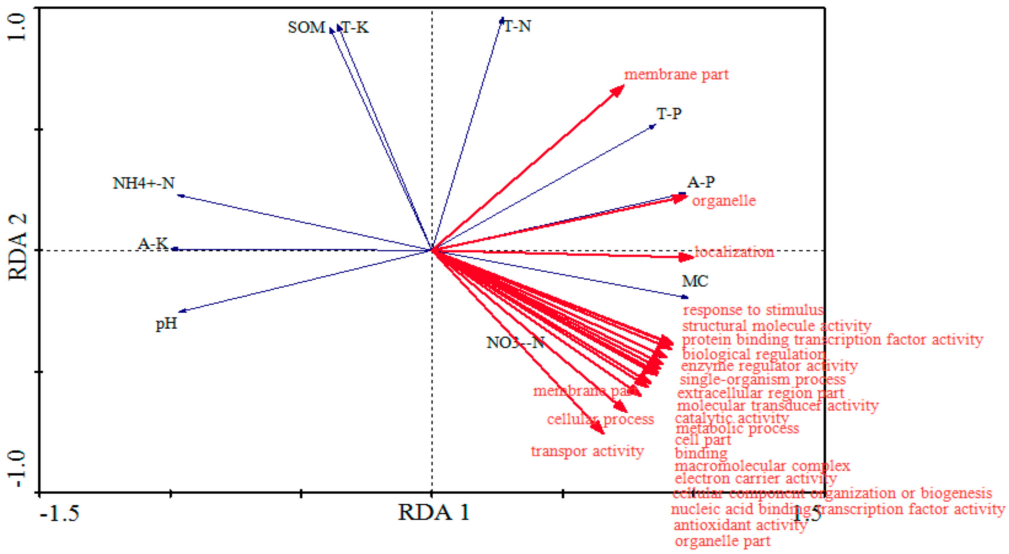

Figure 7. Redundancy analysis (RDA) of soil chemical properties and genes in N cycling, showing the contribution of environmental parameters to variability. Arrows indicate environmental factors and their relative effects on genes of the N cycle.

4. Discussion

4.1. Soil Chemistry Properties

Fertilizers not only improve corn production but also change soil chemical properties [5]. The long-term application of acidifying fertilizers and continuous cropping can cause soil acidification [30]. In long-term fertilized soil, NPK application decreased soil pH and increased soil acidification, whereas organic matter addition buffered acidification [31]. In this paper, compared with the CK treatment, the NPK and NPK+S0.5 treatments significantly decreased soil pH from 7.22 to 6.55, but the S0.5 treatment increased soil pH. This may be because of the fact that after plants absorb the nutrient ions in fertilizer, the hydrogen ion content in the soil increases, which readily causes soil acidification. Meanwhile, soil organic carbon (SOC) and total nitrogen (TN) were highest in the soil

under the NPK+S0.5 treatment. Moreover, fertilizer application influences physical, chemical and biological properties and processes in soils [32]. Previous studies have shown that SOC content was relatively high with maize straw application, and maize straw-supplemented organic matter could optimize the C:N ratio [33]. In this paper, the C:N ratio changed significantly under different fertilizers; each treatment value was higher than the CK treatment, and the highest ratio was found in the soil under the NPK+S0.5 treatment. The NPK+S0.5 treatment had the highest $NH4^+$-N content and the lowest NO_3^--N content, although the opposite trend was observed in the NPK treatment. Agehara et al. [34] argued that soil $NH4^+$-N and NO_3^--N were significantly related to MC and temperature. We found that organic fertilizer application could increase NH_4^+-N content and decrease NO_3^--N (Figure 1).

4.2. Soil Microbiome Community and Function

Sun et al. [35] suggested that environmental factors play important roles in changing microbial community structure and composition. Our research found that NPK+S0.5 had the highest number of metagenome sequences. We analyzed the soil composition (bacteria, fungi, archaea and viruses), and the predominant genes were bacterial genes, which accounted for over 96% of the genes detected by metagenomic taxonomic analysis [36–38]. The dominant phyla were Proteobacteria and Acidobacteria in bacteria [39,40], Ascomycota and Basidiomycota in fungi [41], and Crenarchaeota in archaea [42], and their levels were altered by fertilizer [37]. Moreover, the Proteobacteria, Basidiomycota, Crenarchaeota and Euryarchaeota relative abundances were highest in the NPK treatment and lowest in the S0.5 treatment, and they were negatively related to TN (Table A3). By contrast, Acidobacteria, Ascomycota and Euryarchaeota were lowest in the NPK treatment, and were positively related to TN and negatively related to pH, AK, NH_4^+-N and total phosphorus (TP). In long-term fertilized soil, the pH decreased significantly with inorganic fertilizer, and the C:N ratio was highest in NPK+S0.5; these six factors correlated with the soil microbiome [42,43] (Table A3). Fertilizers influence not only soil chemical properties but also microbiome gene levels. Long-term NPK fertilizer application had a greater effect on microbiome genes than organic fertilizer and organic–inorganic fertilizers. There were 1137 DEGs in NPK vs. CK but 234 and 304 in S0.5 vs. CK and S0.5 vs. NPK+S0.5, respectively (Table A2).

GO and KEGG are well-known to contain DEG classification and functional enrichment information [28,29], showing molecular biological functions, cellular components, and biological processes for describing the functions of gene products [44]. An interesting phenomenon observed in the present study suggested that chemical fertilizer had more effects on nucleic acid transcription and protein transcription than organic fertilizer (Figure 3). This may be related to the significant effects of soil organic carbon on microbial metabolic pathways, which can dissolve organic carbon into CO_2 while providing cells with energy [45]. Chemical fertilizer and organic fertilizer provide different types of nutrition for microorganism activities. Previous studies suggested that fertilizer can improve microbial metabolic activity, increase the number of single organisms, such as microbes, fungi and small animals, and accelerate the decomposition of macromolecular complexes to small complexes [46]. We also found that chemical fertilizer had effects on antioxidant activity, enzyme activity and molecular transducer activity. This may be because chemical fertilizer changed the soil chemical properties, and these metabolic processes had a significant positive correlation with NO_3^--N, AP, and MC (Figure 7). The ribosome pathway was determined to be significantly enriched in each treatment by KEGG pathway analysis, followed by oxidative photosynthesis and carbon metabolism, possibly because fertilizer application affected cation transport and the biosynthesis of organic and amino acids [47]. Moreover, nitrogen chemical fertilizer application could increase environmental information processing (Figure 4), possibly because the N fertilizer had an impact on these processes. Organic fertilizer and chemical fertilizer provided different forms of nutrients to the soil and changed the content of soil organic carbon, active nitrogen and C:N ratio (Figure 1). These changes affected the decomposition, synthesis and transformation of microorganisms [48]. Therefore, compared with the

S0.5 treatment, the NPK treatment had DEGs enriched in the ABC transporter and two-component system pathways.

4.3. The Soil Microbiome Participating in the N Cycle

The N cycle is an important biogeochemical cycle and is almost entirely performed by microbiome redox reactions [49–51]. Fertilization can affect the soil N cycle, in which it plays a very important role [52]. Therefore, it is important to understand how fertilizers affect the parts of the microbiome that participate in the N cycle, especially in long-term fertilized soil. In this study, the nifH gene was less abundant in the CK and NPK treatments and more abundant in the S0.5 and NPK+S0.5 treatments, which might be because maize straw addition increased the soil microbiome carbon and nitrogen resources, which improved the soil microbiome activity to reduce N_2 to NH_4^+ and other nitrogen-containing compounds [53]. Previous research has suggested that certain amounts of fixation microbes belong to Proteobacteria [54] and that NPK+S0.5 and S0.5 treatments decrease the relative abundance of Proteobacteria [55]. Ammonia monooxygenase (Amo) is an important enzyme in ammoxidation, and NPK application can decrease the relative abundance of amoA and amoB genes [56]. Hydroxylamine oxidoreductase (Hao) catalyzes the oxidation of NH_2OH to NO_2^-, and NPK and NPK+S0.5 improved this process. Similarly to results reported by Chen et al. [57], we found that long-term fertilization with nitrogen had significant impacts on the diversity or community of amoA and hao genes. We used narG, nirK, norZ, norB and nosZ genes to reveal denitrification, including NO_3^- to NO_2^-, NO_2^- to NO, NO to N_2O and N_2O to N_2 transformations. We discovered that the NPK and NPK+S0.5 treatments increased the relative abundance of the narG, nosZ, norZ and norB genes and accelerated denitrification (Figure 5). Meng et al. [58] showed that long-term fertilization significantly influenced soil MC, NH_4^+ and NO_3^- and the emission of N_2O, and that these changes were due to long-term N fertilizer application. In addition, fertilizer application had a significant influence on microbiome active processes, especially hydrolase activity acting on carbon–nitrogen bonds, nitrogen compound metabolic processes and carbon–nitrogen ligase activity with glutamine as an amido-N donor (Figure 6).

5. Conclusions

To the best of our knowledge, this study consists of the first attempt to investigate the impact of long-term fertilizer application on the soil microbiome and its function using metagenomic analysis. Fertilizer application changed soil chemical properties: the S0.5 treatment increased soil pH, the NPK inorganic fertilizer decreased either the soil pH or NH_4^+-N, and the NPK+S0.5 treatment also decreased soil pH but increased NH_4^+-N. We obtained 4.391×10^8 clean reads, and the highest number of metagenomic sequences was found in the NPK+S0.5 treatment. The soil microbiome consisted of bacteria, fungi, archaea and viruses, and the bacteria accounted for over 96% of the total genes. The dominant phyla were Proteobacteria, Acidobacteria, Firmicutes (bacteria), Ascomycota, Basidiomycota (fungi) and Crenarchaeota (archaea), and fertilizer application did not change the soil microbiome composition and dominant phyla but changed the relative abundance of the microbiome components. Inorganic fertilizer-supplemented maize straw had more influence on the soil microbiome than other treatments. The genes that were most differentially expressed were enriched in 48 pathways in CK vs. NPK, and we found that organic fertilizer and inorganic fertilizer both had a significant influence on metabolic processes, cellular processes, single-organism processes, binding, catalytic activity and structural molecule activity, cell parts, macromolecules and organelles. The NPK treatment resulted in unique DEGs rich in nucleic acid binding transcription factor activity, protein binding transcription factor activity, antioxidant activity, enzyme regulator activity, molecular transducer activity (molecular function), nucleoid, and extrac ellular region (cellular component). The S0.5 and NPK+S0.5 treatments increased microbial N fixation, and the NPK and NPK+S0.5 treatments decreased amoA and amoB and accelerated denitrification.

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Appendix A

Table A1. Summary of assembled scaffolds data for metagenomic sequencing.

Samples	Counting Numbers ($\times 10^5$)	Total Length (bp) ($\times 10^8$)	Largest Length (bp) ($\times 10^4$)	N50 (bp)	Mapped (%)
CK	1.92 ± 0.23 a	1.34 ± 0.26 b	4.48 ± 0.17 b	716.00 ± 29.87 b	16.56 ± 1.98 b
NPK	2.39 ± 0.14 ab	2.01 ± 0.11 a	6.03 ± 0.26 a	779.67 ± 1.53 a	20.39 ± 1.00 a
NPK+S0.5	2.66 ± 0.10 a	2.16 ± 0.98 a	5.82 ± 0.12 a	758.67 ± 25.42 a	19.81 ± 0.60 a
S0.5	1.82 ± 0.50 a	1.13 ± 0.13 c	4.46 ± 0.11 b	694.00 ± 14.00 b	15.26 ± 2.37 b

The lower case letters 'a', 'b' and 'c' indicate a significant difference ($p < 0.05$) among different samples for each treatment.

Table A2. Functional annotation of DEGs in the general database.

DEG Set	DEG Numbers	High Abundance	Low Abundance	COG	GO	KEGG	NR	Pfam	SwissProt
CK vs. NPK	1137	582	555	295	198	173	626	423	263
CK vs. NPK+S0.5	304	153	151	38	27	16	84	64	39
CK vs. S0.5	234	161	73	18	19	16	64	46	29
NPK vs. S0.5	1581	702	879	464	330	310	885	649	444

Note: COG is a database for homologous classification of gene products; GO is an international standard classification system of gene function database; KEGG is a database for systematic analysis of the metabolic pathways of gene products in cells and their functions; NR is a non-redundant protein database of NCBI database; Pfam is a database with the most comprehensive classification system for protein domain annotation; SwissProt is a database with higer Protein sequence and annotation information quality.

Table A3. The relationship between soil chemistry properties and the dominant microorganisms.

	pH	MC	SOM	AP	A-K	NH_4^+-N	NO_3^--N	T-P	T-K	T-N
Proteobacteria	0.74	−0.66	−0.02	−0.53	0.85	0.72	−0.41	−0.65	−0.23	−0.19
Acidobacteria	−0.82	0.61	0.03	0.63	−0.86	−0.70	0.48	0.73	0.18	0.27
Firmicutes	−0.07	−0.06	−0.67	0.19	−0.06	−0.14	0.63	−0.08	−0.95 *	−0.23
Ascomycota	−0.90	−0.03	−0.04	0.970 *	−0.49	−0.29	0.81	0.87	−0.39	0.60
Basidiomycota	0.95	−0.22	0.19	−0.94	0.70	0.52	−0.90	−0.85	0.43	−0.44
Mucoromycota	−0.76	0.29	−0.52	0.76	−0.66	−0.58	0.99 *	0.58	−0.74	0.14
Crenarchaeota	−0.09	0.63	0.14	−0.16	−0.45	−0.45	−0.24	0.06	0.60	−0.16
Euryarchaeota	−0.07	0.32	0.48	−0.11	−0.20	−0.14	−0.44	0.14	0.84	0.11
Thaumarchaeota	−0.88	0.69	−0.34	0.71	−0.97 *	−0.86	0.82	0.69	−0.27	0.11
Bradyrhizobium	−0.14	−0.03	−0.67	0.25	−0.12	−0.18	0.68	−0.03	−0.96 *	−0.20
Streptomyces	−0.93	−0.09	0.37	0.97 *	−0.44	−0.17	0.52	0.99 **	0.09	0.83
Mycobacterium	−0.76	0.29	−0.52	0.77	−0.67	−0.58	0.99	0.58	−0.74	0.14
Sordaria	0.62	−0.66	−0.10	−0.39	0.77	0.65	−0.24	−0.55	−0.38	−0.15
Fusarium	−0.33	−0.79	0.43	0.59	0.35	0.55	0.18	0.51	−0.11	0.79
Paracoccidioides	0.33	0.79	−0.43	−0.59	−0.35	−0.55	−0.18	−0.51	0.11	−0.79
Halorubrum	−0.19	−0.32	−0.39	0.37	0.07	0.08	0.59	0.12	−0.82	0.10
Halococcus	−0.76	0.29	−0.52	0.76	−0.66	−0.58	0.99 *	0.58	−0.74	0.14
Haloferax	0.43	−0.98	0.70	−0.18	0.93	0.99	−0.63	−0.14	0.38	0.48
Alphabaculovirus	−0.67	−0.30	−0.05	0.83	−0.19	−0.02	0.72	0.67	−0.52	0.57
Jd18virus	0.50	−0.86	0.82	−0.31	0.90	0.96 *	−0.83	−0.19	0.64	0.44
Betabaculovirus	0.738	−0.685	0.011	−0.524	0.863	0.738	−0.424	−0.639	−0.213	−0.17

Notes: * indicates $p < 0.05$, ** indicates $p < 0.01$; MC is the moisture content of soil; SOM is Soil organic carbon content; AP is available phosphorus; AK is available potassium; NH_4^+-N is ammonium nitrogen; NO_3^--N is nitrate nitrogen; TP is soil total phosphorus; TK is soil total potassium; TN is soil total nitrogen.

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