

Article

Changes in Soil Microbial Community Structure and Function Across Cultivation Durations Under Cucumber–Bitter Melon Rotation for Sustainable Greenhouse Production

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Abstract

Greenhouse vegetable production is important for food security, but intensive long-term cultivation can threaten soil sustainability. Although previous studies have reported that cultivation duration alters microbial community structure, research on greenhouse soils with crop rotation involving cucumbers and bitter melons remains rare, especially lacking in predicted microbial functional profiles. Therefore, this study performed 16S rRNA amplicon sequencing on greenhouse soils from four different cultivation years, aiming to evaluate the changes in soil microbial community structure and function due to long-term greenhouse cultivation. Over the first three years of cultivation, both alpha diversity and enzyme activity in the soil increased. Continuous cultivation and routine management were accompanied by marked shifts in the soil microbiota. Specifically, after 9 years, bacterial communities in surface and subsurface soils became highly imbalanced, with the Chao1 index decreasing by 65.64% and 82.46% compared to 3-year soils. Soils with shorter cultivation periods (1 and 3 years) had three times as many biomarkers as those with longer periods (6 and 9 years), and surface soils had nine times more biomarkers than subsurface soils. $\text{NH}_4^+\text{-N}$ and urease were significantly associated with bacterial and archaeal communities. PICRUSt2-based predictions suggested that the 9-year greenhouse soils had a higher inferred abundance of genes associated with certain metabolic processes compared to other cultivation durations, indicating potential functional shifts in the microbial community across the cultivation-duration gradient. These findings provide insights into soil microbial responses to cultivation duration and support sustainable management of greenhouse soils under cucumber–bitter melon rotation.

Keywords: microorganisms; greenhouse soil; cultivation duration; enzyme activity; functional prediction; sustainable agriculture; soil health



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1. Introduction

Compared with traditional open-field vegetable cultivation, greenhouse farming provides plants with suitable growth conditions and offers off-season vegetables, thereby increasing farmers' income. However, the crop rotation system in greenhouse vegetable cultivation eliminates the soil's fallow period. Meanwhile, greenhouse vegetable farming relies on annual fertilizer inputs exceeding 2000 kg N ha^{−1} to support crop rotation and high yields, which is 2–5 times higher than that in open-field farming [1]. Nutrient levels in

greenhouse soils have risen substantially as a result of excessive fertilizer application [2]. In greenhouse tomato production in North China, nitrogen use efficiency is typically less than 10%, with Shouguang showing an even lower efficiency of only 2.4–5.7% [3,4]. In Shouguang, while nitrogen fertilizers are applied to greenhouse vegetable fields, large amounts of organic fertilizers are also used. As the cultivation period increases, these practices lead to nitrate leaching, soil acidification, nutrient excess, and soil degradation, while also increasing production costs [5,6].

Understanding the associations between soil microbial communities and cultivation duration is essential for informing sustainable greenhouse management practices that maintain soil health and productivity. Bacteria and archaea influence soil ecological functions by producing extracellular polymers, releasing intracellular components, and regulating metabolic activities; they are highly sensitive to changes in the soil environment [7,8]. Enzyme activity is a crucial indicator of soil biological function, and its variation is influenced not only by the soil's physicochemical properties but also by the diversity and functionality of microbial communities. Researchers believe that the majority of microbial biomass, activity, and diversity are predominantly distributed in the 0–20 cm soil layer, and nitrogen cycling primarily occurs through the biological activity of the root zone, which typically extends to a depth of 1 m [9,10]. Therefore, studying the changes in microbial community structure in the 0–40 cm soil layer of greenhouse soils during the cultivation period provides valuable insights for safeguarding soil ecosystems and for advancing sustainable greenhouse agriculture.

Previous studies have shown that with the increase in cultivation duration, pH, NH_4^+ -N, and NO_3^- -N levels decline in the rhizosphere as well as in bulk soil, while the levels of organic matter, enzyme activity, and the abundance of bacteria, fungi, and actinobacteria increase [11]. However, conclusions across different studies are not entirely consistent, an inconsistency that may be attributable to variations in study regions, soil types, crop species, and field management practices. Microorganisms, as integral components of soil ecosystems, play a crucial role in processes such as soil organic matter decomposition and the carbon–nitrogen cycle [12]. Studies suggest that cultivation duration is regarded as a key determinant of soil bacterial community composition and diversity [13]. Several studies have shown that bacterial diversity decreases with the increase in cultivation duration and significantly alters the composition of autotrophic microbial communities involved in carbon sequestration ($p < 0.01$) [14,15]. Additionally, soils with longer cultivation durations showed differences in microbial structure, with a reduction in beneficial microorganisms, an increase in pathogenic microbial abundance, and the induction of various plant diseases [16–18].

Currently, research on the temporal changes in soil microbial communities in greenhouse vegetable fields remains limited, with significant differences in findings due to variations in research methods, locations, soil types, and crop species [19,20]. Previous studies have largely concentrated on open-field farmland or on greenhouse soils under monoculture conditions. In particular, there is a lack of studies on greenhouse soils subjected to long-term rotation of cucumber and bitter melon. Shouguang City, as the birthplace of greenhouse agriculture in China, has highly representative soil. Therefore, this study collected greenhouse soil samples at four different cultivation durations (1, 3, 6, and 9 years) from this region, using 16S rRNA high-throughput sequencing to analyze the soil microbial community structure, incorporating network analysis of the microbial community, and predicting various soil functions based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The aim is to provide valuable insights for scholars into the changes in greenhouse soil microbial community structure and function over time and to offer a scientific basis for the protection of greenhouse soil quality.

Our hypothesis is as follows: (1) The physicochemical properties of soil will differ across the cultivation-duration gradient, and these differences will be associated with microbial community structure and enzyme activity; (2) the soil microbial community composition will differ among cultivation durations, potentially manifesting as shifts in the abundance of certain species, which could be linked to variations in soil functions; and (3) the microbial community will exhibit not only annual variations in species richness and diversity, but also demonstrate adaptive functions in response to specific greenhouse conditions (e.g., long-term fertilization, monoculture cropping, etc.).

2. Materials and Methods

2.1. Experimental Site and Soil Sampling Procedure

The soil investigated in this study was taken from a typical greenhouse vegetable cultivation area located in Shouguang, Shandong Province, China (36°53' N, 118°44' E). The region has a warm continental monsoon climate, with an annual average temperature of 14.5°C, total precipitation of 1033.1 mm, and 2534.7 h of sunlight in 2023 (Shandong Statistical Yearbook, 2023, <http://tjj.shandong.gov.cn/> (accessed on 15 March 2024)). According to the World Reference Base for Soil Resources (WRB), this soil is classified as *Cambisols* (*Calcaric, Loamic*). It is a weakly alkaline fluvo-aquic soil and has been cultivated with cucumbers and bitter melons for many years. The fertilizers applied to the greenhouse vegetable soil are primarily compound fertilizers (5000 kg ha⁻¹ per year) and organic fertilizers (chicken manure and rice husks, 15000 kg ha⁻¹ per year). Following a thorough investigation, four greenhouse vegetable plots with consistent cultivation histories and field management practices were selected in this study to minimize, as far as possible, the variation in the results that might arise from the cross-sectional chronosequence design, whose cumulative cultivation years were 1, 3, 6, and 9, respectively. After removing the contaminated surface soil, samples were collected from the surface (0–20 cm, SS) and sub-surface (20–40 cm, DS) layers of the greenhouse vegetable fields using a five-point sampling method. For each field and each soil layer, the five subsamples were thoroughly mixed to form one composite sample, and this process was independently repeated three times to obtain three composite samples per layer per field ($n = 3$). These three composite samples were used for soil physicochemical and enzyme activity analyses. For microbial sequencing, equal amounts of the three composite samples were pooled to form one sequencing sample per layer per field.

2.2. Measurement Parameters and Methods

Soil NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N were determined by KCl extraction followed by UV spectrophotometry. SOC was measured by the dichromate oxidation-spectrophotometric method. Soil urease, sucrase, and catalase activities were determined colorimetrically or by UV spectrophotometry according to standard procedures. Detailed reagent compositions, extraction conditions, and measurement wavelengths are provided in the Supplementary Material (Text S1).

The soil acidity or alkalinity was measured using a pH meter. The calculation method of the soil apparent nitrification ratio (ANR) is as follows [21]:

$$\text{ANR (\%)} = \frac{\text{NO}_3^- \text{-N}}{\text{NO}_3^- \text{-N} + \text{NH}_4^+ \text{-N}} \times 100$$

2.3. Soil Microbial Analyses

The core of 16S rRNA gene sequencing is to investigate the species classification, abundance, and phylogeny of the sample. Three replicate soil samples from the same source were pooled to form a composite sample for analysis. Soil DNA was extracted using

a DNA extraction kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. PCR amplification of the genes was performed using the ABI GeneAmp®9700 PCR instrument (Thermo Fisher Scientific, Waltham, MA, USA), with cycling conditions optimized for the PCR process. Bacterial primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT), together with archaeal primers Arch519F (CAGCCGC-CGCGGTAA) and Arch915R (GTGCTCCCCCGCCAATTCCT), were used for 16S rRNA amplicon sequencing. The amplified products were then used for library preparation with the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Libraries were quantified using Qubit and checked for quality. Sequencing was carried out on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA) with a PE250 configuration. For microbial sequencing, equal amounts of the three composite samples from each field and each soil layer were pooled to form one sequencing sample. Therefore, there was one sequencing sample per cultivation duration per soil layer, and no independent biological replicates were available at the sequencing level.

2.4. Statistical Analysis

All one-way ANOVA tests for soil physicochemical and enzyme activity data were performed with three composite samples per field per layer ($n = 3$). Because microbial community data were based on one sequencing sample per cultivation duration per soil layer, no independent group-level replicates were available for statistical testing of alpha diversity, LEfSe, and PCoA; these analyses are therefore descriptive and exploratory. Co-occurrence network analysis was performed across all samples using Spearman correlation, but the limited number of samples means the network should also be interpreted as exploratory. All statistical tests were conducted in IBM SPSS Statistics v27 (IBM, Armonk, NY, USA), with data organization and basic calculations performed in Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Graphing was conducted using Origin 2017 (OriginLab, Northampton, MA, USA). QIIME2 v2023.5 was used for preprocessing and analysis of microbial community data, and generating correlation matrices to construct networks. Canoco v5.0 (Microcomputer Power, Ithaca, NY, USA) was used to analyze the multivariate correlations between microbial communities, enzyme activity, and soil carbon–nitrogen components. The PICRUST2 v2.5.0 bioinformatics package was used for functional prediction based on the 16S rRNA sequencing data and the KEGG database (<https://www.kegg.jp/>) [22]. Visualization of the microbial co-occurrence networks was performed using ChiPlot (<https://www.chiplot.online> (accessed on 2 March 2026)).

For the co-occurrence network analysis, the top 100 most abundant genera were selected to focus on dominant taxa and reduce network complexity. Spearman correlation coefficients were calculated, and connections with $|r| > 0.6$ and $p < 0.05$ were considered valid. This threshold follows common practice in microbial co-occurrence network studies to focus on strong associations. To account for multiple comparisons, p values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure, and only associations surviving an FDR-adjusted threshold of $p < 0.05$ were kept. It should be noted that relative abundance data are compositional, and excluding genera with zero abundance across samples may introduce bias. Therefore, the network results offer exploratory insights into co-variation patterns and do not constitute direct evidence of biological interactions.

3. Results

3.1. Change in Physical and Chemical Properties of Soil

The results show that the concentrations of NH_4^+ -N and SOC increase with the duration of soil cultivation. After 9 years of SS (DS) cultivation, the concentrations of NH_4^+ -N and SOC increased by 267.09% (736.95%) and 175.50% (80.72%), respectively,

compared to the 1-year soil (Figure 1). NO_2^- -N and NO_3^- -N concentrations peaked in the 3-year SS and were significantly different from those in the other soils ($p < 0.05$). The concentrations of SOC, NH_4^+ -N, NO_2^- -N, and NO_3^- -N in SS were higher than those in DS, and the difference in SOC concentration between SS and DS was greater in soils with longer cultivation durations. Among different soils, enzyme activities differed significantly ($p < 0.05$), with enzyme activities generally higher in SS than in DS. The activities of urease, sucrase, and catalase peaked in the 3-year soil and gradually decreased, with the lowest enzyme activities observed in the 1-year soil. All soils exhibited weak alkalinity, with the pH of SS being lower than that of DS. The pH of the 3-year soil was the lowest, which was significantly different from that of other soils ($p < 0.05$). The ANR was highest in the 1-year soil, showing a 57.70% decrease at its peak with increasing years of cultivation.

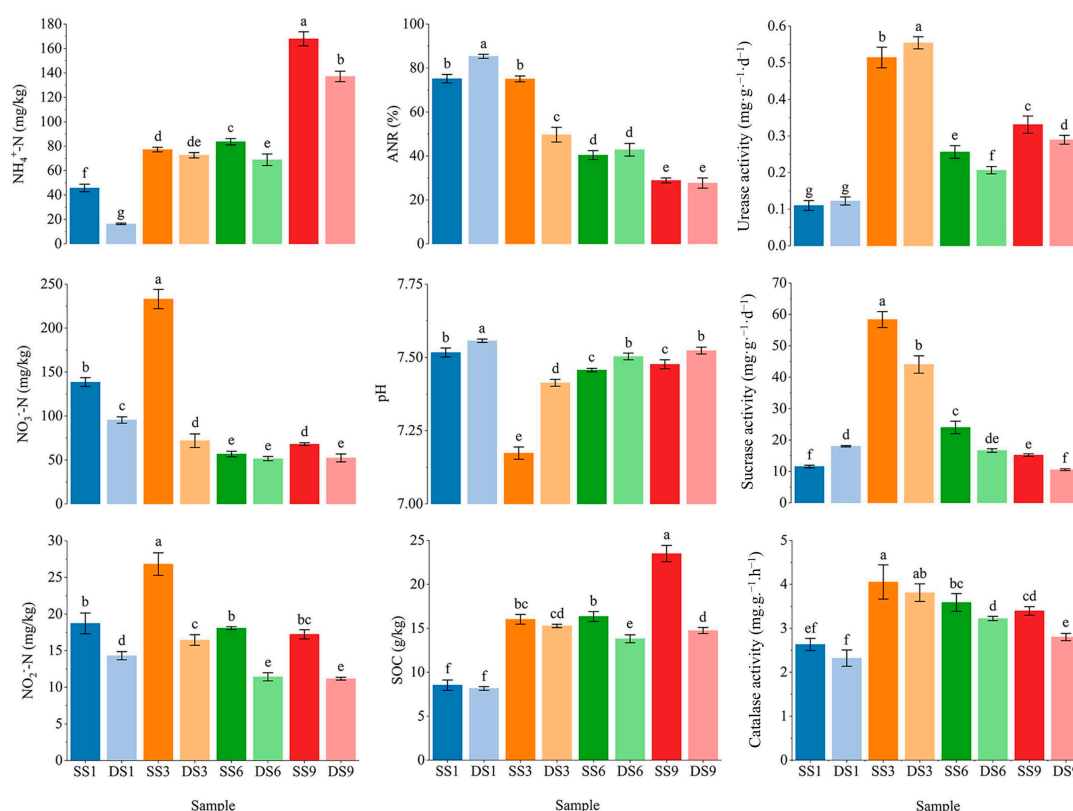


Figure 1. Physicochemical properties of soil in different cultivation years. Lowercase letters denote significant differences among cultivation durations determined by one-way ANOVA and Tukey's HSD test ($p < 0.05$, FDR-adjusted). Error bars represent standard error of the mean ($n = 3$). SS represents 0–20 cm soil layer, DS represents 20–40 cm soil layer, and the number represents the soil cultivation years.

3.2. Differences in Microbial Community Composition

3.2.1. Alpha Diversity Index

The sequencing data were randomly subsampled, and alpha diversity indices were calculated. When the sequencing data reached 49,330, the rarefaction curve leveled off. At this point, further sequencing reads contributed little to the alpha diversity index, which had essentially plateaued. The final results of the alpha diversity indices are shown in Table 1. Microbial species richness, diversity, and evenness were evaluated using the Chao1, Shannon, and Simpson indices, respectively. The results indicated that the bacterial Chao1, Shannon, and Simpson indices in both SS and DS of greenhouse vegetable soils first increased and then decreased as cultivation years increased, all peaking at 3 years of

cultivation. In contrast, the archaeal diversity indices were highest in DS1. Overall, SS3, DS3 and DS1 exhibited the highest microbial diversity and evenness.

Table 1. Microbial alpha diversity index.

Sample	Chao1 Index		Shannon Index		Simpson Index	
	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea
SS1	935.370	27.600	7.178	1.469	0.968	0.374
DS1	1719.905	61.583	9.094	4.310	0.994	0.919
SS3	1509.000	37.200	9.014	2.965	0.994	0.695
DS3	1727.632	60.769	9.256	3.316	0.995	0.786
SS6	971.529	28.000	7.690	2.876	0.982	0.776
DS6	1005.086	31.000	6.462	2.729	0.885	0.790
SS9	518.545	9.000	6.292	1.691	0.932	0.598
DS9	302.941	14.000	4.416	1.752	0.857	0.534

Compared with soils under other cultivation durations, the 9-year soil had a lower alpha diversity index. Compared to the 3-year soil, the Chao1 index in SS and DS after 9 years of cultivation decreased by 65.64% and 82.46%, respectively. This may be because, during the early stages of cultivation, plant root exudates were abundant, providing microorganisms with rich carbon sources and energy, thereby promoting their growth and reproduction. Long-term greenhouse cultivation with cucumber–bitter melon rotation may lead to soil degradation and imbalance of certain microbial communities. The alpha diversity index of DS was generally higher than that of SS after 1 and 3 years of cultivation. This trend reversed after 6 years of soil cultivation, indicating that the greenhouse cultivation model has a greater impact on the microbial community structure of SS in the short term, and in soils with longer cultivation durations; differences were also observed in the DS microbial community structure.

3.2.2. Relative Abundance of Species

For each soil, Figure 2 shows the 10 most abundant microbial taxa at the phylum and genus levels. The major dominant bacterial and archaeal phyla include *Firmicutes*, *Proteobacteria*, *Gemmatimonadota*, *Crenarchaeota*, and *Chloroflexi*. *Proteobacteria* had the highest relative abundance in soils after 3 years of cultivation, while *Chloroflexi* exhibited the highest relative abundance in DS6. *Firmicutes* displayed a “U”-shaped trend in relative abundance across the cultivation-duration gradient. It is noteworthy that the microbial phyla in the 9-year soil were overly dominated by a single phylum, with *Firmicutes* accounting for more than 65% in both SS and DS microbial communities, reaching up to 97.63%. Furthermore, in the 9-year soil, the total relative abundance of phyla outside the top 10 was below 1%, indicating a certain degree of microbial community imbalance after 9 years of cultivation.

In soils cultivated for 1 and 6 years, the dominant genera at the genus level were mainly *Bacillus* and *Candidatus_Nitrososphaera*. The dominant genera in the 3-year soil were *Sphingomonas*, *Candidatus_Nitrososphaera*, and *Candidatus_Nitrocosmicus*. *Alicyclobacillus*, *Bacillus*, *Candidatus_Nitrososphaera*, and *Candidatus_Nitrosotenuis* predominated in the 9-year soil. In greenhouse soils, the dominant archaeal genera were primarily ammonia-oxidizing archaea, suggesting active nitrification in the soil, which could also lead to increased nitrous oxide emissions. The top 10 bacterial genera accounted for more than 40% in SS9 and over 75% in DS9. Combined with the low alpha diversity index of the soil, this further suggests a risk of microbial community imbalance after 9 years of cultivation.

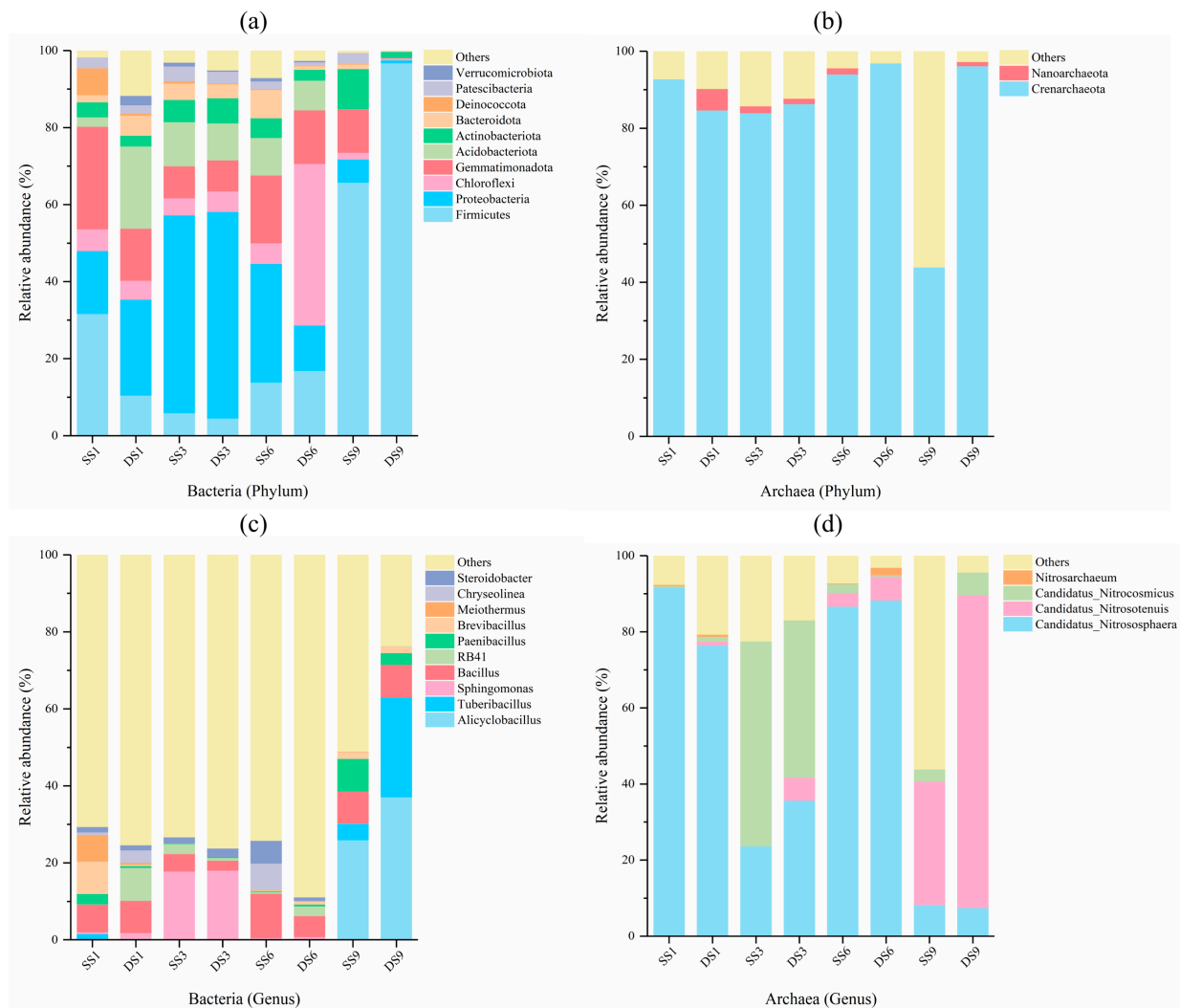


Figure 2. Relative abundance of microorganisms. (a) Bacterial phylum; (b) Archaeal phylum; (c) Bacterial genus; (d) Archaeal genus.

3.3. Statistical Test

3.3.1. Feature Sequence

Microbial marker sequences typically represent specific microbial species, genera, or functional groups. Therefore, the number of marker sequences can reflect species richness and community structural complexity. Figure 3 illustrates the number of shared and unique marker sequences between soils of different cultivation durations. The results indicate that the number of bacterial and archaeal marker sequences was highest in the 3-year soil and lowest in the 9-year soil. Soils under 1 and 6 years of cultivation showed the greatest overlap in bacterial and archaeal marker sequences, with 354 and 10 shared sequences, respectively. Combined with the alpha diversity index and species relative abundance, it can be inferred that the soils with 1 and 6 years of cultivation share certain similarities. The shared bacterial marker sequences among the four soils were 33, while the shared archaeal marker sequences were 0, further indicating that the microbial community structure of greenhouse soils underwent significant changes after prolonged cultivation.



Figure 3. Common and unique feature sequences among soils in different cultivation years: (a) bacteria and (b) archaea.

3.3.2. LDA Effect Size Analysis

LDA Effect Size (LEfSe) analysis was performed as an exploratory approach to identify taxa with differential relative abundance among groups. The length of the bar chart represents the effect size of the biomarker (i.e., LDA score), with biomarkers having an LDA score > 2 being considered reliable. In the phylogenetic tree, concentric circles radiating from the center to the periphery represent taxonomic levels from phylum to species, with species showing no significant difference being uniformly colored yellow. The study compared soils with short (Figure 4a,c) and long (Figure 4b,d) cultivation periods, as well as SS and DS. The results showed that, at the taxonomic levels from phylum to species, 20 biomarkers were identified between soils with short and long cultivation periods (Phylum, 0; Class, 1; Order, 5; Family, 6; Genus, 4; Species, 4), and 27 biomarkers were identified between SS and DS (Phylum, 1; Class, 3; Order, 7; Family, 9; Genus, 6; Species, 1). At progressively finer taxonomic levels, the number of biomarkers increases from phylum to family, indicating that as the taxonomic resolution becomes finer, the environmental adaptability of microorganisms decreases and the influence of cultivation duration and agricultural practices becomes more pronounced.

The microbial diversity in soil under short-term cultivation is relatively high and exhibits significant variation. Over time, the microbial community gradually tends toward stability. *Deinococci*, as biomarkers in the early stages of soil cultivation, exhibit a certain degree of resistance and represent specific groups that increase during adaptation to environmental stresses, such as fluctuations in moisture and temperature. Genera such as *Aneurinibacillus* and *Bacillus_graminis* dominate in soils under long-term cultivation, possibly due to their ability to tolerate the gradually increasing organic load in the soil and their symbiotic relationships with plants. The microbial communities in SS and DS differ significantly, with the former displaying a higher number of biomarkers, predominantly *Actinobacteria*, which possess strong organic matter decomposition capabilities and resistance, enabling them to adapt to the rich organic content and frequent environmental changes in SS. These biomarkers reflect significant changes in microbial community diversity with increasing soil depth in greenhouse soils.

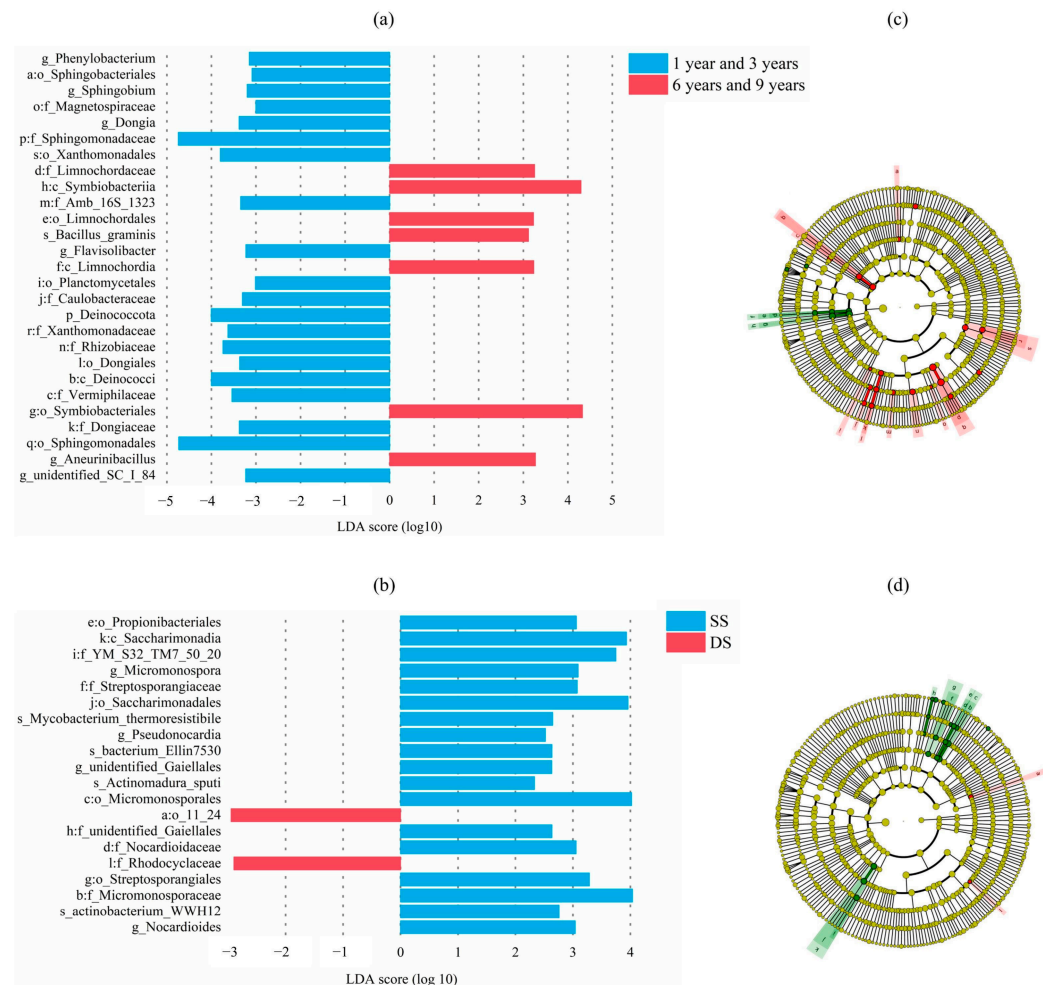


Figure 4. LEfSe analysis of soil microbial communities. **(a)** LDA score distribution of bacterial taxa between short-term (1 and 3 years, blue) and long-term (6 and 9 years, red) cultivation; **(b)** LDA score distribution of bacterial taxa between surface soil (SS, blue) and subsurface soil (DS, red); **(c)** evolutionary branching diagram showing the taxonomic hierarchy of bacterial taxa that differ between short-term and long-term cultivation; **(d)** evolutionary branching diagram showing the taxonomic hierarchy of bacterial taxa that differ between SS and DS. In the branch plots, green and red nodes represent taxa enriched in the corresponding groups, and yellow nodes represent taxa with no significant difference. The lowercase letters in the branch plots correspond to the taxa listed in the bar plots.

3.3.3. Principal Coordinates Analysis

We performed Principal Coordinates Analysis (PCoA) based on the Weighted UniFrac distance and selected the principal coordinate combinations with the highest contribution for graphical representation. In this study, the PCoA principal coordinates for bacteria and archaea explained 74.09% and 75.87% of the variation, respectively, effectively characterizing the sample features (Figure 5). Samples that are closer together in distance indicate more similar species composition; thus, samples with higher community structure similarity tend to cluster together, while those with greater community differences are separated more distantly. The results show that soils cultivated for 3 and 9 years are distinctly separated from other soils. Combined with the LEfSe analysis results, these findings suggest that the cultivation duration has altered the soil microbial community structure. The microbial community structure of DS1 is most similar to that of SS6, further supporting the previous hypothesis that soils cultivated for 1 and 6 years share certain similarities.

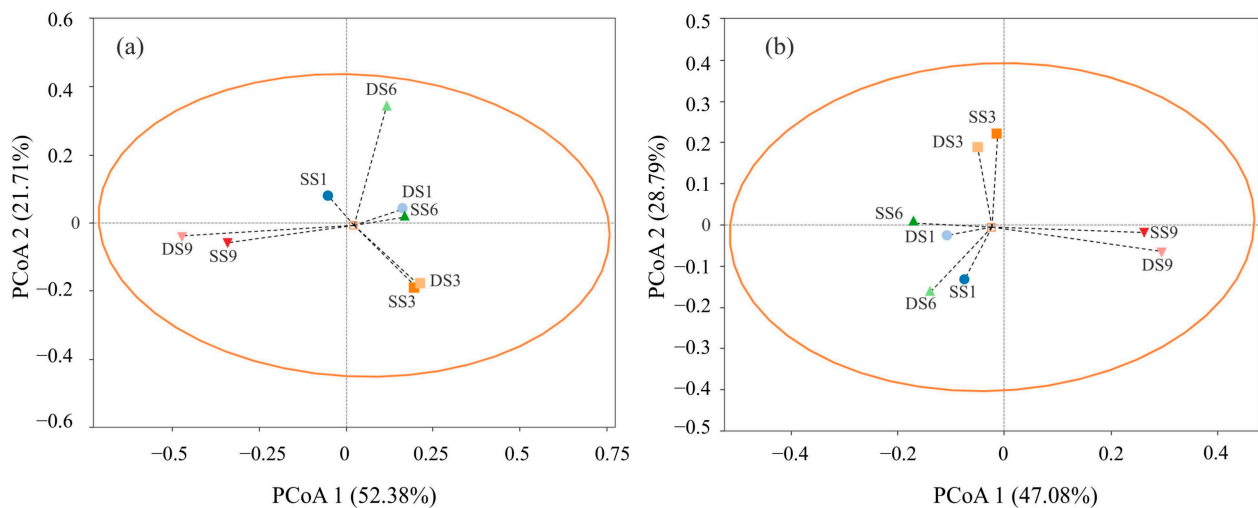


Figure 5. Principal Coordinates Analysis: (a) bacteria and (b) archaea.

3.4. Multidimensional Data Analysis and Microbial Network Research

3.4.1. Redundancy Analysis and Correlation Analysis

The two principal components, RDA1 and RDA2, explained 37.77% (at the genus level: 34.21%) and 22.66% (at the genus level: 26.03%) of the variance in microbial phylum-level community structure in response to environmental factors, respectively, accounting for a total of 60.43% (at the genus level: 60.24%) of the variation (Figure 6). NH_4^+ -N and urease were the factors most strongly associated with bacterial and archaeal communities in greenhouse soils in the RDA ordination (Table 2). LEfSe analysis indicated that the more refined the taxonomic classification level, the greater the environmental influence. According to Figure 6b, the 3-year soil samples are distributed along the direction of urease, while the 9-year soil samples are distributed along the direction of NH_4^+ -N, indicating that NH_4^+ -N and urease have the greatest influence on the genus-level microbial community composition in these two soils.

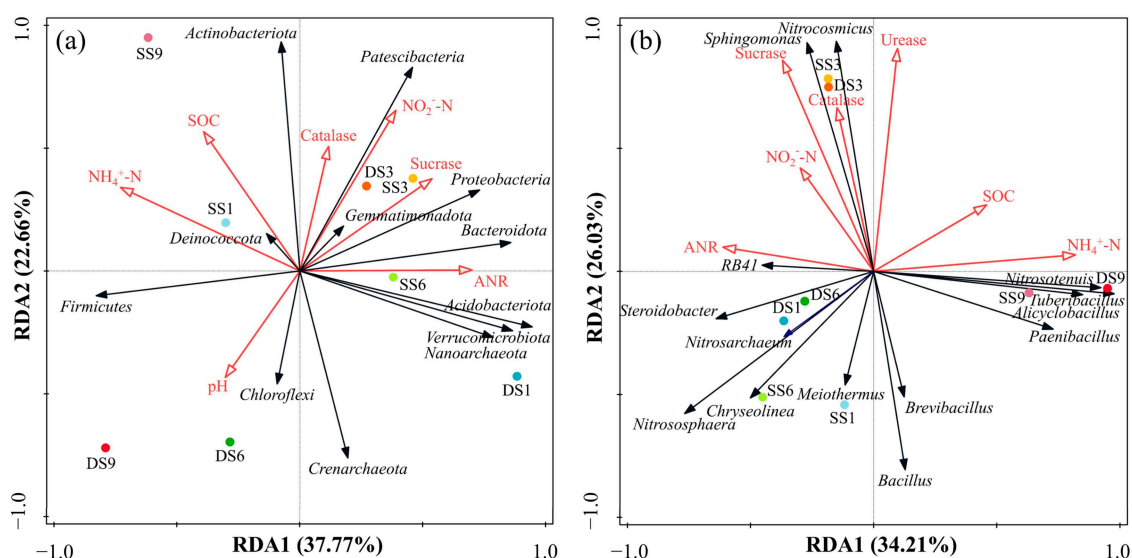


Figure 6. Redundancy analysis at the (a) phylum and (b) genus levels. Red arrows represent environmental factors, black arrows represent microbial taxa, and different colored points represent different soil samples. SS represents the 0–20 cm soil layer, DS represents the 20–40 cm soil layer, and the numbers represent the cultivation years.

Table 2. The significance of constraint axis eigenvalues and environmental factors for redundancy analysis.

		Phylum	Genus
Eigenvalues	Axis1	0.3777	0.3421
	Axis2	0.2266	0.2603
	Axis3	0.1767	0.1664
	Axis4	0.1038	0.1198
p	NH ₄ ⁺ -N	0.022	0.038
	Urease	>0.05	0.018

We further analyzed the interactions between environmental factors and the dominant genera. Genera with a relative abundance of zero in a given sample were excluded from the analysis. The results show that soil enzyme activity is significantly negatively correlated with pH ($p < 0.05$), indicating that the weak alkalinity of the soil inhibits enzyme activity (Figure 7). The concentration of SOC is significantly positively correlated with NH₄⁺-N ($p < 0.01$), while pH is significantly negatively correlated with soil NO₂⁻-N and NO₃⁻-N. There is a strong positive correlation between soil urease, catalase, and sucrase ($p < 0.05$). Urease is a positive factor promoting the growth of *Sphingomonas* and *Brevibacillus* ($p < 0.01$), as well as *Bacillus* and *Candidatus_Nitrososphaera* ($p < 0.05$). Sucrase is significantly positively correlated with *Brevibacillus* and *Sphingomonas* ($p < 0.05$). This is because urease and sucrase decompose urea and sucrose, releasing nutrients available to microorganisms. Additionally, *Bacillus* and *Brevibacillus* have the ability to produce urease. The concentration of NH₄⁺-N is significantly positively associated with *Candidatus_Nitrososphaera*, *Paenibacillus*, and *Steroidobacter* ($p < 0.05$). *Candidatus_Nitrososphaera* is an ammonia-oxidizing archaeon, while *Paenibacillus* has nitrogen-fixing capabilities.

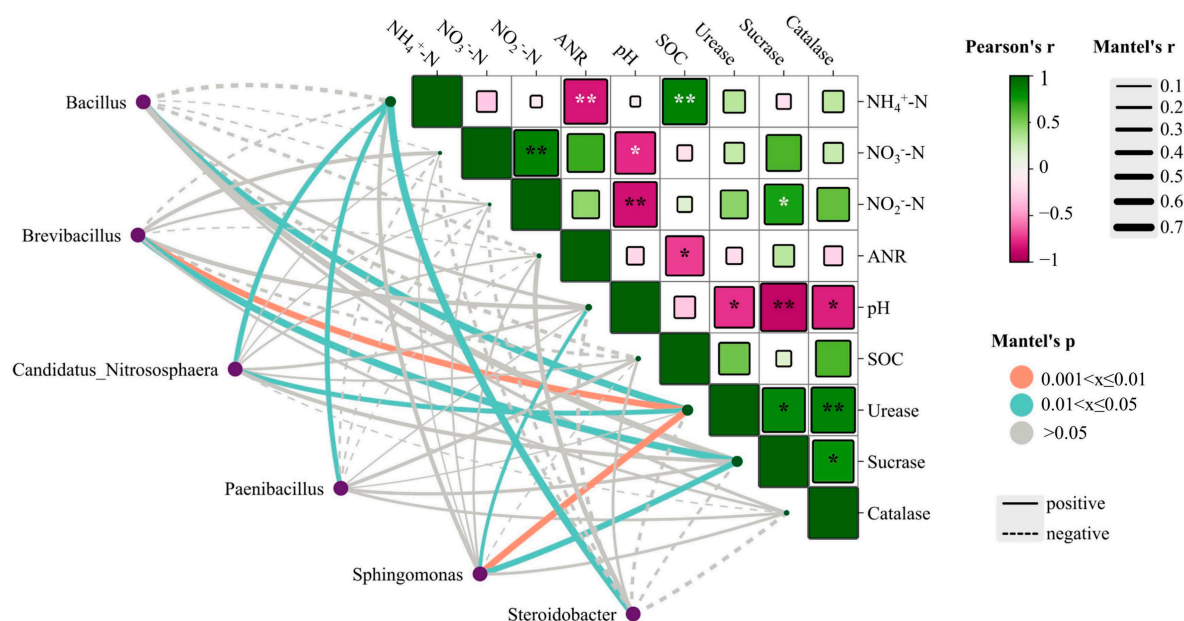


Figure 7. Correlation analysis of environmental factors and their effects on dominant bacteria. In the heatmap, the color of each square represents the Pearson correlation coefficient (Pearson's r), and the size of each square represents the Mantel's r value. * $p < 0.05$, ** $p < 0.01$. The color of the connection line represents the p value under the Mantel test, the thickness of the connection line represents the r value under the Mantel test, and the solid and dashed lines indicate positive and negative correlations, respectively.

3.4.2. Microbial Co-Occurrence Network

Based on the relative abundance results of soil microorganisms from different cultivation years, the top 100 microorganisms at the genus level were selected to calculate the

Spearman correlation coefficient. Then, connections with a correlation coefficient greater than 0.6 or less than -0.6 , and with significance ($p < 0.05$), were selected as valid connections for the network diagram, resulting in 52 microorganisms at the genus level of bacteria and archaea (Figure 8). The network diagram can reveal the modular structure of the microbial community, provide clues about its potential ecological functions, and identify potential associations or co-occurrence patterns among microbial taxa. From the co-occurrence network, it can be seen that *Proteobacteria* and *Bacteroidota*, as dominant phyla in soil bacteria, showed the strongest positive association, suggesting that their roles may be complementary in breaking down organic matter and in nutrient transformation. However, this interpretation requires further experimental validation.

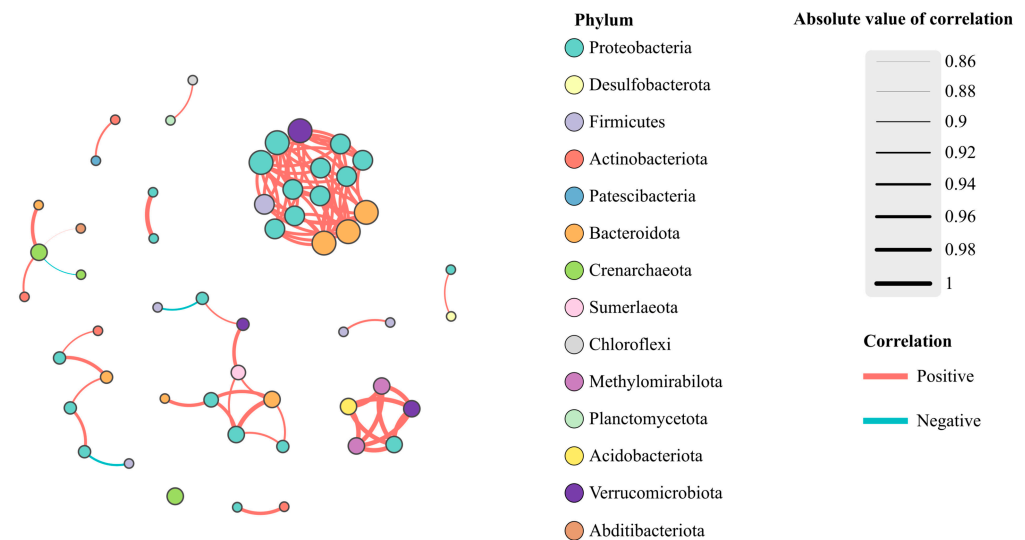


Figure 8. Co-occurrence network of microorganisms. The more interactions between communities, the larger the network node, the thickness of the connection line represents the correlation, and the color of the connection line represents the positive and negative correlation.

The strongest positive associations were observed between *Proteobacteria*, *Bacteroidota*, *Acidobacteria*, *Methylomirabilota*, and *Verrucomicrobiota*, suggesting that these phyla may co-occur in more complex material metabolism processes in greenhouse soils. Through Figure 2, we observed that during the 9 years of continuous cultivation, the relative abundance of *Proteobacteria* in the soil first increased and then decreased, while the relative abundance of *Firmicutes* first decreased and then increased. The co-occurrence network suggested that this phenomenon may be associated with a negative association between *Proteobacteria* and *Firmicutes*. Finally, no statistical relationship was observed between the relative abundance of phyla and the strength of their interactions.

3.5. Microbial Function Prediction

Based on 16S sequencing data, functional predictions were made using the KEGG database, which provides extensive information on metabolic pathways involving various elements, such as nitrogen, carbon, and sulfur, all of which are critical for the functional predictions of soil microbial communities. The top 35 functions were selected according to their abundance ranking and database annotations, and a heatmap was then constructed using their corresponding abundance information for each sample. Clustering was performed at various functional levels (Figure 9).

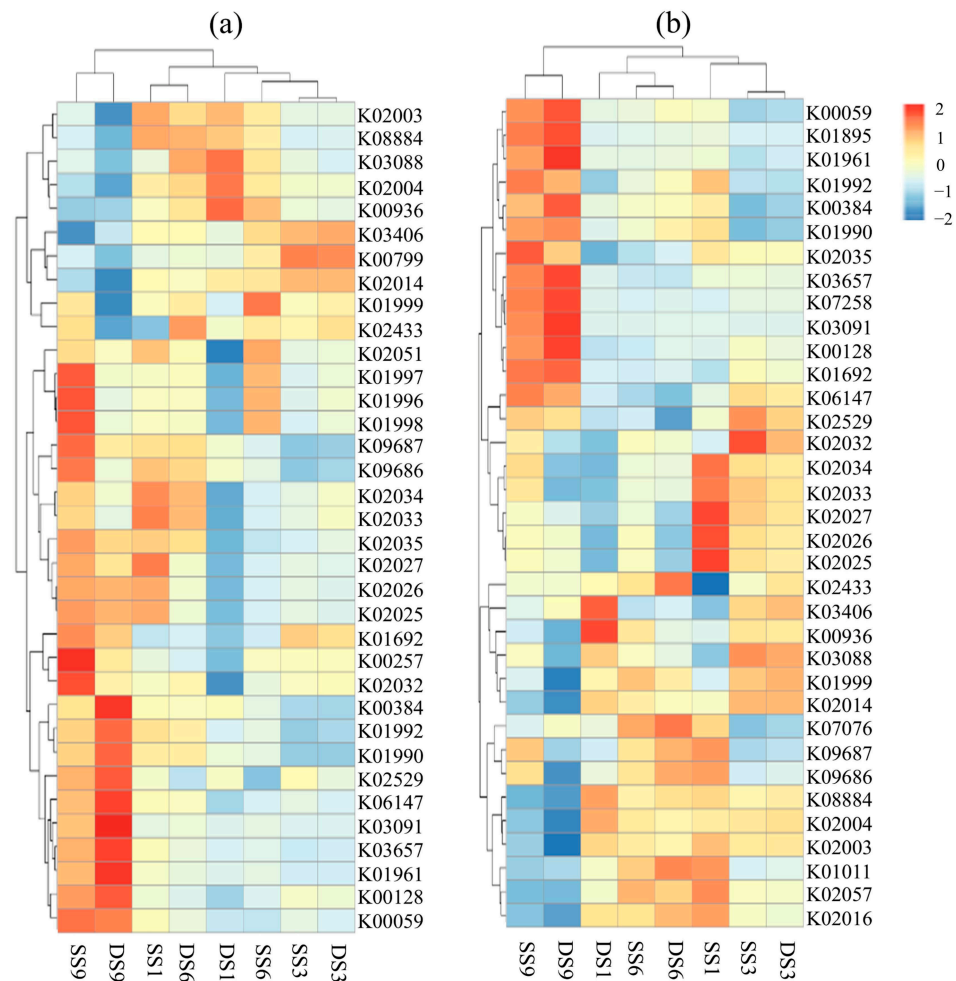


Figure 9. Prediction of microbial function: (a) bacteria and (b) archaea.

Although the microbial community exhibited imbalances in soils cultivated for 9 years, the PICRUSt2-based predictions suggested a higher inferred abundance of genes associated with various metabolic processes, implying greater predicted functional complexity. For example, K00384, K06147, and K00257 correspond to thioredoxin reductase, ATP-binding cassette transporter, and acyl-ACP dehydrogenase, respectively. In the 1-year soil, the core functional genes were more abundant, with the representative function being RNA polymerase sigma-70 factor (K03088). In the 3-year soil, the predicted functions included iron complex outer membrane receptor protein (K02014) and peptide/nickel transport system ATP-binding protein (K02032), which may suggest a potential for enhanced antioxidant capacity and chemotactic ability, though this requires experimental validation. In the 6-year soil, the dominant functional genes, primarily thiosulfate/3-mercaptopyruvate sulfurtransferase (K01011), may indicate a shift in predicted metabolic functions.

Figure 10 shows the PICRUSt2-predicted KEGG pathway annotation and the number of genes predominantly found in greenhouse soil. This prediction suggests that the most prominent microbial functions in greenhouse soil are amino acid metabolism, membrane transport, and carbohydrate metabolism. This may suggest that the relevant microorganisms have a potential for strong adaptability when facing fertilization, plant residues, or other external changes. In contrast, functional genes related to the sensory system and cell communication are relatively fewer, suggesting that the environment inside the greenhouse is stable. This implies that microorganisms in greenhouse soil may allocate more energy and resources to metabolic functions closely associated with their growth, reproduction, and survival activities, rather than responding intensively to environmental signals.

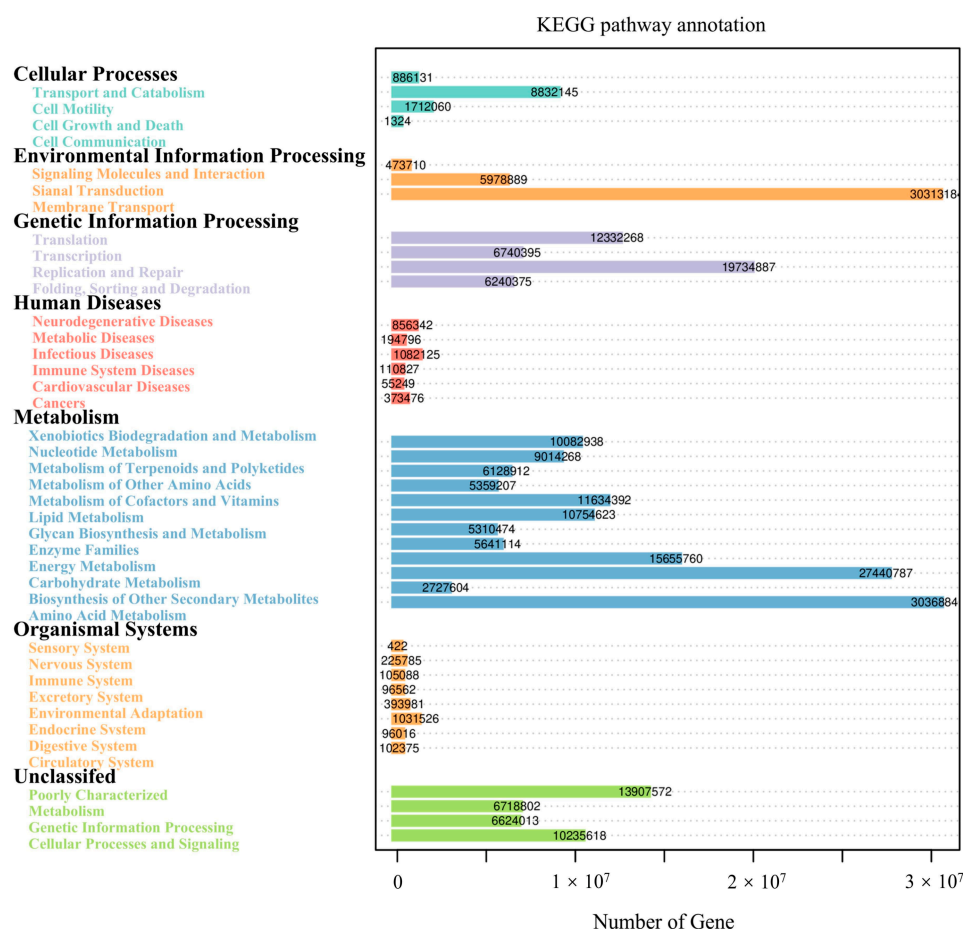


Figure 10. Predicted KEGG pathway annotation based on PICRUSt2 analysis.

4. Discussion

4.1. Cultivation Duration Effects on Soil Physicochemical Properties and Enzyme Activity

SOC is not only a key indicator of soil fertility but also plays a crucial role in soil microbial activity, soil structure, and water retention. Several studies have shown that long-term cultivation increases the stock of organic matter in the soil [23,24]. In this study, soils with longer cultivation durations had higher SOC and $\text{NH}_4^+\text{-N}$ concentrations (Figure 1), especially in soils cultivated for 9 years. This pattern suggests that longer cultivation durations were associated with the build-up of inorganic nitrogen as well as organic carbon in greenhouse soils. The annual application of large amounts of organic fertilizer is likely one of the main reasons for the higher SOC concentrations in soils with longer cultivation durations. Plant residues from cucumber and bitter melon also break down at the soil surface, serving as an additional source of SOC [25]. A significant positive correlation was observed between SOC and $\text{NH}_4^+\text{-N}$ ($p < 0.01$) (Figure 7), indicating that SOC contributes importantly to the nitrogen cycle by providing a carbon source for microorganisms, thereby facilitating nitrogen transformation.

In different soils, the decrease in carbon and nitrogen content with soil depth may be attributed to reduced root growth and decreased organic matter input from root residues, as most of the crop root biomass (>50%) is concentrated within the 0–20 cm depth, which contributes to nutrient accumulation in SS [26,27]. Plant roots stimulate enzyme activity [28], and in SS, where plant roots are more developed, nutrient accumulation is greater, leading to generally higher enzyme activity compared to DS, particularly in soils with longer cultivation periods. $\text{NH}_4^+\text{-N}$ generates H^+ and $\text{NO}_3^-\text{-N}$ during nitrification, leading to a decrease in soil pH. Increased urease activity facilitates the hydrolysis of urea, subse-

quently producing more $\text{NH}_4^+\text{-N}$. Therefore, in this study, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$, and urease activity are significantly negatively correlated with soil pH (Figure 7). This explains why the soil pH in the third year of cultivation is lower than in other soils, as it has the highest urease activity, $\text{NO}_3^-\text{-N}$, and $\text{NO}_2^-\text{-N}$ concentrations. The pH of SS is significantly lower than that of DS, indicating that the greenhouse vegetable cultivation model poses a risk of soil acidification, particularly in SS.

However, not all soil indicators showed consistent trends across the cultivation-duration gradient. We observed that the concentrations of $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ peaked after 3 years of cultivation (Figure 1). The observed peak in $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations at 3 years suggests that nitrogen mineralization and transformation may be more active at this stage, while the lower concentrations in later years could be associated with a decline in nitrogen cycling efficiency. However, this interpretation is based on correlational evidence, and the underlying mechanisms require further investigation. Genera such as *Ramlibacter*, *Paraburkholderia*, and *Alsobacter* in the *Proteobacteria* phylum possess urease decomposition, nitrate reduction, and nitrogen fixation functions [29–31]. In the 3-year soils, the relative abundance of *Proteobacteria* in the bacterial community exceeded 50%, with two ammonia-oxidizing archaea at the genus level dominating the archaea community (Figure 2). Its urease activity was also higher than that in other soils, indicating that the 3-year soils may have stronger nitrogen fixation and transformation functions.

Soils cultivated for 1 and 3 years exhibit higher ANR, indicating stronger nitrogen transformation capabilities and a higher risk of nitrate nitrogen leaching. The higher enzyme activity after 3 years of cultivation reflects a more active microbial community and an increased rate of organic matter decomposition. The urease activity of SS was lower than that of DS after 1 and 3 years of cultivation, but this trend reversed after 6 years of cultivation (Figure 1), suggesting that fertilization during short-term cultivation has a greater impact on SS, and excessive nitrogen fertilization may inhibit urease activity. Although organic matter gradually accumulates with an increase in cultivation duration, the decline in functional enzyme activities may be attributable to shifts within the microbial community.

4.2. Effects of Cultivation Duration and Soil Properties on Microbial Community Structure

Recent studies have found that in greenhouse vegetable fields, the alpha diversity index increases with the length of cultivation during the process of growing melons. In these soils, *Proteobacteria* and *Acidobacteriota* exhibited the highest relative abundance [32]. The soils selected for this study are primarily used for growing bitter melon and cucumber. During the first three years of cultivation, the soil alpha diversity index, the number of characteristic sequences, and enzyme activity showed an upward trend (Table 1), suggesting that short-term cultivation increases microbial activity due to the combined effects of nutrient inputs and plant root systems. After 3 years of cultivation, the soil alpha diversity index began to decline. The differences in the results between studies may be attributed to variations in soil texture, crop types, and management practices. PCoA analysis showed a clear distinction between the soils from 3 and 9 years of cultivation and those of other soils (Figure 5), suggesting that community composition varied with cultivation duration.

Firmicutes dominated the 9-year soil (Figure 1), suggesting that the microbial community structure differed substantially among cultivation durations, with a trend toward dominance by fewer species in the oldest fields. This finding agrees with earlier reports, which indicated that extended cultivation periods may be linked to changes in soil microbial community composition [14]. A decline in microbial community diversity reflects soil health degradation, particularly in greenhouse systems characterized by long-term fertilization and intensive crop rotation. Previous studies have indicated that *Firmicutes* exhibit higher activity in eutrophic and nutrient-imbalanced environments, and in this study, the

dominance of *Firmicutes* in the soil was also associated with the application of chicken manure [10,33]. RDA results suggested that the excessive dominance of *Firmicutes* is closely related to long-term organic matter accumulation, nutrient surplus, and inorganic nitrogen content in the soil. *Acidobacteriota* are involved in the decomposition of organic nitrogen and other organic compounds, releasing inorganic nitrogen and phosphorus available for plant uptake. Their abundance was lower in soils with longer cultivation durations and is extremely low in the 9-year soil, as their growth is inhibited in eutrophic environments [34].

In agricultural soils, the predominant phyla are *Actinobacteriota* (35–39%), *Proteobacteria* (26–33%), *Acidobacteriota* (11–13%), and *Verrucomicrobiota* (3–6%), while phyla such as *Firmicutes* (3–6%), *Bacteroidota* (2–3%), *Gemmatimonadota* (1–2%), *Nitrospirae* (1%), *Chloroflexi* (1–2%), and *Planctomycetota* (0–1%) are less common [35]. *Actinobacteria* can inhibit the invasion of pathogenic bacteria, and certain microorganisms in *Planctomycetota* can produce antibiotics, which help control the spread of soil pathogens and protect soil health [36]. However, *Actinobacteriota* was markedly less abundant in the greenhouse soils examined here than in agricultural soils. Further analysis revealed that in soils prior to 6 years of cultivation, the abundance of *Planctomycetota* showed a trend of initially increasing and then decreasing, with relative abundances ranging from 0.8% to 3.8%. In contrast, in the 9-year SS, it was only 0.01%, and in DS, it was 0 (Figure 1). These observations suggest that long-term cultivation may potentially affect the biological suppression capacity of soil, but direct evidence linking these changes to soil disease resistance is lacking and requires further experimental validation.

LEfSe analysis revealed that the number of biomarkers in SS was nine times higher than that in DS (Figure 4), and the alpha diversity indices of DS1 and DS3 were higher than those of SS1 and SS3 (Table 1), further demonstrating that short-term cultivation in greenhouse soils has a greater impact on the SS microbiome. This conclusion has been validated in the study of Zhang et al. [10]. The LEfSe analysis also suggested that the microbial community exhibited greater changes under short-term cultivation (Figure 4a,c). However, this phenomenon changed in soils after 6 and 9 years of cultivation, suggesting that in soils with longer cultivation durations, intensive fertilization and continuous planting were associated with differences in microbial community structure of DS. The reduction in microbial diversity may be attributed to the substantial nitrogen residues, as mentioned earlier. During greenhouse vegetable cultivation in Shouguang City, nitrogen use efficiency was only 2.4–5.7%, and the low nitrogen use efficiency resulted in nitrogen residues, which had a more pronounced impact on the diversity of the SS microbiome during short cultivation periods [37].

Different environmental factors influence microbial growth and metabolic activity, thereby affecting the composition and distribution of microbial communities. A significant positive correlation ($p < 0.05$) between catalase and microbial community richness and diversity was observed in the RDA (Figure 6). Catalase is an antioxidant enzyme that regulates cellular redox balance in soil ecosystems and helps plants resist various stresses. This enzyme is particularly important for bacteria living in oxygen-rich environments, as it helps them survive oxidative stress induced by reactive oxygen species [38,39]. Soil pH was inversely related to the majority of dominant phyla and various enzyme activities, indicating that the slightly alkaline soil in the greenhouse suppresses microbial activity.

As shown in Figure 7, urease exhibited a significant positive correlation with *Bacillus*, *Candidatus_Nitrososphaera* ($p < 0.05$), *Brevibacillus*, and *Sphingomonas* ($p < 0.01$). This correlation may be attributable to the urease-producing capacity of *Bacillus* and *Brevibacillus*. The mechanisms linking $\text{NH}_4^+\text{-N}$ and soil microorganisms are relatively complex. *Candidatus_Nitrososphaera* belongs to the phylum *Nitrososphaerota* and is generally regarded as an ammonia-oxidizing archaeon. Its relative abundance was significantly

positively correlated with $\text{NH}_4^+\text{-N}$, which in turn was negatively correlated with nitrogen-fixing phyla such as *Proteobacteria* and *Bacteroidota*. Previous studies have shown that increases in soil biological nitrogen fixation rates and nitrogen-fixing community activity are closely associated with decreases in $\text{NH}_4^+\text{-N}$ concentrations [40]. In summary, genera such as *Bacillus*, *Candidatus_Nitrososphaera*, and *Sphingomonas* exhibit significant variations with changes in cultivation years and soil environments and can be considered indicator species of soil environmental changes. By integrating bacterial and archaeal communities, enzyme activities, environmental variables, and predicted functional potential across two soil depths, this study provides a more comprehensive view of how greenhouse soil ecosystems vary with cultivation duration, extending previous studies that focused on individual dimensions. Predictions indicate that long-term greenhouse cultivation may lead to microbial community imbalance and reduced enzyme activity, threatening the sustainability of greenhouse vegetable production. Maintaining crop rotation and optimizing fertilization are of great significance for soil health and long-term sustainability.

4.3. Microbial Co-Occurrence Network and Functional Prediction

The complexity of the soil microbial community network relationships is crucial for maintaining soil ecosystem functions. In this study, *Proteobacteria* dominate the co-occurrence network (Figure 8). This is because *Proteobacteria* include numerous bacteria closely associated with environmental changes, such as nitrifying bacteria, denitrifying bacteria, reducing bacteria, and some pathogens. These bacteria are involved in nitrogen cycling, sulfur cycling, and carbon decomposition in soils. Studies suggest that a near-neutral or alkaline soil pH is the optimal condition for the survival of *Bacteroidota*. *Bacteroidota* can secrete a range of carbohydrate-active enzymes (CAZymes) directed against the structurally diverse polysaccharides in soil. *Actinobacteriota* participate in organic matter decomposition and carbon fixation in soils, and also possess certain functions in disease prevention [15,41].

Proteobacteria and *Bacteroidota* showed a positive association in the co-occurrence network. *Proteobacteria* primarily participate in nitrogen cycling in soils, while *Bacteroidota* mainly focus on organic matter decomposition. This pattern may indicate potential complementary ecological roles in soil nutrient cycling. The relative abundance of these phyla is highest in soils that have been cultivated for three years, indicating a stronger stability and adaptability of the ecosystem in 3-year soils. As the dominant phyla in soils, *Proteobacteria* and *Firmicutes* showed a negative association. This may be because *Firmicutes* exhibit higher activity in nutrient-rich environments, and due to the functional similarities between the two (such as organic matter decomposition), the continuous input of chicken manure and the accumulation of SOC were associated with opposing abundance trends between *Proteobacteria* and *Firmicutes*.

The number of shared characteristic sequences of bacteria and archaea in the four soils was 33 and 0, respectively (Figure 3), indicating that the composition of the soil microbial community differed considerably among cultivation durations. Consistent with this compositional shift, the PICRUSt2-based predictions described below suggested potential changes in soil microbial functions after years of cultivation. Although the microbial community structure of greenhouse soils is somewhat disrupted with increasing years of cultivation, its functionality tends to become more diverse and complete as the cultivation period extends (Figure 9). Based on the annotation of dominant functional information in KEGG for each soil, the abundance of the putative ABC transport system ATP-binding protein and phosphonate transport system substrate-binding protein in 1-year soil indicates the rapid response of microorganisms to external resources (e.g., phosphorus). Genes such as threonine protein kinase and RNA polymerase sigma-70 factor suggest that the microorganisms in 1-year soil are engaged in basic transcription and signal transduction.

The relatively low urease, catalase, and sucrase activities recorded in this soil suggest that microbial functions in 1-year soil are primarily focused on basic metabolism and rapid environmental adaptation.

Enzymes such as glutathione S-transferase in 3-year soil suggest that the microbial community has likely encountered a certain degree of environmental stress (e.g., oxidative stress or toxic substances) and has enhanced its antioxidant and detoxification abilities during this process. The increase in methyl-accepting chemotaxis protein indicates that microorganisms are gradually optimizing their resource acquisition methods and adjusting migration and orientation in response to environmental changes. This suggests that the microbial community in 3-year soil has entered an adaptation phase, with increasingly complex metabolic processes and exhibiting more sophisticated functions such as antioxidation, detoxification, and chemotaxis.

The abundance advantage of the branched-chain amino acid transport system substrate-binding protein and thiosulfate/3-mercaptopyruvate sulfurtransferase in the 6-year soil suggests that these microorganisms may have optimized their metabolism under conditions of high competition or resource abundance. The increase in iron complex transport system substrate-binding proteins and proteins involved in their metabolism indicates that the microbial community is more efficient in acquiring and utilizing key elements such as Fe in the soil. The microbial community has become more specialized, with its metabolic network shifting toward refined and efficient resource acquisition, demonstrating dependence on specific resources such as Fe and amino acids. Annotation data from the 9-year soil showed a higher predicted abundance of genes associated with certain metabolic processes, including ATP-dependent DNA helicase, ATP-binding cassette, and thioredoxin reductase. These predicted patterns may suggest a potential for more complex mechanisms related to repair, stress response, and resource acquisition, but further validation is needed.

Figure 10 reflects that Amino Acid Metabolism, Membrane Transport, and Carbohydrate Metabolism are the most prominent functions of microorganisms in greenhouse soils. The prominence of these functions reflects the active role of microorganisms in maintaining their basic physiological activities in greenhouse soils. They adapt to the soil environment and acquire the necessary resources for survival through these metabolic processes. In contrast, functional genes related to the Sensory System and Cell Communication are less abundant, suggesting that microorganisms in greenhouse soils may not need to sense complex external signals or engage in intercellular communication extensively. This may be due to the relatively stable greenhouse environment, where changes in soil conditions are minimal.

4.4. Limitations of This Study

This study employed a cross-sectional temporal design, comparing independent plots with different cultivation years rather than tracking changes in the same plots over time—an approach that inherently limits causal inference. Moreover, the selected plots were strictly matched in terms of management history, thereby controlling for plot-specific differences to a considerable extent. Despite these limitations, the findings provide valuable baseline data for understanding how long-term greenhouse cultivation affects soil microbial communities.

In addition, microbial sequencing was performed on one pooled sample per cultivation duration per soil layer. The lack of independent sequencing replicates means that all microbial community analyses, including alpha diversity, LEfSe, PCoA, and co-occurrence network analysis, are descriptive and exploratory and cannot support statistical inference. Future studies should include independent field replicates and separate sequencing of each replicate.

5. Conclusions

The results indicate that soils with longer cultivation durations had higher SOC and $\text{NH}_4^+\text{-N}$ concentrations, while carbon and nitrogen content, as well as enzyme activity, were lower in deeper soils than in surface soils. This suggests that heavy fertilization and continuous cropping were associated with the chemical properties of greenhouse soils. In this study, 16S rRNA gene sequencing and functional predictions were performed on greenhouse soils from four different cultivation years. Among the cultivation durations studied, the soil alpha diversity index, number of characteristic sequences, and enzyme activity showed an increasing trend, suggesting that shorter cultivation durations were associated with higher microbial activity, potentially reflecting the combined influences of nutrient input and plant roots. Furthermore, $\text{NH}_4^+\text{-N}$ ($p < 0.05$) and urease ($p < 0.05$) were the most significant factors associated with bacterial and archaeal communities in greenhouse soils.

Proteobacteria and *Bacteroidota* showed a positive association in the nutrient cycling process of greenhouse soils. In contrast, there was a negative association between *Proteobacteria* and *Firmicutes*, as *Firmicutes* exhibit higher activity in eutrophic environments. With continuous input of chicken manure and the accumulation of SOC, *Firmicutes* showed an opposing abundance trend to that of *Proteobacteria*. *Firmicutes* dominate in soils after 9 years of cultivation, and PCoA reveals a clear distinction between soils cultivated for 9 years and other soils. These patterns suggest that community composition varied with cultivation duration, with potential implications for community balance. Nevertheless, we observed that the predicted functional profiles appeared to become more diverse across the cultivation-duration gradient. Based on PICRUST2-based predictions, the 1-year soil was inferred to be dominated by genes related to basic metabolism and rapid environmental adaptation, while the 9-year soil showed a higher predicted abundance of genes associated with more complex metabolic processes, suggesting potential functional shifts across the cultivation-duration gradient. In conclusion, this study deepens our understanding of the differences in microbial community structure and function in greenhouse soil systems across different cultivation durations, and provides a scientific basis for the sustainable management of greenhouse soils under cucumber–bitter melon rotation, emphasizing the need to balance productivity and soil health.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su181910072/s1>. Text S1. Detailed procedures for the measurement of soil physicochemical properties and enzyme activities.

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