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Long-Term Organic-Mineral Fertilization Reshapes Soil Microbiomes, Nitrogen Cycling, and Tea Quality

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Abstract

Continuous fertilization may modify soil nutrient pools and microbial communities in tea plantations, but their joint relationships with nitrogen-cycling genes and tea-leaf composition remain incompletely characterized. Soil properties, tea quality, and shotgun metagenomic profiles were examined after 16 years of four field management regimes: no fertilization (CK), chemical fertilization (CF), organic manure (OM), and combined organic–mineral fertilization (OMN). Relative to CK, OMN had higher soil organic matter (73.30%), total nitrogen (76.12%), total phosphorus (140.45%), alkali-hydrolyzable nitrogen (128.20%), available phosphorus (182.35%), available potassium (93.89%), and nitrate nitrogen (313.86%). OMN also increased tea-shoot free amino acids (29.60%) and catechins (21.96%) relative to CK, whereas caffeine (13.45%) and water-extract content (6.73%) were lower. Microbial richness indices (ACE, Chao1, and observed species) were higher under OMN than CK by 22.46%, 18.24%, and 22.90%, respectively. Community composition differed among treatments (ANOSIM: R = 1, $p = 0.001$). Of 32,733 annotated nitrogen-cycling genes, 21,874 were shared among treatments, while OMN had the largest treatment-specific gene set (1912). Soil nutrient properties covaried with nitrogen-cycling gene profiles. These endpoint observations indicate distinct soil, microbial, and tea-chemical characteristics under sustained fertilizer regimes; metagenomic gene profiles describe functional potential rather than measured nitrogen-transformation rates.

Keywords: *Camellia sinensis*; functional genes; microbial diversity; nutrient management; shotgun metagenomics; soil fertility

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1. Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is an economically important perennial crop that is widely cultivated in the acidic soils of subtropical and tropical regions. Southern China is one of the principal tea-producing regions worldwide, where intensive fertilizer application

is commonly used to maintain tea yield and quality [1,2]. However, the long-term and imbalanced use of mineral fertilizers may aggravate soil acidification, disrupt nutrient stoichiometry, and reduce the sustainability of tea production systems [3,4]. Developing fertilization strategies that maintain soil fertility and tea quality while minimizing soil degradation is therefore essential for the sustainable management of tea plantations [5].

Soil microorganisms play central roles in organic matter decomposition, nutrient mineralization, and nitrogen transformation. Microbially mediated processes, including nitrogen fixation, ammonification, nitrification, denitrification, and nitrate reduction, collectively determine soil nitrogen availability and potential nitrogen losses. These processes involve multiple functional genes, such as *nifH* for nitrogen fixation, *amoA* for ammonia oxidation, *narG* and *napA* for nitrate reduction, *nirK* and *nirS* for nitrite reduction, and *nosZ* for nitrous oxide reduction [6,7]. Fertilization can alter the abundance and composition of microbial communities by changing soil pH, nutrient availability, carbon inputs, and substrate stoichiometry, thereby modifying the genetic potential for nitrogen cycling [8,9]. However, metagenomic gene abundance represents functional potential and should not be interpreted as direct evidence of gene expression, enzyme activity, or actual nitrogen-transformation rates.

Organic and mineral fertilizers differ substantially in their effects on soil microbial habitats. Mineral fertilizers provide readily available nutrients but may intensify soil acidification when applied continuously, whereas organic fertilizers supply both nutrients and carbon substrates and can improve soil organic matter content and microbial resource availability [10,11]. The combined application of organic and mineral fertilizers may balance immediate nutrient supply with longer-term improvements in soil fertility and has been associated with shifts in microbial community composition and functional potential [12]. Nevertheless, these responses are strongly influenced by soil type, crop species, fertilization history, and environmental conditions.

Tea plantations differ from annual croplands because of their perennial root systems, continuous rhizosphere inputs, repeated fertilizer application, and characteristically acidic soils. These features may produce distinct microbial responses to long-term fertilization. Although previous studies have examined individual soil properties or microbial groups in tea plantations, relatively few have simultaneously evaluated soil fertility, tea quality, microbial community structure, and nitrogen-cycling functional genes in long-term field experiments on acidic red soils. In particular, it remains unclear whether organic–mineral fertilization produces distinct microbial taxonomic and functional profiles compared with mineral or organic fertilization alone and how these profiles are associated with soil properties and tea quality.

The single sampling campaign at the end of the 16-year experiment captures the soil and microbial conditions associated with the accumulated management history and the interval since the most recent fertilizer application.

Therefore, based on a 16-year field fertilization experiment in a tea plantation, this study compared four fertilization regimes: no fertilization, conventional mineral fertilization, organic fertilization, and combined organic–mineral fertilization. The objectives were to (1) evaluate the effects of long-term fertilization on soil physicochemical properties and tea quality components; (2) characterize changes in soil microbial diversity and community composition using shotgun metagenomic sequencing; (3) compare the abundance and composition of nitrogen-cycling functional genes among fertilization regimes; and (4) assess the relationships among soil properties, microbial taxa, nitrogen-cycling functional profiles, and tea quality traits. We hypothesized that organic fertilizer inputs, particularly when combined with mineral fertilizer, would improve soil nutrient status and produce

distinct microbial taxonomic and nitrogen-cycling functional profiles relative to mineral fertilization alone.

2. Materials and Methods

2.1. Experimental Site and Design

A 16-year field experiment was carried out at the Jiangxi Economic Crop Research Institute, China (28°22′ N, 116°00′ E). The region is characterized by a humid subtropical monsoon climate, with an average annual temperature of 18.9 °C and precipitation of 1645.3 mm during 2009–2025. The tea cultivar 'Fuding Dabai' was grown throughout the experiment, and all fertilizer regimes were maintained annually without interruption.

The soil originated from Quaternary red clay and was identified as an Ultisol under the USDA Soil Taxonomy. Its particle-size distribution comprised 19.1% sand, 32.6% silt, and 48.3% clay, corresponding to a clay texture in the USDA classification system. The experimental soil was acidic and exhibited limited cation-exchange and buffering capacities, together with a comparatively low organic matter content, which is characteristic of red-soil tea plantations in southern China. Baseline physicochemical properties and particle-size composition of the 0–20 cm soil layer were measured before treatment establishment in 2009 and are reported in Table 1.

Table 1. Initial physicochemical properties and particle-size distribution of the 0–20 cm soil layer before treatment establishment in 2009.

Soil Property	Baseline Value	Unit
Bulk density	1.1	g cm^{-3}
pH	5.2	-
Soil organic matter (SOM)	18.1	g kg^{-1}
Total nitrogen (TN)	1.1	g kg^{-1}
Total phosphorus (TP)	0.4	g kg^{-1}
Total potassium (TK)	3.9	g kg^{-1}
Alkali-hydrolyzable nitrogen (AN)	115.0	mg kg^{-1}
Available phosphorus (AP)	15.9	mg kg^{-1}
Available potassium (AK)	64.0	mg kg^{-1}
Sand	19.1	%
Silt	32.6	%
Clay	48.3	%
Soil texture	Clay	-

Note: SOM, soil organic matter; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AN, alkali-hydrolyzable nitrogen; AP, available phosphorus; AK, available potassium. Soil textural class was determined using the USDA classification system.

The experiment comprised four fertilizer regimes: an unfertilized control (CK), mineral fertilizer alone (CF), chicken manure alone (OM), and a combination of chicken manure and mineral fertilizer (OMN). Details of the fertilizer materials, nutrient concentrations, application rates, and calculated annual nutrient inputs are provided in Table 2.

Treatments were assigned in a randomized block arrangement with three independent field replicates. Individual plots covered 667 m^2 and were separated by buffer areas to minimize nutrient movement between adjacent treatments. Except for fertilizer application,

management operations—including pruning, weed control, and pest management—were applied consistently across all plots in accordance with standard local practices.

Table 2. Experimental design of different fertilization treatments in the red-soil tea plantation.

Treatment	Fertilization Mode	Application Method	Nutrient Input (N-P_2O_5-K_2O, kg ha^{-1})	Source
CK	No fertilization	-	-	-
CF	Conventional chemical fertilization	Locally recommended compound fertilizer applied as basal fertilizer in mid-October each year, incorporated into the tea rows at a depth of 20 cm.	300-60-120	Xinyangfeng Agricultural Technology Co., Ltd., Jingmen, Hubei, China
OM	Application of organic fertilizer only	Chicken manure (4.7–2.6–2.1, N-P_2O_5-K_2O) applied at a rate of 5100 kg·ha^{-1} as basal fertilizer in mid-October each year, incorporated into the tea rows at a depth of 20 cm.	240-133-107	Lvtun Biotech Co., Ltd., Pingxiang, Fujian, China
OMN	Combined organic–inorganic fertilization	Mixture of compound fertilizer (18–8–12) at 300 kg ha^{-1} and chicken manure (4.7–2.6–2.1) at 4200 kg ha^{-1} applied in mid-October each year, incorporated into the tea rows at a depth of 20 cm.	251-133-124	Tea-Friendly Agricultural Technology Co., Ltd., Yichang, Hubei, China

Chicken manure contained 4.7% N, 2.6% P_2O_5, and 2.1% K_2O. The corresponding annual nutrient inputs were approximately 240–133–107 kg ha^{-1} (N-P_2O_5-K_2O) under OM and 197–109–88 kg ha^{-1} from manure under OMN; the latter also received 54–24–36 kg ha^{-1} from compound fertilizer. All figures represent total nutrient inputs rather than immediately plant-available nutrients (Table 2).

2.2. Sample Collection and Processing

In April 2025, approximately six months after the October 2024 basal fertilization, soil and tea shoots were sampled once from each field plot. Tea shoots comprising one bud and two leaves were pooled within plots, microwave-fixed, rolled, and oven-dried for chemical analysis.

Soil was sampled from the 0–20 cm depth at five locations within each plot using an S-shaped route. After removing surface litter, the five cores were pooled and mixed thoroughly to produce approximately 1 kg of composite material per plot. Each plot-level

composite was treated as an independent biological replicate, yielding 12 samples in total. Samples were transferred to sterile polyethylene bags, kept cool during transport, and processed immediately upon arrival at the laboratory. Roots, stones, plant fragments, and other visible materials were manually removed before the soil was sieved through a 2 mm mesh. Each homogenized sample was then separated into three subsamples. Fresh soil maintained at 4 °C was used to measure pH, NH_4^+-N, and NO_3^--N. A second subsample was frozen at −80 °C for DNA extraction and shotgun metagenomic sequencing. The third was air-dried, finely ground, and sieved to 0.149 mm for routine physicochemical analyses. Sampling and initial processing were completed within the same day to limit variability arising from handling and storage.

2.3. Determination of Soil Physicochemical Properties

Soil chemical properties were analyzed using established agrochemical methods. Soil pH was recorded with an FE28 digital pH meter (Mettler Toledo, Shanghai, China) in a 1:2.5 soil-to-water suspension (w/v). Soil organic matter (SOM) was quantified by potassium dichromate oxidation with external heating, whereas total nitrogen (TN) was measured by Kjeldahl digestion.

Following NaOH fusion, total phosphorus (TP) was determined by the molybdenum–antimony colorimetric procedure using a UV-2600 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), and total potassium (TK) was measured by flame photometry with an FP640 instrument (Shanghai Precision Scientific Instrument Co., Ltd., Shanghai, China). Alkali-hydrolyzable nitrogen (AN) was analyzed by alkaline diffusion. Available phosphorus (AP) was extracted with 0.5 mol L^{-1} $NaHCO_3$ and quantified using the molybdenum–antimony colorimetric method. Available potassium (AK) was extracted with 1.0 mol L^{-1} ammonium acetate and measured using the FP640 flame photometer. Ammonium nitrogen (NH_4^+-N) was determined by the indophenol blue colorimetric assay, while nitrate nitrogen (NO_3^--N) was quantified by ultraviolet spectrophotometry using the UV-2600 instrument. All nutrient contents are reported on a dry-soil basis.

2.4. Determination of Tea Quality Parameters

Dried tea material was milled and sieved to 0.5 mm before analysis. Soluble sugars were recovered with 80% ethanol and quantified by the anthrone assay. Glucose was used for calibration, and absorbance was recorded at 620 nm with a UV-2600 UV–visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Flavonoids were extracted in 70% ethanol and analyzed through the $NaNO_2$-$Al(NO_3)_3$-NaOH reaction, using rutin as the reference compound and measuring absorbance at 510 nm. Free amino acids were assayed in accordance with GB/T 8314–2013. Tea extracts were reacted with ninhydrin in a boiling-water bath, after which absorbance was recorded at 570 nm against a glutamic acid calibration curve.

Caffeine was measured by ultraviolet spectrophotometry at 274 nm following GB/T 8312–2013. For catechin analysis, 70% methanol extracts were separated on a reversed-phase C18 column and quantified at 278 nm using an Agilent 1260 Infinity II high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA), in accordance with GB/T 8313–2018. Water extract was determined following GB/T 8305–2013. Briefly, 3.0 g of ground tea was heated with 450 mL of boiling distilled water for 45 min. A 50 mL portion of the filtered extract was then evaporated and dried to constant weight at 103 ± 2 °C in a DHG-9070A oven (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China). All measurements were expressed on a dry-weight basis, and each treatment mean was derived from three independent field replicates.

2.5. Microbiome Sequencing and Bioinformatic Analysis

2.5.1. DNA Extraction and Quality Assessment

Twelve frozen soil samples, comprising four fertilization treatments with three independent field replicates per treatment, were subjected to shotgun metagenomic sequencing. Total microbial genomic DNA was extracted from approximately 0.5 g of each soil sample using a commercial soil DNA extraction kit according to the manufacturer's instructions. DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and DNA integrity was evaluated by 1% agarose gel electrophoresis. Only DNA samples exhibiting intact bands and satisfying the concentration and purity requirements of the sequencing provider were used for library construction [13].

2.5.2. Library Construction and Metagenomic Sequencing

Qualified genomic DNA was fragmented to an average insert size of approximately 350 bp using a Covaris M220 focused ultrasonicator (Covaris, Woburn, MA, USA). Paired-end sequencing libraries were constructed by end repair, Y-shaped adapter ligation, magnetic-bead purification, fragment selection, and PCR enrichment according to the sequencing provider's standard protocol. Library concentration and fragment-size distribution were assessed before sequencing. Qualified libraries were subjected to paired-end shotgun metagenomic sequencing on an Illumina NovaSeq platform (Illumina Inc., San Diego, CA, USA).

2.5.3. Sequence Quality Control and Metagenomic Assembly

Raw reads were assigned to individual samples on the basis of their barcode sequences and processed with fastp v0.23.0. Reads containing adapter contamination, low-quality bases, or an excessive proportion of ambiguous nucleotides were discarded. Sequences originating from the host were identified by alignment to the reference genome with BWA v0.7.17 and removed. The resulting high-quality, non-host dataset was subsequently used for metagenomic assembly.

Quality-filtered reads were assembled using MEGAHIT v1.2.9, retaining contigs $\geq$ 500 bp. Open reading frames were predicted with Prodigal v2.6.3 in metagenomic mode; predicted coding sequences <100 bp were excluded. Predicted genes were clustered into a non-redundant catalog using CD-HIT v4.6.1, and clean reads from each sample were mapped to the representative genes using SOAPaligner v2.21. Gene abundance was normalized using transcripts per million (TPM), which accounts for both gene length and sequencing depth. For gene *i*, TPM was calculated as follows: $TPM_i = (r_i/L_i)/\Sigma_j(r_j/L_j) \times 10^6$. where r_i is the number of reads mapped to gene *i*, and L_i is the length of gene *i* in kilobases.

Taxonomic annotation of the non-redundant gene catalog was performed using DIAMOND v2.0.13 in BLASTP mode against the NCBI non-redundant protein database released on 7 October 2024. An E-value threshold of 1×10^{-5} was used for sequence alignment. For each query sequence, matches with E-values within tenfold of the best hit were retained, and taxonomic assignments were determined using the lowest common ancestor algorithm. The abundance of each microbial taxon was calculated by summing the normalized abundances of all genes assigned to that taxon. Taxon-level abundance was obtained by aggregating the normalized abundances of genes assigned to the same taxonomic group. Relative-abundance profiles were subsequently constructed from the domain to species levels to describe microbial community composition.

Species-level taxonomic profiles were used to calculate ACE, Chao1, observed species (Sobs), Shannon, Simpson, and Pielou indices for individual field plots. Bray–Curtis dis-

similarities were visualized by principal coordinate analysis (PCoA). The three field plots per treatment were the independent biological replicates.

Functional annotation of the non-redundant gene catalog was performed using DIAMOND v2.0.13 against the Clusters of Orthologous Groups database released in 2020 and the Kyoto Encyclopedia of Genes and Genomes database released on 7 October 2024. KEGG Orthology identifiers were assigned to the predicted genes, and genes annotated to the KEGG nitrogen metabolism pathway (map00910) were extracted for subsequent analyses. The investigated nitrogen-cycling processes included biological nitrogen fixation, nitrification, denitrification, assimilatory nitrate reduction, dissimilatory nitrate reduction to ammonium, and ammonium assimilation. The abundance of each nitrogen-cycling KO was calculated by summing the normalized abundances of all non-redundant genes assigned to the corresponding KEGG Orthology identifier. Gene abundances belonging to the same nitrogen-transformation process were subsequently summed to generate process-level functional profiles. Within-sample relative abundances were used for comparisons of functional composition among treatments, whereas the normalized gene-abundance matrix was used for multivariate analysis. Gene abundances within the same nitrogen transformation process were summed to obtain process-level functional profiles.

Nitrogen-cycling genes were summarized by annotated functional category. The Venn diagram shows the overlap of detected gene-catalog entries across treatments; detection-based overlap does not establish treatment-exclusive biological functions. Redundancy analysis (RDA) was used to evaluate covariation between soil physicochemical properties and nitrogen-cycling functional profiles. Before RDA, the functional-gene abundance matrix was Hellinger-transformed, and the soil physicochemical variables were centered and standardized. Collinearity among explanatory variables was evaluated using pairwise correlations and variance inflation factors. Highly collinear variables were excluded before model fitting. The significance of the overall RDA model, constrained axes, and retained environmental variables was evaluated using 999 permutations. The adjusted coefficient of determination was also calculated.

2.6. Statistical Analysis

Microsoft Excel 2019 was used for data organization, whereas statistical analyses were performed in SPSS 26.0 (IBM Corp., Armonk, NY, USA) and R 4.2.2. Soil properties and tea-quality variables were subjected to one-way ANOVA, with treatment means separated by Tukey's honestly significant difference test. Before ANOVA, data were checked for normality and equality of variances using the Shapiro–Wilk and Levene tests, respectively. Values are reported as means ± standard deviations based on three independent field replicates.

Treatment effects on the relative abundance of each nitrogen-cycling gene group were assessed with the Kruskal–Wallis test. When the omnibus test was significant, Dunn's test was applied for pairwise comparisons. To control for multiple testing across gene groups, the resulting *p*-values were corrected by the Benjamini–Hochberg false discovery rate method. Statistical significance was defined as an adjusted *p*-value (q-value) below 0.05.

Bray–Curtis principal coordinate analysis, redundancy analysis, and ANOSIM were implemented in R 4.2.2 with the vegan and phyloseq packages. Permutation tests with 999 iterations were used to assess the overall RDA model, constrained axes, and retained environmental predictors. Relationships between dominant microbial taxa and measured tea-quality traits were examined using Spearman's rank correlations. The *p*-values for the full set of taxon–trait comparisons were jointly corrected by the Benjamini–Hochberg procedure, with $q < 0.05$ indicating statistical significance.

3. Results

3.1. Soil Properties and Tea Quality Parameters

3.1.1. Soil Physicochemical Properties

Table 3 shows differences in soil chemical properties among fertilization treatments at the final sampling. Relative to CK, OMN had higher SOM, TN, TP, AN, AP, AK, and NO_3^--N by 73.30%, 76.12%, 140.45%, 128.20%, 182.35%, 93.89%, and 313.86%, respectively ($p < 0.05$). SOM was also higher under CF (74.46%) and OM (75.41%) than CK, although mineral fertilizer was not a direct source of organic carbon. CF had higher TN, TP, and TK than CK, while OM had higher TK. Soil pH ranged from 4.26 to 4.60, with OM lower than CK; the remaining pairwise pH contrasts were not significant according to the reported letter groupings. Extractable NH_4^+-N was lower under CF and OM than CK, whereas OMN and CK did not differ significantly. The OM treatment had the lowest mean NH_4^+-N (8.86 mg kg^{-1}), despite receiving manure, indicating that soil mineral N concentrations at sampling did not simply track the form or amount of N supplied.

Table 3. Soil physicochemical properties under different fertilization treatments.

Treatment	pH	SOM (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	TK (g kg^{-1})	AN (mg kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})
CK	4.60 ± 0.21 a	24.16 ± 2.52 b	1.34 ± 0.18 b	0.89 ± 0.06 b	8.81 ± 0.42 b	136.40 ± 11.79 b	224.47 ± 15.50 b	123.83 ± 7.88 c	32.97 ± 12.12 a	6.42 ± 3.34 c
CF	4.34 ± 0.15 ab	42.15 ± 6.31 a	2.49 ± 0.07 a	2.62 ± 0.54 a	12.39 ± 0.49 a	220.38 ± 14.74 ab	593.25 ± 87.30 a	306.35 ± 77.54 a	13.95 ± 5.58 b	42.23 ± 5.81 a
OM	4.26 ± 0.06 b	42.38 ± 18.42 a	2.17 ± 0.75 b	0.87 ± 0.60 b	11.59 ± 0.59 a	192.55 ± 55.94 ab	144.67 ± 130.19 b	132.20 ± 12.05 bc	8.86 ± 2.43 b	8.05 ± 0.56 c
OMN	4.35 ± 0.12 ab	41.87 ± 3.66 a	2.36 ± 0.50 a	2.14 ± 0.80 a	9.13 ± 0.70 b	311.26 ± 113.72 a	633.78 ± 236.11 a	240.09 ± 84.43 ab	33.84 ± 4.83 a	26.57 ± 9.96 b

Note: CK: No fertilization; CF: Conventional chemical fertilization; OM: Application of organic fertilizer only; OMN: Combined organic–inorganic fertilization. Values are means ± SD ($n = 3$). Different lowercase letters within a column indicate significant differences at $p < 0.05$. pH: soil pH; SOM: soil organic matter; TN: total nitrogen; TP: total phosphorus; TK: total potassium; AN: alkali-hydrolyzable nitrogen; AP: available phosphorus; AK: available potassium; NH_4^+-N: ammonium nitrogen; NO_3^--N: nitrate nitrogen. The soil data presented in Table 3 represent soil properties measured at the time of microbial sampling in Year 16.

3.1.2. Tea Quality Parameters

Tea chemical composition varied among treatments (Table 4). Soluble sugars were higher under OMN than OM (43.69 versus 38.78 mg g^{-1}; $p < 0.05$). Free amino acids and catechins were higher under OMN than CK, whereas caffeine, total flavonoids, and water-extract content were lower. Responses therefore differed among measured constituents.

Table 4. Effects of different fertilization treatments on tea quality parameters.

Treatment	Total Soluble Sugars (mg g^{-1})	Total Flavonoids (mg g^{-1})	Caffeine (mg g^{-1})	Total Free Amino Acids (mg g^{-1})	Catechins (mg g^{-1})	Water Extract (%)
CK	40.70 ± 2.26 ab	113.22 ± 4.48 a	27.81 ± 0.07 a	15.34 ± 0.71 b	55.06 ± 0.70 c	44.58 ± 0.86 a
CF	40.62 ± 1.66 ab	109.82 ± 2.01 a	26.14 ± 0.66 b	14.74 ± 0.79 b	53.67 ± 1.15 c	42.65 ± 1.21 ab
OM	38.78 ± 1.78 b	93.98 ± 3.81 b	27.02 ± 1.27 ab	19.00 ± 0.86 a	57.42 ± 0.82 b	43.21 ± 1.81 ab
OMN	43.69 ± 2.42 a	92.28 ± 1.62 b	24.07 ± 0.78 c	19.88 ± 1.46 a	67.15 ± 1.10 a	41.58 ± 1.18 b

Note: CK: No fertilization; CF: Conventional chemical fertilization; OM: Application of organic fertilizer only; OMN: Combined organic–inorganic fertilization. Values are means ± SD ($n = 3$). Different lowercase letters within a column indicate significant differences at $p < 0.05$.

3.2. Responses of Soil Microbial Diversity and Community Structure

3.2.1. Microbial Alpha Diversity

Species-level metagenomic profiles showed higher richness under OMN than CK, with ACE, Chao1, and Sobs increasing by 22.46%, 18.24%, and 22.90%, respectively (Figure 1; $p < 0.05$). Shannon diversity and Pielou evenness were higher under OMN than OM by 8.18% and 6.38%, respectively ($p < 0.05$). The Simpson index was 94.12% higher under OM than CK ($p < 0.05$). Thus, richness and evenness indices showed different responses to the fertilization regimes.

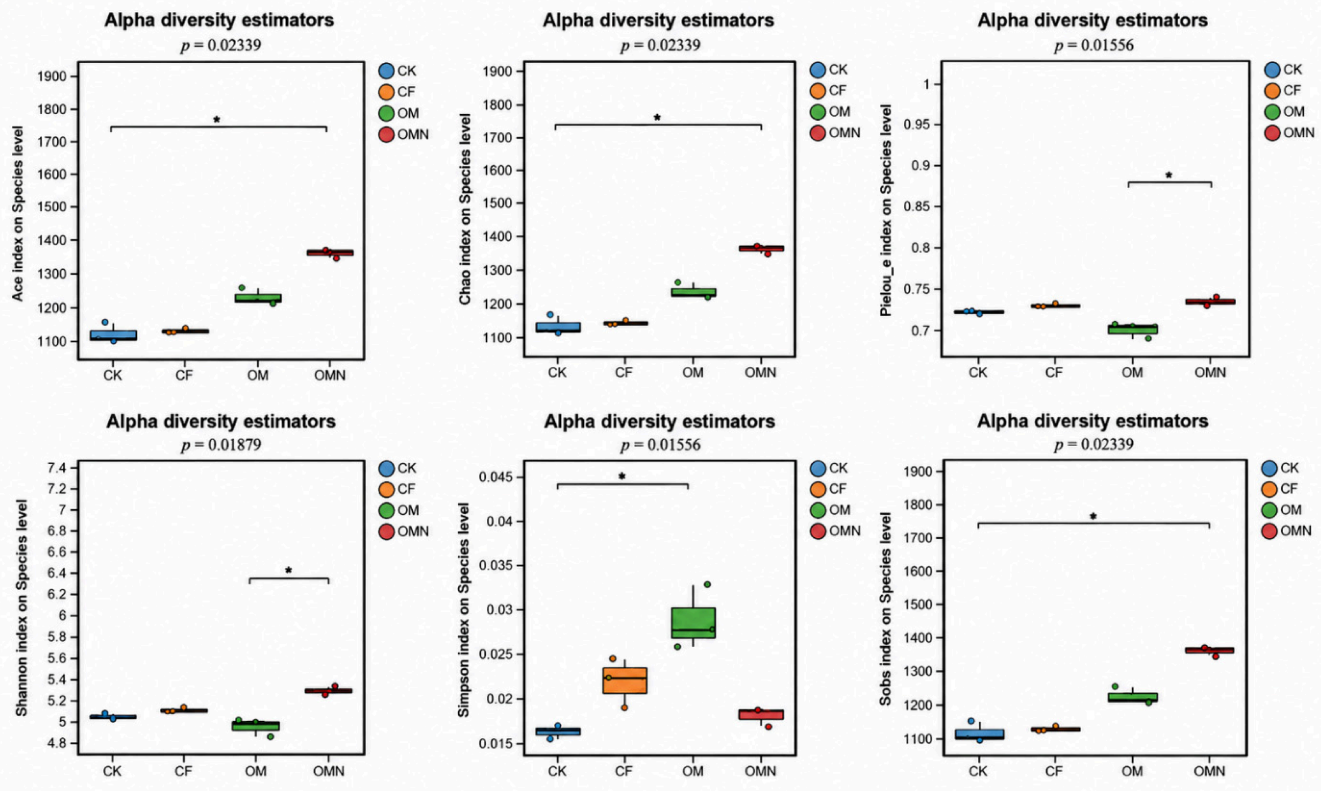


Figure 1. Alpha-diversity indices of microbial communities at the species level under different fertilization treatments. CK, no fertilization; CF, chemical fertilization; OM, organic manure; OMN, combined organic–mineral fertilization. * indicates a statistically significant difference at $p < 0.05$.

3.2.2. Microbial Beta Diversity

PCoA based on Bray–Curtis dissimilarities separated the treatment groups (Figure 2). The first two coordinates represented 58.18% and 24.86% of the variation, respectively. The overall ANOSIM yielded R = 1.000 and $p = 0.001$ (999 permutations).

3.2.3. Functional Gene Overlap and Microbial Composition

Across the treatments, 32,733 annotated nitrogen-cycling gene-catalog entries were detected; 21,874 (66.83%) occurred in all four treatment sets (Figure 3). OMN had the largest treatment-specific detected set (1912 entries). These counts describe detection overlap, not exclusive functional capabilities.

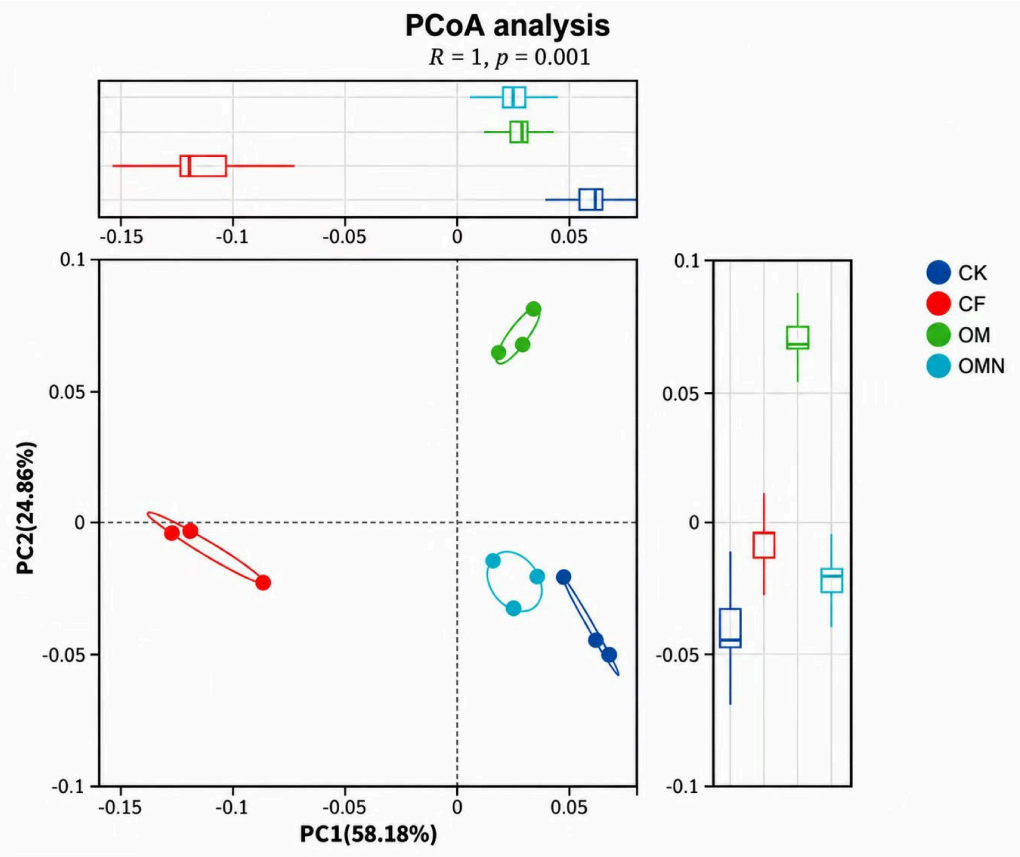


Figure 2. Principal coordinate analysis (PCoA) of soil microbial community composition based on Bray–Curtis dissimilarities. Each point represents an independent field plot. CK, no fertilization; CF, chemical fertilization; OM, organic manure; OMN, combined organic–mineral fertilization.

The soil microbial communities were dominated by Actinomycetota, Pseudomonadota, Acidobacteriota, and Chloroflexota across all fertilization treatments (Figure 4). Together, these four phyla accounted for the majority of the microbial community, although their relative contributions varied among treatments. Actinomycetota showed a higher relative abundance under CF, OM, and OMN than under CK, whereas Pseudomonadota was more abundant under CF and OMN than under CK and OM. In contrast, Acidobacteriota and Chloroflexota accounted for larger proportions of the community under CK than under the fertilized treatments, particularly CF. Candidatus_Dormiibacterota showed its highest relative abundance under OM, while Nitrososphaerota, Bacillota, Candidatus_Binatota, Candidatus_Methylomirabilota, and Planctomycetota occurred at relatively low abundances across all treatments. Overall, the fertilizer treatments differed in the proportional composition of the dominant microbial phyla, with conventional mineral fertilization and combined organic–mineral fertilization showing higher relative proportions of Actinomycetota and Pseudomonadota, whereas the unfertilized soil retained comparatively higher proportions of Acidobacteriota and Chloroflexota. These patterns indicate compositional shifts in the soil microbial community rather than changes in total microbial abundance.

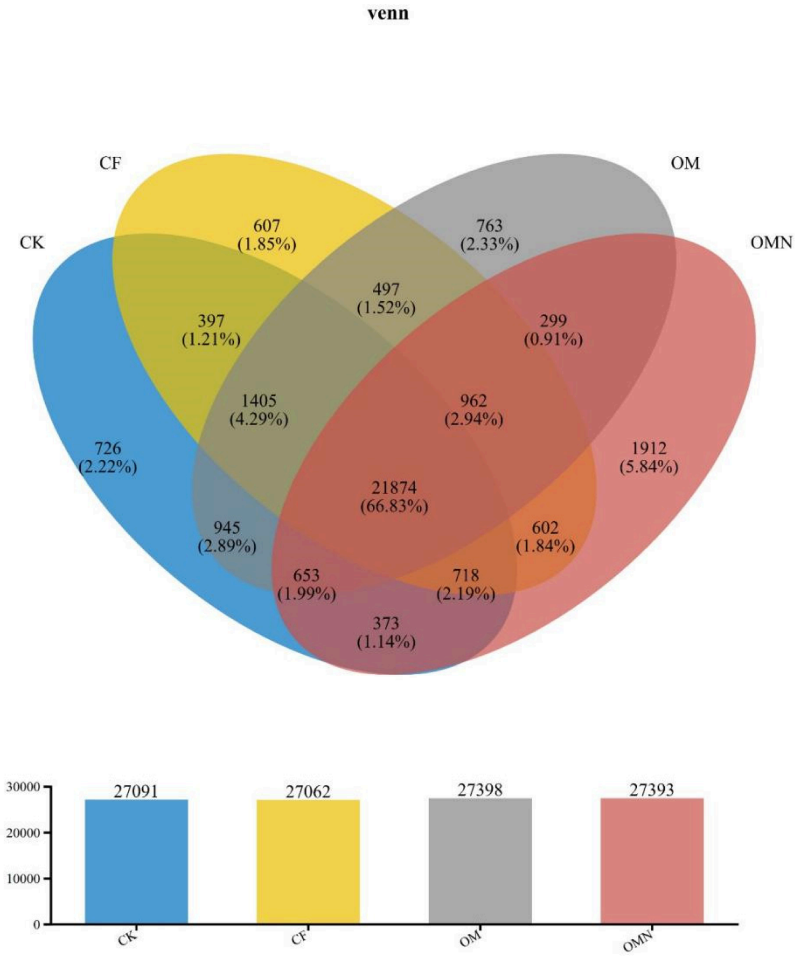


Figure 3. Shared and unique distributions of nitrogen-cycling functional genes under different fertilization treatments. Note: CK, no fertilization; CF, chemical fertilizer; OM, organic manure; OMN, combined organic–inorganic fertilization. Different colored areas represent the number and proportion of shared and unique functional genes among treatments, and the bar chart below shows the total gene count for each treatment.

3.2.4. Abundance of Nitrogen-Cycling Functional Genes

The relative abundances of ten nitrogen-cycling gene groups differed among fertilization treatments (Figure 5; Kruskal–Wallis test, $p < 0.05$). The OM treatment showed the highest mean proportions of glnA/GLUL, ncd2/npd, and GLU/gltS, whereas OMN had the highest proportions of narG/narZ/nxrA, gltB, and GDH2. In contrast, nirK and GLUD1_2/gdhA were most abundant under CK, while narH/narY/nxrB showed the highest mean proportion under CF. Overall, the fertilization treatments differed in the relative distribution of nitrogen-cycling genes, with organic and combined organic–mineral fertilization being associated with distinct functional gene profiles.

3.3. Associations Between Soil Properties and Microbial Profiles

The first two RDA axes accounted for 56.03% and 28.68%, respectively, of the variation reported in the ordination output (84.71% combined; Figure 6). CK was associated with soil pH and NH_4^+-N, whereas CF was associated with AP, TP, AN, AK, and NO_3^--N. These ordination patterns describe associations and do not establish causal effects.

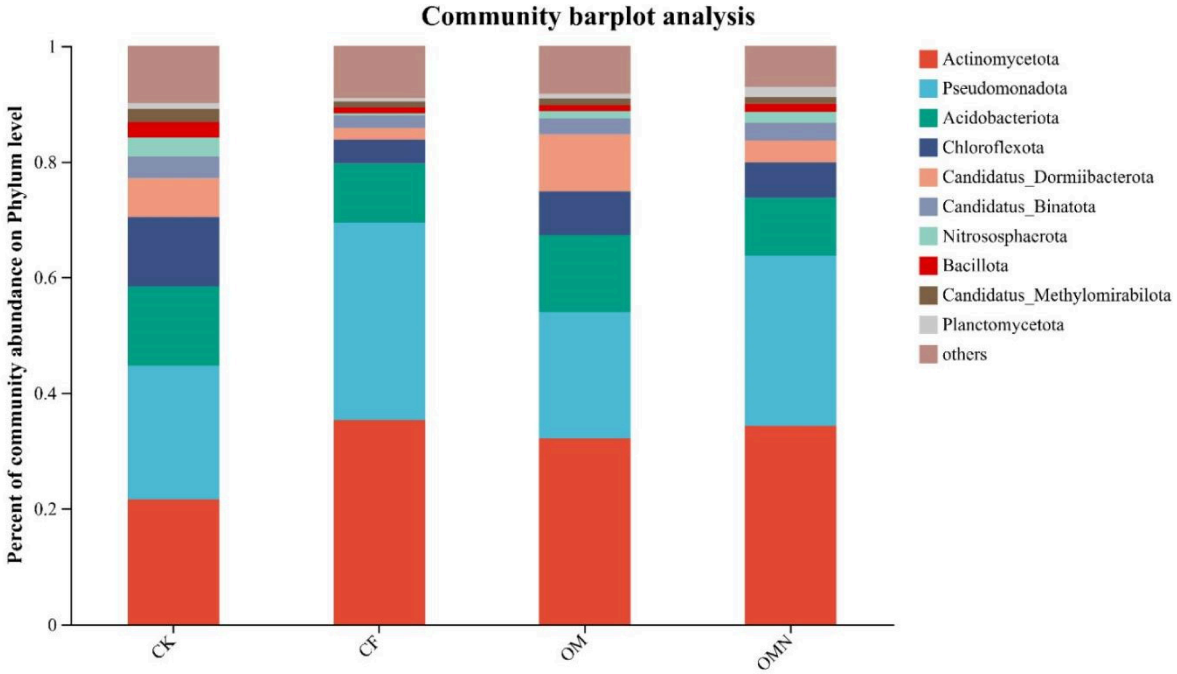


Figure 4. Relative abundance of dominant microbial phyla under different fertilization treatments; taxa below 1% are grouped as "others". CK, no fertilization; CF, chemical fertilizer; OM, organic manure; OMN, combined organic–inorganic fertilization.

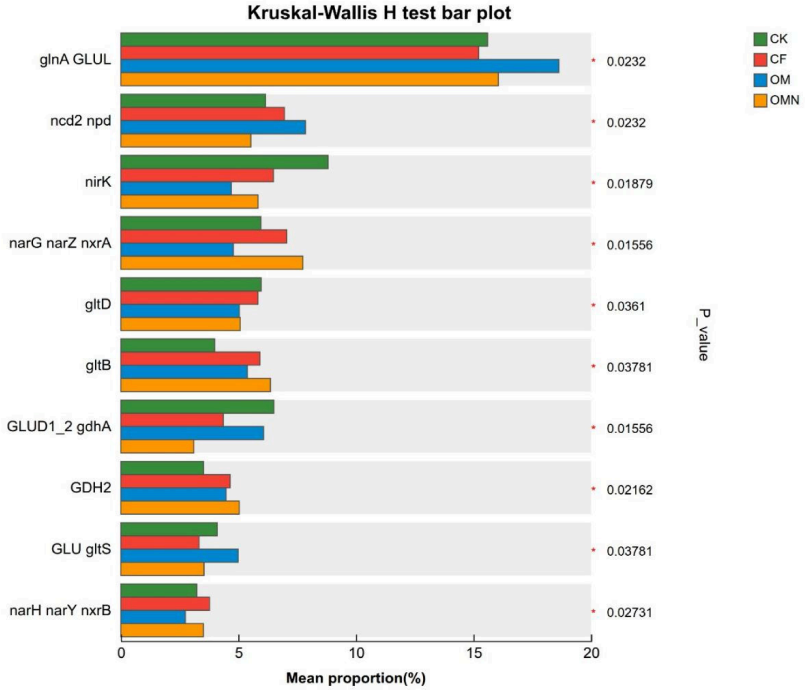


Figure 5. Mean relative proportions of annotated nitrogen-cycling gene groups across fertilization treatments. The right-hand *p* values are from omnibus Kruskal–Wallis tests. CK, no fertilization; CF, chemical fertilizer; OM, organic manure; OMN, combined organic–inorganic fertilization.

3.4. Correlations Between Soil Microbial Communities and Tea Quality Components

Spearman correlations showed associations between selected microbial taxa and tea-quality variables (Figure 7). Trebonia, Gaiella, Rhodanobacter, Pseudonocardia, and Candidatus Sulfotelmatobacter displayed different directions of association with individual traits. These exploratory correlations do not establish direct microbial regulation of tea chemistry.

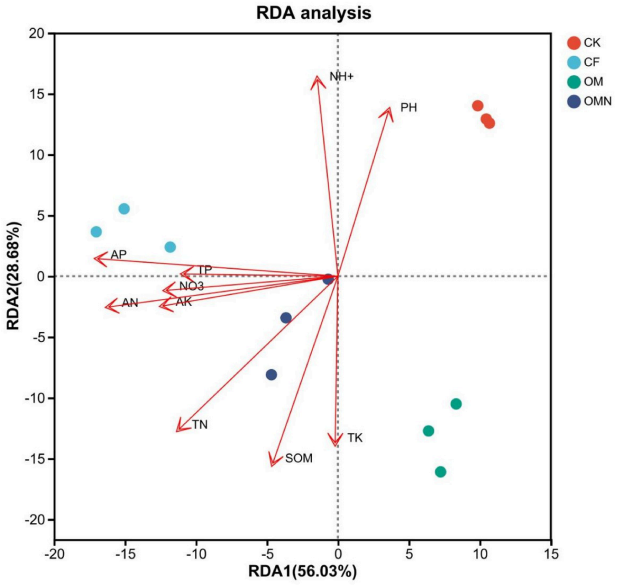


Figure 6. Redundancy analysis showing the covariation between soil physicochemical variables and nitrogen-cycling functional profiles across fertilizer treatments. Before ordination, functional-gene abundances were Hellinger-transformed, and soil variables were centered and scaled. Environmental predictors exhibiting strong collinearity were removed. Statistical support for the complete model, constrained axes, and retained predictors was assessed by 999 permutations. Red vectors denote the selected soil variables, with their orientation indicating the direction of association with the functional profiles. CK, unfertilized control; CF, mineral fertilizer; OM, organic manure; OMN, combined organic–mineral fertilization.

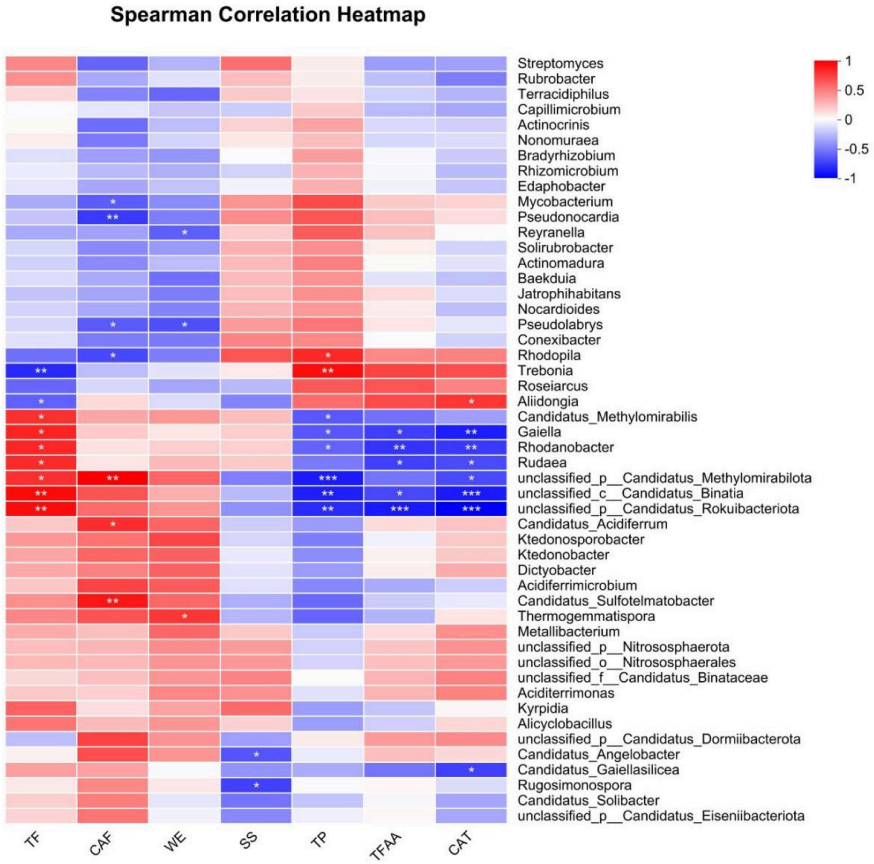


Figure 7. Spearman correlations between microbial taxa and tea quality traits. Red and blue indicate positive and negative correlations, respectively, and color intensity represents the magnitude of the

correlation coefficient. *p*-values from all taxon–trait correlations were adjusted using the Benjamini–Hochberg false discovery rate procedure. *, $q < 0.05$; **, $q < 0.01$; ***, $q < 0.001$. TF, total flavonoids; CAF, caffeine; WE, water extract; SS, soluble sugars; TFAA, total free amino acids; CAT, catechins.

4. Discussion

4.1. Responses of Soil Fertility and Tea Quality to Long-Term Organic Inputs

After 16 years of contrasting fertilizer management, soil nutrient pools differed among treatments (Table 3). OMN had higher SOM, TN, TP, AN, AP, AK, and NO_3^--N than CK, while CF and OM also showed higher SOM than CK. The latter observation is important because chemical fertilizer does not directly supply organic carbon: the SOM increase under CF may involve differences in plant-derived carbon inputs, litter and root turnover, or organic matter stabilization, none of which was measured directly. Organic manure supplies both carbon and nutrients, whereas mineral fertilizer primarily supplies nutrients in more immediately available forms. Differences in nutrient supply, mineralization, immobilization, and retention may therefore contribute to the contrasting soil properties observed here. Previous studies have reported changes in soil carbon storage following manure addition, with the magnitude depending on soil conditions and management duration [14,15].

Differences in soil nutrient concentrations are consistent with contrasting fertilizer sources and nutrient loading. Because the treatments were not nutrient-equivalent, effects of fertilizer form cannot be separated from effects of total nutrient input.

The measured tea constituents responded differently: OMN was associated with higher free amino acids and catechins but lower caffeine, flavonoids, and water-extract content than CK (Table 4). No single constituent adequately represents overall tea quality. Previous studies have also reported that organic amendments, either alone or in combination with mineral fertilizers, can alter tea yield and quality, with the responses depending on amendment type, application rate, and application duration [16–19].

4.2. Effects of Long-Term Fertilization on Microbial Community Structure and Nitrogen-Cycling Potential

Long-term fertilization significantly altered both microbial α-diversity and community composition in the tea-plantation soil. Compared with CK, OMN significantly increased the ACE, Chao, and Sobs indices, indicating that combined organic–inorganic fertilization supported greater species richness. However, the responses of diversity and evenness were more treatment-specific: the Shannon and Pielou indices were higher under OMN than under OM, rather than consistently higher than those under CK, while the Simpson index showed a different response pattern. These results suggest that OMN primarily expanded the number of detectable microbial taxa, whereas its effect on their relative-abundance distribution depended on the diversity metric considered. The higher richness under OMN may be associated with the simultaneous provision of readily available mineral nutrients and chemically diverse organic substrates [20,21]. Organic manure can provide microorganisms with substrates differing in decomposability, while mineral fertilizer may alleviate nutrient limitation during organic-matter decomposition. Together, these inputs may increase resource heterogeneity and create a wider range of ecological niches than a single fertilizer source. Long-term experiments in other agricultural soils have similarly shown that organic and mineral fertilizers exert distinct effects on microbial diversity and community structure, with combined fertilization frequently supporting a more complex or stable microbial community than mineral fertilization alone [22–25]. These richness, diversity, and evenness patterns are shown in Figure 1.

The PCoA and overall ANOSIM indicated treatment-associated differences in community composition (Figure 2). However, the ordination and ANOSIM results alone cannot distinguish shifts in group centroids from differences in within-group dispersion.

The taxonomic responses were consistent with a shift from relatively nutrient-limited communities under CK toward communities associated with greater resource availability under fertilization. Broad shifts in soil microbial community composition following increased nutrient availability have likewise been reported across biomes and fertilization gradients [26–28]. Actinomycetota showed higher relative abundance in the fertilized treatments than in CK, while Pseudomonadota was comparatively enriched under CF and OMN. Members of Actinomycetota include taxa capable of degrading structurally complex organic compounds, whereas many Pseudomonadota respond rapidly to increased concentrations of labile carbon and mineral nutrients [29]. Their higher relative abundances under fertilization may therefore reflect altered resource quantity and quality. Similar shifts toward nutrient-responsive microbial groups have been observed in long-term mineral and organic fertilization experiments [30–32]. In contrast, Acidobacteriota and Chloroflexota accounted for larger proportions of the community under CK than under the fertilized treatments, particularly relative to CF. Acidobacteriota are frequently associated with acidic and comparatively nutrient-poor soils, and their relative decline following fertilization is broadly consistent with reduced resource limitation [33]. Chloroflexota also showed a higher relative abundance under CK, but this phylum encompasses metabolically diverse lineages; therefore, its response should not be interpreted solely through a simple oligotrophic–copiotrophic classification. Similarly, the highest relative abundance of Candidatus Dormiibacterota under OM indicates a specific response to the organic-fertilizer environment, but its ecological role cannot be determined from phylum-level relative abundance alone. Importantly, all of these results describe proportional changes. An increase in relative abundance does not necessarily indicate an increase in absolute cell number because total microbial biomass was not quantified. Treatment-associated differences in the dominant microbial phyla and genera are presented in Figure 4.

Fertilization also affected the composition of the nitrogen-cycling gene repertoire. Of the 32,733 nitrogen-cycling genes detected, 21,874 were shared among all four treatments, accounting for approximately two-thirds of the total gene set. This large shared fraction indicates that the soils retained a common core of nitrogen-cycling functional potential despite 16 years of contrasting nutrient management. Nevertheless, OMN contained substantially more treatment-specific genes than CK, CF, or OM. The larger unique gene set under OMN is consistent with its higher microbial richness and may indicate that combined inputs supported a broader range of taxa carrying different nitrogen-metabolism genes. This does not necessarily mean that nitrogen cycling was faster under OMN, because gene detection is influenced by taxonomic composition, sequencing depth, assembly quality, and annotation thresholds. Moreover, the presence of a gene indicates metabolic potential rather than its transcription or contribution to an in situ process. The shared and treatment-specific gene repertoires are summarized in Figure 3.

The relative-abundance patterns of individual gene groups further suggested that fertilizer regimes selected for different nitrogen-metabolism strategies. The higher proportions of *glnA/GLUL* and *GLU/gltS* under OM, together with higher *gltB* and *GDH2* under OMN, are consistent with differences in the genetic potential for ammonium assimilation and glutamate metabolism. Organic inputs provide both carbon substrates and organically bound nitrogen, potentially increasing microbial demand for nitrogen assimilation during biomass production. However, different ammonium-assimilation pathways operate under different substrate concentrations and energetic conditions; therefore, the contrasting distributions of glutamine synthetase-, glutamate synthase-, and glutamate dehydrogenase-related genes

may reflect differences in microbial taxa and resource availability rather than a uniform increase in nitrogen retention [34]. Direct measurements of microbial biomass nitrogen, isotope-labelled nitrogen incorporation, gene transcription, or enzyme activity would be required to demonstrate enhanced microbial nitrogen immobilization. The functional gene-group profiles are presented in Figure 5.

Genes annotated as *narG/narZ/nxrA* and *narH/narY/nxrB* were most abundant under OMN and CF, respectively. These genes encode homologous subunits that may be assigned to nitrate reductases or nitrite oxidoreductases, making their ecological interpretation dependent on genomic context and the organisms carrying them. Their enrichment therefore indicates a shift in the genetic potential for nitrate or nitrite transformation but does not, by itself, distinguish nitrate reduction from nitrite oxidation. The higher relative abundance of nirK under CK similarly indicates greater representation of copper-containing nitrite-reductase genes, but it cannot be taken as evidence of higher denitrification or N_2O production. Functional-gene abundance may correlate with nitrogen-transformation processes under some conditions, but relationships between gene abundance and measured process rates are strongly affected by soil pH, oxygen status, carbon availability, temperature, and gene expression [35,36].

OMN exhibited higher microbial richness and the largest treatment-specific detected nitrogen-gene set, but these patterns do not demonstrate increased nitrogen-transformation rates, nitrogen-use efficiency, or reduced nitrogen losses. Such inferences require process measurements or gene-expression evidence.

4.3. Relationships Among Soil Properties, Microbial Profiles, and Tea Quality

The RDA results revealed close associations between soil chemical properties and nitrogen-cycling functional profiles, with the first two axes explaining 84.71% of the constrained variation. CK was positively associated with soil pH and NH_4^+-N, CF was aligned primarily with AP, TP, AN, AK, and NO_3^--N, and OMN was more closely associated with TN and SOM. In contrast, OM showed relatively weak alignment with the measured soil variables. These treatment-specific relationships indicate that long-term fertilization generated distinct soil nutrient environments that were accompanied by different microbial functional profiles. Soil pH is widely recognized as an important predictor of bacterial community composition because it imposes physiological constraints on microbial taxa and indirectly regulates nutrient solubility, substrate availability, and metal toxicity [37–39]. Large-scale investigations have further demonstrated that soil microbial communities are jointly structured by pH, organic carbon, nutrient availability, climate, and vegetation rather than by a single environmental variable [38,39]. In the present study, soil pH did not differ significantly among fertilization treatments. Therefore, although CK was directionally associated with pH in the RDA ordination, pH alone cannot explain the treatment-related separation of nitrogen-cycling profiles. The association of OMN with SOM and TN suggests that sustained carbon inputs and nitrogen accumulation contributed to the distinct microbial functional profile under combined fertilization. Greater SOM availability can increase the quantity and diversity of microbial carbon substrates, while changes in TN and mineral nitrogen availability may modify microbial nitrogen demand and select for different nitrogen-assimilation and nitrate-transformation strategies [40]. By contrast, the close alignment of CF with AP, TP, AN, AK, and NO_3^--N is consistent with the cumulative supply of readily available mineral nutrients. Long-term observations in tea plantations have similarly shown that changes in carbon and nitrogen availability are closely associated with variation in bacterial community structure and functional composition [41]. Organic and conventional management can also produce distinct bacterial community structures by altering soil nutrient availability and the resource environment, even when changes in

α-diversity are relatively small [42]. The corresponding soil-property ordination is shown in Figure 6.

RDA describes covariation rather than causal effects. Several soil nutrients are correlated, and the number of independent plots is small relative to the number of explanatory variables. Accordingly, the axis percentages and environmental vectors should not be interpreted as independent contributions of individual soil properties.

Several non-exclusive processes could produce these relationships. Soil microorganisms participate in organic-matter decomposition and nutrient mineralization and may consequently alter the forms and quantities of nitrogen, phosphorus, and other elements available for tea-plant uptake [42]. Changes in plant nutrient status may subsequently affect the allocation of carbon and nitrogen among free amino acids, caffeine, catechins, flavonoids, and other phenolic compounds. Previous studies have demonstrated significant relationships among soil carbon, nitrogen and phosphorus pools, microbial biomass, plant nutrient status, and major tea-quality components [43]. In particular, microbial biomass carbon, microbial biomass phosphorus, soil nitrogen, and plant-tissue nutrient concentrations have been associated with variations in tea polyphenols, amino acids, catechins, caffeine, and water extracts [43]. Studies comparing ancient and modern tea plantations have likewise found coordinated variation in soil fertility, microbial diversity, community composition, and tea-leaf biochemical characteristics [44].

The relationship between soil microorganisms and tea quality may also be bidirectional. Microbial communities can affect plant nutrient availability, but tea plants simultaneously modify the surrounding soil through root exudation, litter deposition, and rhizodeposition. These plant-derived compounds can provide microbial substrates or impose selective pressures on microbial taxa [45]. Large-scale investigations of ancient tea plantations have shown that geography, soil properties, tea plants, and management history jointly structure soil bacterial and fungal communities [46]. In aged tea plantations, the accumulation of polyphenols and catechin-related compounds has also been associated with reduced abundance of some plant-growth-promoting bacteria and enrichment of other microbial groups capable of tolerating or transforming phenolic compounds [45]. Thus, the correlations detected in the present study may reflect not only microbial effects on tea nutrition but also feedbacks of tea metabolites on the soil microbial community.

The ecological roles of the individual genera identified here should nevertheless be interpreted conservatively. Correlation with a tea-quality compound does not demonstrate that a taxon directly synthesizes, degrades, or regulates that compound. For example, a microbial genus may correlate with caffeine or catechin content because both respond to the same fertilizer-induced change in soil nitrogen availability, rather than because the microorganism directly affects the corresponding metabolic pathway. Similar correlation-based studies in tea plantations have identified relationships among soil nutrients, bacterial taxa, tea mineral nutrition, and biochemical quality, but these studies also emphasize the complexity of separating direct microbial effects from shared environmental responses [43]. Recent tea-soil experiments have likewise shown that bacterial community characteristics and tea-quality traits vary simultaneously across soil types and nutrient environments, without necessarily establishing direct microbial regulation of individual metabolites [40]. The taxon–tea-quality associations are summarized in Figure 7.

Relative-abundance data are compositional, so changes in one taxon can affect the apparent proportions of others. With only three plots per treatment and numerous taxon–trait comparisons, Figure 7 correlations should be considered exploratory rather than evidence of direct microbial regulation.

Because all samples were collected at a single endpoint, cumulative management effects cannot be separated from responses to the latest fertilizer application. Unequal nutrient

loading, limited replication, and the absence of process-rate measurements constrain causal interpretation. Repeated sampling and absolute microbial or nitrogen-transformation measurements would strengthen future inference.

5. Conclusions

After 16 years of contrasting fertilizer management, the four treatments differed in soil nutrient status, microbial community structure, nitrogen-cycling gene profiles, and tea-shoot chemistry. OMN showed higher concentrations of several soil nutrients and greater microbial richness than CK, together with higher free amino acids and catechins but lower caffeine, flavonoids, and water-extract content. Most detected nitrogen-gene entries were shared among treatments. These endpoint associations suggest that combined organic–mineral fertilization warrants further evaluation for acidic red-soil tea plantations, but unequal nutrient inputs and the absence of functional-rate measurements preclude attribution to fertilizer form alone or inference of increased nitrogen-cycling activity.

Author Contributions: L.-X.W. drafted the manuscript. X.-F.J. conceived and supervised the study, provided critical revisions, and secured funding. Q.L. and Y.-X.Z. performed the experiments and collected the data. W.-L.M., J.L. (Jin Li), and C.L. assisted with data analysis and interpretation. J.-G.Z., Q.-Y.S., and J.L. (Jun Le) contributed to sample preparation and laboratory work. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The raw metagenomic sequencing data presented in this study are openly available in the NCBI Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1537721 (accessed on 10 October 2026), under BioProject accession number PRJNA1537721. The other data supporting the findings of this study are included in the article or are available from the corresponding author upon reasonable request.

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