

## Article

# Insights into Soil-Driven Microbial Succession and Regulation in *Phallus indusiatus*

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## Highlights

### What are the main findings?

- Soil nutrients and microbial structures shift dynamically across *Phallus indusiatus* growth stages.
- Key taxa (*Phallus*, *Massilia*, *Bryobacter*) correlate significantly with stage-specific nutrient fluctuations.

### What are the implications of the main findings?

- Phased nutrient and pH management prevents late-stage depletion and soil acidification.
- Targeted microbial inoculation and pathogen inhibition optimize soil health for high yields.

## Abstract

*Phallus indusiatus* is a prestigious macro-fungus with both nutritional and medicinal significance. However, its industrial development is limited by low yields and inconsistent quality, largely due to an incomplete understanding of the underlying soil microecological mechanisms. In this study, field experiments were conducted to measure soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), total potassium (TK), and pH across different growth stages. High-throughput sequencing was further employed to characterize the dynamic successions of bacterial and fungal communities. The results revealed a continuous depletion of SOC throughout the growth cycle, with a marked decrease in TN during the ovoid stage, whereas TP, TK, and pH showed increasing trends. Bacterial abundance followed a fluctuating “increase–decrease–increase” pattern, reaching its lowest level during the ovoid stage; similarly, fungal abundance initially decreased and subsequently increased, also attaining its minimum at the ovoid stage. Based on these stage-specific soil dynamics, targeted management strategies are proposed, including the application of basal carbon fertilizers supplemented with low-concentration phosphorus and potassium, the integration of slow-release nitrogen fertilizers, and the inoculation of functional microbes such as *Massilia*, *Acidobacteriaceae*, and *Terriglobales*. Dynamic regulation of soil pH is also recommended. This study provides a theoretical framework and technical guidance for the sustainable and high-efficiency cultivation of *P. indusiatus* and contributes to the broader development of the edible fungus industry.

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## 1. Introduction

*Phallus indusiatus*, taxonomically classified under the phylum Basidiomycota, class Agaricomycetes, order Phallales, family Phallaceae, and genus *Phallus*, is colloquially renowned as the “Veiled Lady” due to its distinctive net-like indusium [1]. As a prestigious macro-fungus with both nutritional and medicinal significance, *P. indusiatus* is rich in polysaccharides, proteins, amino acids, dietary fibers, and essential minerals. Furthermore, it exhibits remarkable antioxidant and immunomodulatory biological activities [2], garnering extensive attention from the food, nutraceutical, and pharmaceutical industries. Characterized by a brief cultivation cycle and high productivity, this species demonstrates significantly stronger environmental adaptability than other *Phallus* fungus varieties, flourishing in diverse substrates including relatively nutrient-poor sandy soils [3]. In recent years, its cultivation scale has expanded rapidly across bamboo-producing regions and under-forest economic zones, emerging as a cornerstone industry for enhancing rural incomes in mountainous areas and optimizing the integrated utilization of forestry resources. However, at present, the rapid expansion of cultivation areas and the prolongation of continuous monocropping have accentuated several critical challenges, including unstable yields, soil nutritional imbalances, microbial community dysbiosis, and strain degeneration, among others [4,5]. These issues constitute the primary bottlenecks restricting the sustainable development of the industry. In the artificial cultivation of *P. indusiatus*, the application of casing soil is a pivotal stage for inducing primordium formation and ensuring the systematic development of fruiting bodies. The micro-ecological environment of the casing layer directly determines the uniformity of mushroom emergence, biological efficiency, and the commercial quality of the fruiting bodies [6,7]. Nevertheless, current production practices are frequently hampered by inconsistent casing soil quality, imbalanced microbial structures, and inefficient nutrient supply, leading to significant yield fluctuations and unstable product quality. To address these critical limitations, investigating synergistic regulation strategies involving specialized fertilization and functional microbial inoculants under casing cultivation conditions is of direct and urgent scientific and industrial significance for optimizing the soil microenvironment and promoting sustainable industrial growth.

Soil exhibits stage-specific functional roles throughout the four developmental phases of *P. indusiatus*: the mycelial, primordium, ovoid, and harvest stages. During the mycelial stage, the nutritional matrix of the casing soil induces upward mycelial colonization. Concurrently, mycelial secretions synergize with beneficial bacteria, establishing a foundational microecological scaffold for subsequent development [8,9]. The primordium stage represents the critical physiological transition from vegetative to reproductive growth. Within the casing layer, available nutrients accelerate mycelial proliferation and bolster stress resistance, while the massive expansion of beneficial bacterial populations triggers the initiation of primordia [10]. During the ovoid stage, microbially mediated decomposition of organic matter in the casing soil facilitates nutrient uptake via the rhizomorphic network, thereby driving the maturation of the “fungal eggs” [11]. During the harvest stage, the total microbial abundance reaches its peak across the entire cultivation cycle, with bacteria becoming the dominant group and the fungal community structure tending toward stability. Collectively, these observations underscore that the morphogenesis of *P. indusiatus* is intrinsically linked to soil nutrient availability and microbial community dynamics. Nutritional deficiencies or dysbiosis in soil microecology can lead to significant yield reductions or, in severe instances, the complete inhibition of fructification.

Currently, research on nutritional management and the application of microbial inoculants during the growth stages of edible fungi has primarily focused on species cultivated with casing soil, such as *Stropharia rugosoannulata*, *Agaricus bisporus*, *Morchella* spp, and *Phallus dongsun*. Studies have demonstrated that employing a mixed casing substrate of sawdust, cattle manure, and soil during *S. rugosoannulata* cultivation can enhance soil cellulase activity, thereby accelerating the degradation of hemicellulose, cellulose, and lignin, which ultimately increases mushroom yield [12]. In *Morchella* cultivation, the application of wood ash not only alters soil physicochemical properties and reshapes the microbial community but also improves both the yield and quality of the mushrooms [13]. Furthermore, the introduction of synergistic bacterial functional groups—specifically potential copiotrophic, denitrifying, and nitrogen-fixing bacteria—promotes the decomposition of nutrients within exogenous nutrient bags and sustains soil nitrogen homeostasis, thereby boosting the productivity and yield of *Morchella* species [14]. Similarly, inoculating the cultivation substrate of *A. bisporus* with *Azospirillum lipoferum* has been shown to increase yield. This bacterium fixes atmospheric nitrogen, secretes phytohormones, mobilizes soil nutrients, and optimizes the rhizosphere-like microecological environment during the casing cultivation process [15]. Additionally, Ge et al. reported that utilizing a woody substrate-soil covering method for *P. dongsun* cultivation increases the contents of soil organic carbon, total nitrogen, and magnesium ions, ensuring stable and high yields [16]. Collectively, these findings underscore that the strategic application of microbial inoculants and the selection of appropriate casing substrates are of paramount importance for enriching soil nutrients and optimizing microbial community structures, which are essential for improving mushroom quality and achieving sustainable yield enhancement.

This study systematically investigated the imbalances in soil microbial communities and nutrient profiles, as well as their dynamic coupling mechanisms throughout the entire growth cycle of *P. indusiatus*. Furthermore, practical cultivation strategies applicable to commercial production were proposed, thereby providing both theoretical insights and technical support for the sustainable development of the *P. indusiatus*.

## 2. Materials and Methods

### 2.1. Experimental Site and Cultivation Environmental Conditions

The casing cultivation experiment of *P. indusiatus* was conducted in a greenhouse at Anhui Science and Technology University, Fengyang County, Chuzhou City, Anhui Province, China. Environmental parameters within the plastic greenhouse, including soil temperature (22–25 °C), air relative humidity (80–90%), and incubation conditions (complete darkness), were strictly controlled to maintain stable growth conditions throughout the cultivation period.

### 2.2. Casing Soil Formula and Casing Cultivation Process

The casing soil consisted of a mixture of 75% local red loam and 25% peat soil. Soil collected prior to inoculation served as the bare soil control (CK). Immediately after spawning, the cultivation substrate was covered with a 2–3 cm layer of finely prepared casing soil. One week after inoculation, white mycelia emerged around the spawn blocks and gradually extended into the casing layer. Soil samples were collected at four representative developmental stages of *P. indusiatus*. The mycelial stage (Pi1) was characterized by complete colonization of the soil surface by mycelia around day 30. The primordium stage (Pi2), observed around day 40 and lasting for approximately 10 days, was characterized by the formation of dense mycelial bundles and spherical primordia (0.5–1 mm in diameter) on the soil surface. The ovoid stage (Pi3) was characterized by the formation of fungal eggs with diameters of approximately 4–4.5 cm within the soil layer around day 60

(with a phase duration of 20 days). The harvest stage (Pi4) was defined as the period immediately following fruiting body harvest.

### 2.3. Soil Sample Collection and Preprocessing at Different Growth Stages

To ensure rigorous statistical evaluation and eliminate technical replication bias, three spatially independent cultivation plots were designated as true biological replicates for each developmental stage. Soil samples were collected using a five-point sampling method within each experimental plot. Prior to sampling, the upper 1 cm of surface soil was carefully removed. Intact soil cores were subsequently collected using a soil auger (5 cm internal diameter) in combination with a 100 cm<sup>3</sup> cutting ring. All samples were carefully cleared of *P. indusiatus* mycelia, primordia, fruiting bodies, gravel, and visible plant and animal residues. The soil samples were subsequently homogenized and passed through an 80-mesh sieve prior to physicochemical and microbial analyses.

### 2.4. Determination of Soil Physicochemical Properties

Soil organic carbon (SOC) content was determined using the potassium dichromate oxidation–external heating method (NY/T 1121.6–2006) [17,18]. Total nitrogen (TN) was analyzed using the Kjeldahl method in accordance with LY/T 1228–2015 [19,20]. Total phosphorus (TP) was determined using the alkali fusion–molybdenum antimony colorimetric method (GB/T 9837–1988) [21], whereas total potassium (TK) was quantified using the alkali fusion–flame photometric method (NY/T 87–1988) [22]. Soil pH was determined using the potentiometric method (soil-to-water ratio of 1:2.5, *w/v*) according to NY/T 1121.2–2006 [23,24]. All measurements were performed in triplicate, and the mean values were used for subsequent statistical analyses.

### 2.5. DNA Extraction, Library Preparation, and High-Throughput Sequencing

Total genomic DNA was extracted from 0.5 g of fresh soil using the TGuide S96 Magnetic Bead-Based Soil Genomic DNA Extraction Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. DNA concentration and purity were determined by using a Synergy HTX microplate reader (GeneCompany Limited, Hong Kong, China) and a 1× dsDNA HS Working Solution kit (Yeasen Biotechnology, Shanghai, China). For bacterial community profiling, the V3–V4 hypervariable regions of the 16S rRNA gene were amplified using the universal primer pair 338F (5'-ACTCCTACGG-GAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). For fungal community profiling, the internal transcribed spacer 1 (ITS1) region was amplified by using the primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3'). Amplicon libraries were constructed following standard Illumina library preparation protocols and sequenced using the Illumina MiSeq platform (2 × 300 bp) by Nanjing Tsingke Biotechnology Co., Ltd. (Nanjing, China).

### 2.6. Bioinformatic Processing

Raw sequencing reads in FASTQ format were subjected to stringent quality-control procedures prior to downstream analyses. Initially, low-quality reads were filtered and trimmed using Trimmomatic (v0.33, <https://github.com/usadellab/Trimmomatic>) [25] with a 50 bp sliding-window approach, and reads with an average quality score below 20 within the sliding window were truncated. Primer sequences were subsequently identified and removed using Cutadapt (v1.8.3, <http://cutadapt.readthedocs.org/>) [26], with a maximum allowable error rate of 20% and a minimum overlap length of 15 bp. Sequence length filtering was further performed to retain fragments ranging from 350–490 bp for the bacterial 16S rRNA V3–V4 regions and 300–500 bp for the fungal ITS regions. Subsequent bioinformatic analyses, including sequence denoising, paired-end read merging,

and chimera removal, were conducted using the DADA2 package implemented in R (v3.1.1). Specifically, the filterAndTrim function was applied with a maximum expected error (EE) threshold of 2. Error-rate models were estimated using the learnErrors function, followed by sequence denoising with the DADA2 algorithm. Paired-end reads were merged using the parameters minOverlap = 18 bp and maxMismatch = 4 bp. Chimeric sequences were identified and removed using the removeBimeraDenovo function under the “consensus” mode. The resulting high-quality sequences were used for subsequent downstream analyses. After quality control, quantification, and normalization of the DNA libraries, the obtained high-quality sequences were identified as Amplicon Sequence Variants (ASVs) by using the DADA2 pipeline. To process the taxonomic classification of ASVs, the representative sequences of each ASV were generated and aligned against the Silva database (Release 138, <https://www.arb-silva.de/documentation/release-138/>) for bacteria and UNITE database (Release 8.0, <https://unite.ut.ee/>) for fungi. The raw sequencing datasets generated in this study were deposited in the NCBI BioProject database under accession numbers PRJNA1467461 (16S *rRNA* gene sequences and ITS sequences to be released upon publication).

### 2.7. Statistical Analysis

Statistical analyses were performed using Excel 2016 and SPSS 26.0 (IBM Corp., Armonk, NY, USA), while graphical visualizations were generated using Origin 2021 (OriginLab Corp., Northampton, MA, USA). To characterize soil microbial diversity across different developmental stages of *P. indusiatus*, taxonomic annotation of amplicon sequence variants (ASVs) was conducted using a Naive Bayes classifier combined with sequence alignment against the Silva reference database. This approach enabled high-resolution taxonomic assignment of microbial sequences and facilitated the comprehensive characterization of bacterial and fungal community compositions at multiple taxonomic levels. Species abundance tables were generated using the QIIME 2 platform (2020.6.0, <https://qiime2.org>). Microbial community structures were subsequently visualized using R-based analytical tools, including a Permutational Multivariate Analysis of Variance (PERMANOVA/Adonis) based on the Bray–Curtis distance matrix to test community variations, a Redundancy Analysis (RDA) using the *vegan* package (2.3-0, <https://cran.r-project.org/web/packages/>) to evaluate the driving effects of soil environmental variables, Venn diagrams for identifying shared and unique ASVs among developmental stages, as well as stacked bar plots illustrating microbial compositions at the phylum and genus levels. Correlations between soil physicochemical properties and microbial community dynamics were evaluated using Spearman's rank correlation analysis and visualized through heatmaps generated in Origin 2021 and Excel 2016. In addition, microbial co-occurrence networks were constructed based on Spearman's correlation coefficients to explore potential interspecific interactions within the microbial communities. Only statistically significant and robust correlations ( $p < 0.05$ ) were retained for network topology analysis. Gemini 1.5 Pro (<https://deepmind.google/technologies/gemini/>) and GPT-5.5 (<https://openai.com/gpt-5.5/>) were solely utilized for language polishing and grammatical revisions.

## 3. Results

### 3.1. Dynamics of Soil Physicochemical Properties

As summarized in Table 1, soil physicochemical properties exhibited distinct successional dynamics throughout the developmental stages of *P. indusiatus* cultivation. At the Pi1, TN content reached its maximum level, corresponding to 109.60% of the initial soil value. During the Pi2, TP increased markedly and peaked at 173.91% of the baseline level.

Subsequently, at the Pi3, TK attained its highest concentration, accounting for 112.98% of the initial soil content. By the Pi4, SOC declined to its lowest level, with a cumulative reduction of 49.80% relative to the initial soil. Similarly, TN decreased to its minimum value at the Pi4, representing a 24.29% reduction compared with the baseline. In contrast, TP and TK contents at the Pi4 remained higher than their initial levels, reaching 113.04% and 108.78% of the baseline, respectively. Meanwhile, soil pH increased substantially to 134.03% of the initial value. Overall, SOC exhibited a continuous declining trend from Pi1 to Pi4, whereas TN and TP showed an initial increase followed by a gradual decrease. In contrast, TK and soil pH displayed consistent upward trends throughout the cultivation period. Notably, significant variations in SOC, TN, TP, TK, and pH were observed across different developmental stages, which indicated pronounced stage-specific characteristics in soil nutrient transformation and cycling during *P. indusiatus* cultivation.

**Table 1.** Dynamics of Soil Physicochemical Properties During Different Developmental Stages of *P. indusiatus*.

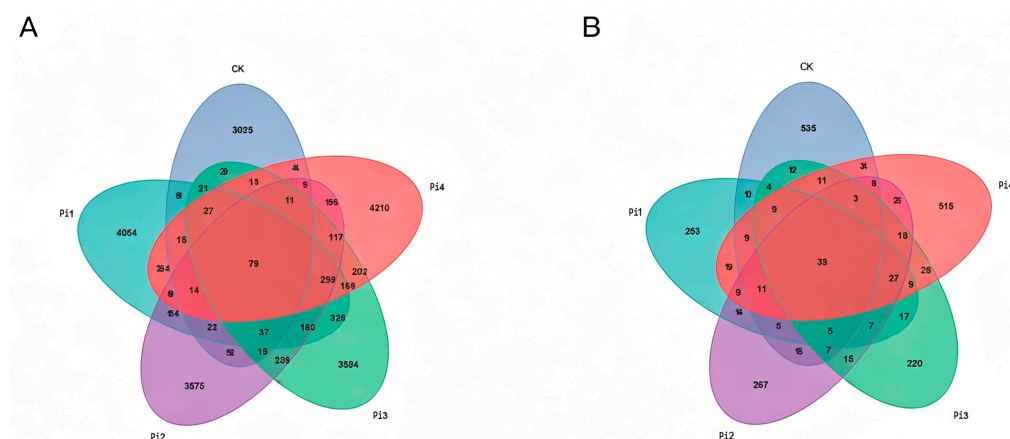
| Growth Stage | Soil Organic Carbon (SOC) Content g/kg | Total Nitrogen (TN) Content g/kg | Total Phosphorus (TP) Content g/kg | Total Potassium (TK) Content mg/kg | pH Value      |
|--------------|----------------------------------------|----------------------------------|------------------------------------|------------------------------------|---------------|
| CK           | 113.43 ± 2.17 a                        | 1.77 ± 0.04 b                    | 0.23 ± 0.01 b                      | 11.48 ± 0.12 c                     | 5.23 ± 0.17 e |
| Pi1          | 100.20 ± 1.32 b                        | 1.94 ± 0.08 a                    | 0.27 ± 0.01 a                      | 12.30 ± 0.26 b                     | 5.61 ± 0.13 d |
| Pi2          | 81.571 ± 1.17 c                        | 1.88 ± 0.05 a                    | 0.4 ± 0.01 b                       | 12.36 ± 0.23 a                     | 5.82 ± 0.21 c |
| Pi3          | 79.33 ± 0.93 d                         | 1.55 ± 0.02 c                    | 0.28 ± 0.01 c                      | 12.97 ± 0.13 b                     | 6.35 ± 0.19 b |
| Pi4          | 56.49 ± 1.15 e                         | 1.34 ± 0.04 d                    | 0.26 ± 0.01 d                      | 12.49 ± 0.26 b                     | 7.01 ± 0.18 a |

Note: CK, bare soil stage; Pi1, mycelial Stage; Pi2, primordium Stage; Pi3, ovoid Stage; Pi4, harvest Stage. Different lowercase letters (a–e) indicate significant differences among developmental stages. Stages sharing the same lowercase letter are not significantly different, whereas stages with different lowercase letters differ significantly at  $p < 0.05$ .

### 3.2. Soil Microbial Diversity Across Different Growth Stages

#### 3.2.1. Stage-Specific Dynamics of Bacterial and Fungal Taxa

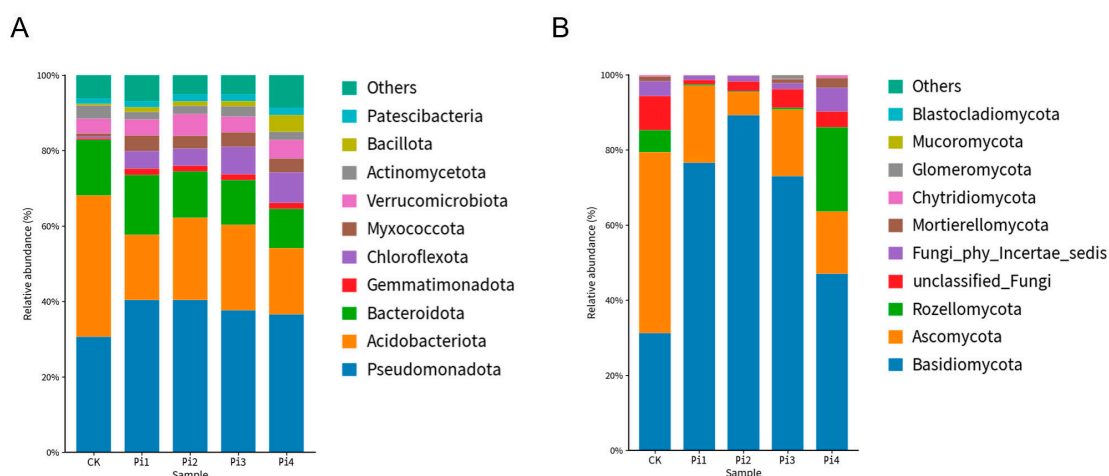
Venn diagrams constructed based on the Operational Taxonomic Units (OTUs) of soil samples were utilized to intuitively illustrate the shared and unique bacterial and fungal taxa across various growth stages. This approach facilitates the assessment of community similarity and divergence, while identifying core microbial cohorts and stage-specific signatures (Figure 1). The results indicated that the lowest bacterial species richness was observed during the bare soil stage (CK), with all subsequent cultivation stages exhibiting significantly higher richness than the initial soil. In particular, bacterial richness reached its maximum during Pi4, accounting for 139.17% of the initial bacterial richness (Figure 1A). In contrast, fungal species richness was highest at the CK stage and declined to its lowest level during Pi3, at which the number of fungal taxa represented only 41.12% of the baseline value (Figure 1B). Overall, distinct successional patterns in microbial richness were observed throughout the cultivation cycle of *P. indusiatus*. Specifically, bacterial richness exhibited a dynamic trend characterized by an initial increase, followed by a decline and subsequent recovery, whereas fungal richness displayed a U-shaped pattern characterized by an initial decrease followed by gradual recovery.



**Figure 1.** Venn diagram. Each circle in the figure represents a sample group. The numbers in the overlapping parts of the circles represent the number of OTUs shared between the sample groups, and the numbers in the non-overlapping parts represent the number of OTUs unique to the sample groups (bacteria (A) and fungi (B)). CK, bare soil stage; Pi1, mycelial Stage; Pi2, primordium Stage; Pi3, ovoid Stage; Pi4, harvest Stage.

### 3.2.2. Diversity of Soil Microbial Communities Across Different Growth Stages

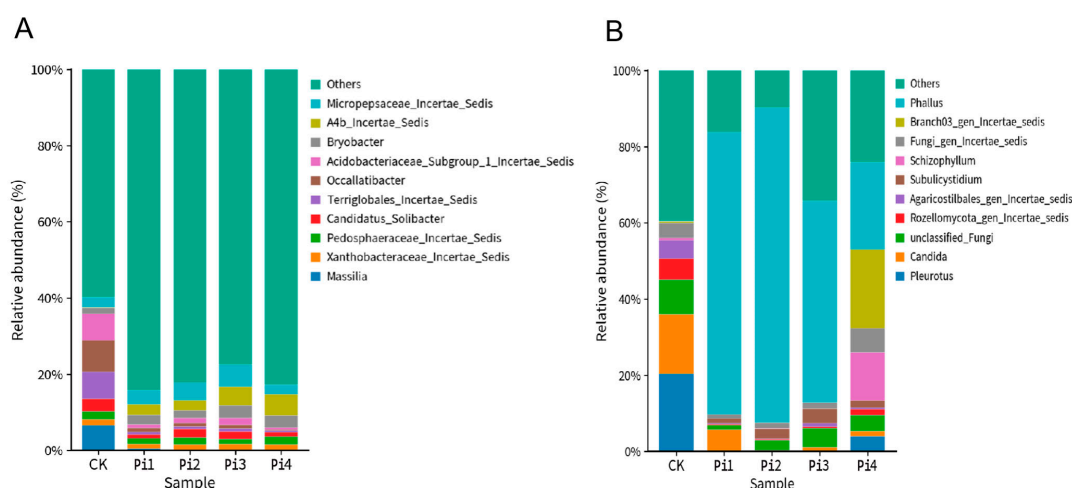
Figure 2A depicts the phylum-level composition of soil bacterial communities across the different developmental stages of *P. indusiatus*. The six most dominant phyla (*Pseudomonadota*, *Acidobacteriota*, *Bacteroidota*, *Chloroflexota*, *Verrucomicrobiota*, and *Myxococcota*) collectively accounted for over 85% of the total relative abundance. Distinct stage-specific successional patterns were observed. During the bare soil stage (CK), the abundances of *Pseudomonadota*, *Myxococcota*, *Chloroflexota*, and *Verrucomicrobiota* were minimal, whereas *Acidobacteriota* reached its maximum. Upon transition to Pi1, the abundance of *Acidobacteriota* declined to its lowest level, while *Bacteroidota* and *Myxococcota* peaked. In Pi2, *Pseudomonadota* exhibited its highest relative abundance, indicating its predominance during early fruiting body development. Finally, at Pi4, the abundance of *Bacteroidota* decreased to a minimum, whereas *Chloroflexota* and *Verrucomicrobiota* attained their maximum levels.



**Figure 2.** Relative abundance of microbiota at the phylum level in *P. indusiatus* ((A) bacteria at the phylum level; (B) fungi at the phylum level). CK, bare soil stage; Pi1, mycelial Stage; Pi2, primordium Stage; Pi3, ovoid Stage; Pi4, Harvest Stage.

At the fungal phylum level, Figure 2B shows that the community was dominated by Basidiomycota, Ascomycota, and Chytridiomycota, which together accounted for over 80% of the total relative abundance. Specifically, during the bare soil stage (CK), the abundance of Ascomycota reached its maximum, whereas Basidiomycota was at its lowest. This pattern shifted markedly by Pi2, when Basidiomycota peaked in relative abundance, coinciding with the minimum levels of both Ascomycota and Chytridiomycota. By Pi4, Chytridiomycota reached its peak abundance, while the abundance of Ascomycota remained lower than that observed in the initial CK stage. In contrast, the other two dominant phyla exhibited higher abundances in the CK stage than in the final harvest stage. These results indicate that, throughout the cultivation cycle of *P. indusiatus*, Basidiomycota followed a “rise–then–fall” trajectory, whereas Ascomycota and Chytridiomycota displayed a V-shaped successional trend, characterized by an initial decline followed by a subsequent recovery in the later stages.

At the genus level, the soil microbial community exhibited distinct successional dynamics across the developmental stages of *P. indusiatus*. As illustrated in Figure 3A, the bacterial community displayed varying abundance patterns. During the bare Soil Stage (CK), the genera *Acidobacteriaceae\_subgroup\_1\_incertae\_sedis*, *Terriglobales\_incertae\_sedis*, and *Massilia* were the most prevalent; however, their abundances declined continuously, reaching their lowest levels by Pi4. In contrast, the abundance of *Bryobacter* peaked during Pi3. Meanwhile, *Micropepsaceae\_incertae\_sedis* declined to its minimum level by Pi4.

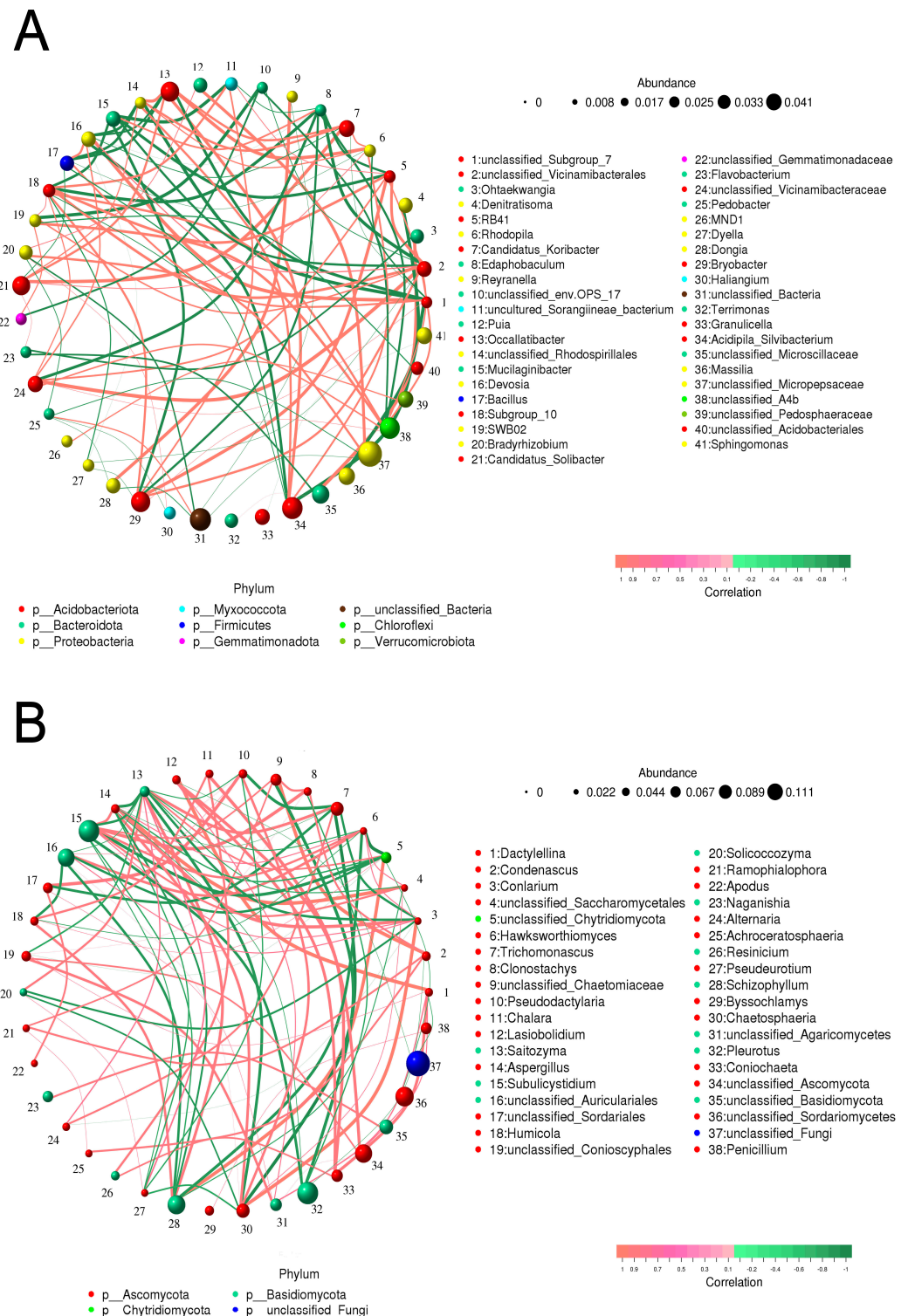


**Figure 3.** Relative abundance of microbiota at the genus level in *P. indusiatus* ((A) bacteria at the genus level; (B) fungi at the genus level). CK, bare soil stage; Pi1, Mycelial Stage; Pi2, Primordium Stage; Pi3, Ovoid Stage; Pi4, Harvest Stage.

Regarding the fungal community (Figure 3B), notable genus-level shifts occurred throughout the cultivation cycle. During the CK stage, the community was dominated by *Pleurotus* and *Candida*, with a substantial presence of *Schizophyllum*. Upon transition to Pi1, the genus *Phallus* proliferated sharply, accounting for over 70% of the total fungal sequence reads, while the abundance of *Schizophyllum* began to decrease. At Pi2, *Phallus* reached its maximum relative abundance, whereas *Pleurotus* declined to its lowest level. During Pi3, the abundance of *Phallus* decreased markedly, and *Schizophyllum* became undetectable. By Pi4, *Phallus* no longer maintained absolute dominance, with its relative abundance decreasing to approximately 30%, while *Schizophyllum* concurrently reached its peak abundance.

### 3.2.3. Co-Occurrence Network Analysis

Topological analysis of the soil bacterial co-occurrence network revealed that the dominant nodes were primarily affiliated with the phyla Acidobacteriota, Bacteroidota, Proteobacteria, Myxococcota, Chloroflexota, and Verrucomicrobiota. Among these, Acidobacteriota, Bacteroidota, and Proteobacteria exhibited the highest relative abundances and occupied central positions within the network. Furthermore, strong intra- and inter-phylum correlations were observed among the genera within these taxa, suggesting complex and highly integrated ecological associations (Figure 4A).

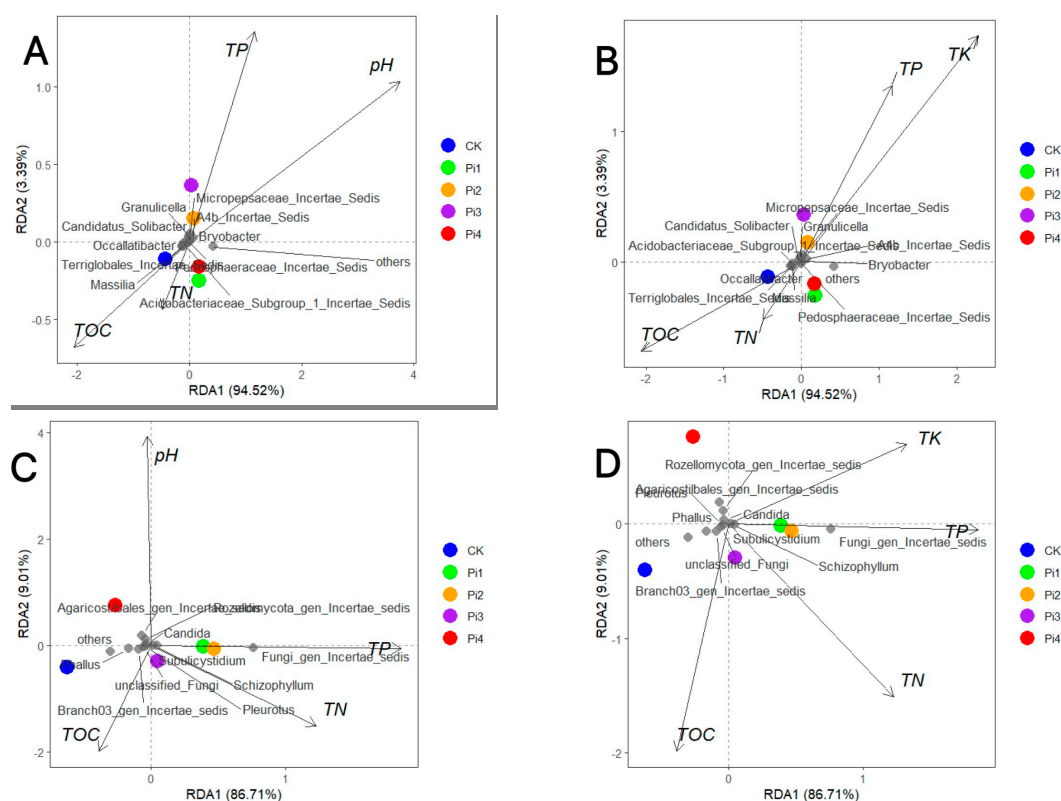


**Figure 4.** Cooccurrence network analysis of bacteria (A) and fungi (B). The different colors of the circle represent different genera of bacteria or fungi. The top 50 genera were shown ( $p < 0.05$ ). When a correlation exists between any two genera, they are visually connected by a line within the network. The thicker the line, the stronger the correlation. The larger the circle, the higher the abundance of the species.

For the fungal community, the network topology was predominantly composed of nodes belonging to the phyla Ascomycota, Basidiomycota, and Chytridiomycota. Ascomycota emerged as the most influential group, maintaining a dominant presence within the network. Most fungal genera exhibited significantly positive correlations, with only a minority displaying antagonistic (negative) interactions, indicating that synergistic cooperation is the primary mode of interaction within the fungal community. Notably, several high-abundance taxa, such as Node 28 (*Schizophyllum*) and Node 32 (*Pleurotus*), demonstrated extensive connectivity with numerous other species. These taxa were identified as keystone groups, playing pivotal roles in maintaining the stability and structural integrity of the soil fungal community network (Figure 4B).

### 3.2.4. Driving Effects of Soil Physicochemical Properties on Microbial Community Successions

To further elucidate the macro-scale relationships between soil physicochemical properties and microbial community dynamics across successive developmental stages, multivariate Redundancy Analysis (RDA) models were constructed (Figure 5). In the cultivation soil of *P. indusiatus*, bacterial and fungal taxa exhibited significant, group-specific responses and differential spatial correlations with environmental variables at distinct growth phases. The first two canonical axes captured a cumulative variance of 97.91% for the bacterial community (Figure 5A,B) and 95.72% for the fungal community (Figure 5C,D). Crucially, PERMANOVA testing robustly validated these patterns, demonstrating distinct clustering for the bacterial community ( $F = 15.842$ ,  $R^2 = 0.8637$ ,  $p < 0.001$ ) and the fungal community ( $F = 67.52$ ,  $R^2 = 0.9643$ ,  $p < 0.001$ ).



**Figure 5.** Redundancy analysis (RDA) reflecting the relationships between soil physicochemical properties and dominant microbial communities at the genus level across different growth stages. Panels (A,B) display bacterial assemblages, whereas panels (C,D) illustrate fungal assemblages. Colored circles indicate samples from distinct developmental stages: CK (blue), Pi1 (green), Pi2 (orange), Pi3 (purple), and Pi4 (red). Black lines with arrows indicate soil environmental variables (TOC, total organic carbon; TN, total nitrogen; TK, total potassium; TP, total phosphorus; pH). Gray circles represent core microbial genera. The statistical significance of temporal variations in microbial structures was validated by PERMANOVA, demonstrating distinct clustering for the bacterial community ( $F = 15.842$ ,  $R^2 = 0.8637$ ,  $p < 0.001$ ) and the fungal community ( $F = 67.52$ ,  $R^2 = 0.9643$ ,  $p < 0.001$ ).

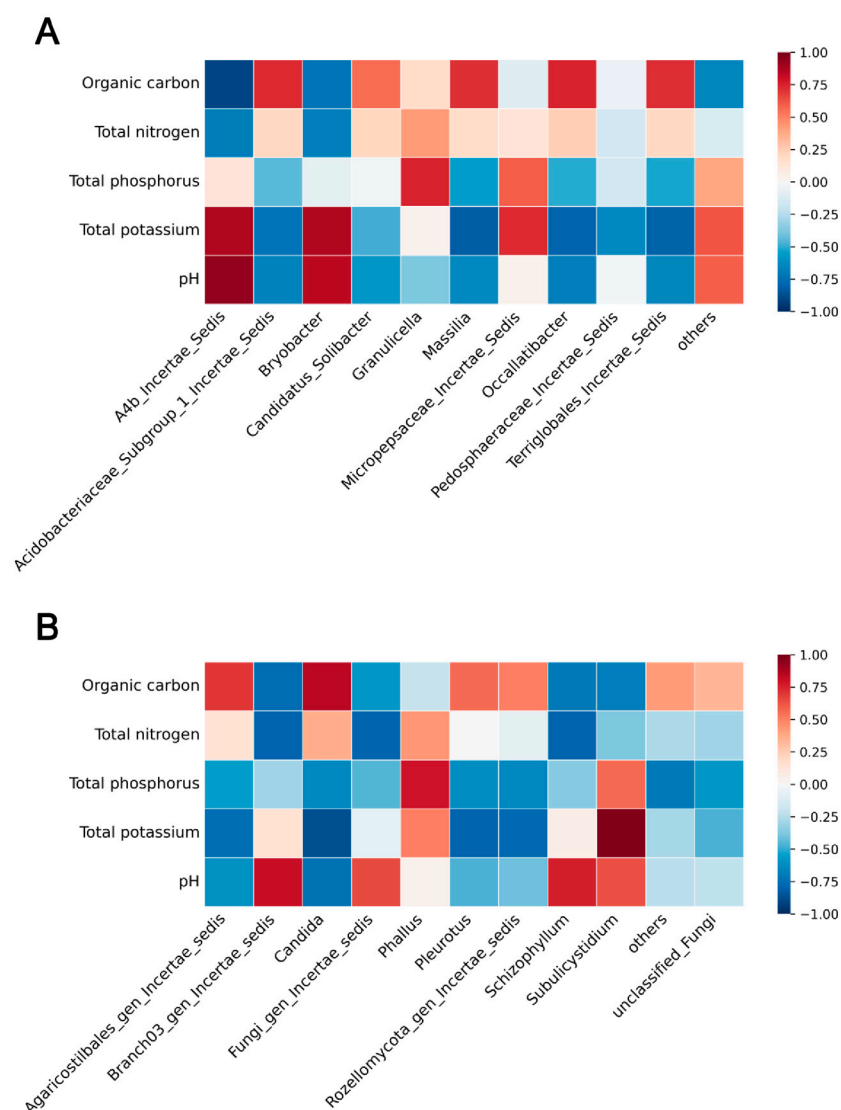
Specifically, TOC content was strongly positively correlated with the bacterial vectors of *Massilia*, *Terriglobales\_incertainae\_sedis*, and *Occallatibacter* (Figure 5A,B), as well as the fungal genus *Phallus* (Figure 5C,D), with all vectors pointing tightly toward the unplanted control stage, indicating their high organic matter dependence during the pre-cultivation phase. TN showed a significant positive correlation with *Phallus* and the fungal clusters of *Subulicystidium*, *Schizophyllum*, and *Pleurotus* during the Pi1 and Pi2 stages, but exhibited a complete inverse correlation relative to the late reproductive phases. TP was positively associated with *Micropepsaceae\_incertainae\_sedis* and *Granulicella* during Pi3, but was negatively correlated with *Massilia* and *Candida* in early growing periods. Regarding potassium gradients, TK exhibited severe multicollinearity with pH, with both parameters displaying synchronized, elongated vectors projecting exclusively toward the final harvesting stage. In addition, soil pH was profoundly positively correlated with *Micropepsaceae\_incertainae\_sedis* and *Granulicella* (Figure 5A), alongside the fungal opportunistic colonizers *Agaricostilbales\_gen\_incertainae\_sedis* and *Rozellomycota\_gen\_incertainae\_sedis* (Figure 5C), exhibiting an acute selective enrichment effect driven by substrate alkalization during Pi4.

Overall, bacterial and fungal taxa demonstrated substantial variations in their responses to the five environmental factors, reflecting their highly dynamic ecological niches from early vegetative growth to late reproductive maturation. Notably, *Massilia* and *Phallus* maintained consistent response directions across key carbon parameters during the initial phases (CK–Pi1). In contrast, *Micropepsaceae\_incertainae\_sedis* and *Agaricostilbales\_gen\_incertainae\_sedis* maintained matched positive responses to substrate alkalization (pH) and maturation (TK) specifically during the late-stage transitions (Pi3–Pi4). These findings suggest that bacteria and fungi perform distinct yet complementary ecological roles within the soil ecosystem during *P. indusiatus* cultivation, driven by their differential sensitivities to changing environmental conditions across the full life cycle.

### 3.2.5. Correlation Analysis Between Environmental Factors and Microbial Communities

To further elucidate the relationships between soil physicochemical properties and microbial community dynamics across developmental stages, a Spearman correlation heatmap was constructed (Figure 6). In the cultivation soil of *P. indusiatus*, bacterial and fungal taxa exhibited significant, group-specific responses and differential correlations with environmental variables. Specifically, SOC content was significantly positively correlated with the bacterial genera *Acidobacteriaceae\_subgroup\_1\_incertainae\_sedis*, *Massilia*, and *Terriglobales\_incertainae\_sedis*, as well as the fungal genus *Candida*. TN showed a significant positive correlation with *Phallus* but a negative correlation with *Schizophyllum*. TP was negatively correlated with *Massilia* and the fungal genera *Candida* and *Pleurotus*. TK exhibited positive associations with both *Bryobacter* (bacteria) and *Schizophyllum* (fungi). In addition, soil pH was positively correlated with *Schizophyllum* but negatively correlated with *Candida*. Overall, bacterial and fungal taxa demonstrated substantial variations in their responses to the five environmental factors. Notably, *Bryobacter* and *Schizophyllum*

maintained consistent response directions across three key parameters: TN, TK, and pH. These findings suggest that bacteria and fungi perform distinct ecological roles within the soil ecosystem during *P. indusiatus* cultivation, driven by their differential sensitivities to changing environmental conditions.



**Figure 6.** Heat map of the correlation between soil physicochemical properties and bacterial (A) and fungal (B) communities in soil during different growth stages of *P. indusiatus*. The maps intuitively illustrate the associations between soil factors and microbial communities. Red indicates positive correlation, and blue denotes negative correlation. The color intensity reflects the strength of correlation.

#### 4. Discussion

Casing soil is an essential factor for ensuring the successful transition of *P. indusiatus* from vegetative growth to reproductive development. Soil physicochemical properties and microbial community structures not only undergo dynamic shifts throughout the cultivation cycle [27] but also exert profound effects on fungal growth, disease incidence, yield, and quality. Previous studies by Duan et al. demonstrated that intercropping *P. indusiatus* with sugarcane can mitigate soil nitrogen loss, enhance soil fertility, and optimize microbial communities and metabolomes, thereby effectively improving soil health [28]. Furthermore, Sun et al. [29] confirmed that carbon (C), nitrogen (N), and phosphorus (P) act as key regulators of microbial metabolism. In line with this, our redundancy

analysis (RDA) further demonstrated that these soil physicochemical properties were the primary environmental drivers shaping the microbial community structures. Therefore, a systematic analysis of the dynamic patterns of soil nutrients and microbial communities across different developmental stages is essential for identifying soil-related challenges during cultivation. Such insights facilitate the development of evidence-based management strategies, providing both a theoretical framework and technical support for the high-yield cultivation of *P. indusiatus*.

Organic carbon (OC) serves as the primary energy source and carbon skeleton provider for *P. indusiatus*, while also functioning as a key indicator of soil fertility [30]. The total quantity, composition, and mineralization rate of SOC directly determine the nutrient supply potential throughout the entire growth cycle. In this study, *P. indusiatus*, as a saprophytic fungus, was found to continuously decompose SOC during its growth, thereby accelerating the mineralization of soil organic matter [31] to support fungal development. This is consistent with the findings of Duan et al. [28] who reported that the metabolism of *P. indusiatus* significantly drives OC decomposition and alters the carbon pool. Furthermore, Lei et al. [32] demonstrated that cultivating *P. indusiatus* using a substrate mixture of *Cenchrus fungigraminus* and spent mushroom substrate (SMS) significantly increases SOC content and subsequent yields. Therefore, augmenting the cultivation substrate with organic amendments (such as decomposed bamboo chips, mushroom residue, or straw) prior to planting represents a viable strategy for enhancing SOC. Our results also revealed that SOC content is significantly and positively correlated with microbial taxa, including *Acidobacteriaceae\_subgroup\_1\_incertae\_sedis*, *Massilia*, and *Terriglobales\_incertae\_sedis*, in agreement with previous studies [32]. *Terriglobales\_incertae\_sedis* primarily drives the mineralization and decomposition of SOC [33], whereas members of *Acidobacteriaceae* [34] and *Massilia* facilitate the continuous transformation of crop residues into SOC [35]. Taken together, these studies imply the potential feasibility of optimizing substrate formulations by incorporating carbon-rich organic matrices, such as decomposed bamboo chips, spent mushroom substrates, and straw prior to cultivation to elevate the soil organic carbon content, which holds crucial theoretical value for constructing a high-yield substrate environment for *P. indusiatus*.

Nitrogen is a fundamental constituent of proteins, nucleic acids, chitin, and extracellular enzymes in the mycelial cells of *P. indusiatus*, serving as an essential nutrient for its growth and development. In this study, total nitrogen (TN) levels during the Pi1 and Pi2 stages were significantly higher than those observed in the bare soil, ovoid, and harvest stages. Notably, TN at the Harvest stage fell below initial baseline levels, reflecting distinct stage-specific nitrogen demands. The elevation of TN during the Mycelial stage may be attributed to microbial nitrogen fixation in the soil [36], whereas the subsequent decline during the Pi2 and Pi3 stages suggests substantial nitrogen uptake to support fruiting body maturation [37]. The significant positive correlation between the genus *Phallus* and TN further underscores that nitrogen is a critical driver of mycelial expansion [38] and primordium differentiation. However, nitrogen requirements appear to follow a nuanced regulatory mechanism. Hennicke et al. demonstrated in *Cyclocybe aegerita* that organic nitrogen supplementation during the transition from vegetative to reproductive growth can significantly enhance mycelial biomass and primordium numbers, while promoting earlier fruiting [39]. Conversely, Sakamoto reported that excessive nitrogen concentrations can inhibit primordium formation in various edible fungi, indicating that lower nitrogen environments are more conducive to primordium induction [27]. Therefore, a stage-specific nitrogen management strategy is highly recommended for *P. indusiatus* cultivation. Maintaining a relatively stable and sustained low-concentration nitrogen environment during the early mycelial phase helps avoid microbial community imbalances triggered by excessive vegetative growth, whereas the high-efficiency supply of readily available

nutrients during the bud and egg stages is key to supporting fruiting body development and community stability. *Schizophyllum* abundance exhibited a significant negative correlation with total nitrogen. An excessive abundance of this genus may lead to localized soil nitrogen depletion and micro-ecological imbalances, thereby elevating the risks of associated soil-borne diseases [40,41]. Consequently, when such weed molds emerge, properly supplementing nitrogen fertilizers to restore the soil's nitrogen supply capability is recommended to sustain the substrate's micro-ecological balance and suppress the excessive expansion.

Phosphorus is intrinsically involved in energy metabolism (ATP formation) and the maintenance of cell membrane structural integrity [42]. In this study, TP content peaked during Pi2 and subsequently declined, indicating that this phase represents a critical period for phosphorus demand. Consequently, maintaining adequate phosphorus levels within the substrate is essential for guaranteeing successful primordium differentiation and bud growth [43]. However, excessive TP accumulation can exacerbate soil acidification [44] which in turn inhibits mycelial expansion, primordium formation, and fruiting body development in *P. indusiatus*. Under such conditions, the negative correlations observed between TP and the genera *Massilia* [45], *Candida* [46], and *Pleurotus* have important ecological implications. *Massilia* functions as a phosphate-solubilizing bacterium, capable of converting insoluble phosphorus (organic and calcium-bound P) into bioavailable forms [45]. Meanwhile, *Candida* and *Pleurotus* can actively sequester and store phosphorus under nutrient-limiting conditions, indirectly supporting the growth of *P. indusiatus* [46,47]. The blind and excessive application of phosphorus fertilizers during *P. indusiatus* cultivation may lead to production disbenefits, perturb the substrate micro-ecological balance, and induce diseases. Therefore, when soil phosphorus levels are excessively high, a theoretical strategy would involve promoting functional taxa like *Massilia* to regulate phosphorus forms and alleviate negative micro-ecological effects [48]. Conversely, under phosphorus-deficient conditions where *Candida* and *Pleurotus* may actively compete with *P. indusiatus* for limited nutrients, micro-ecological modulation should pivot toward introducing or activating beneficial consortia, such as *Bacillus* species, to maintain microbial equilibrium, secure phosphorus availability, and suppress potential soil-borne pathogens [49].

Potassium plays a pivotal role in regulating osmotic pressure, activating essential enzymes, and facilitating carbohydrate transport [50]. In this study, total potassium (TK) levels were lowest during the bare soil stage (CK) and remained elevated throughout subsequent developmental phases, with the highest consumption observed during Pi3. This trend indicates a continuous pattern of potassium uptake and utilization throughout the growth cycle of *P. indusiatus*. These findings underscore that a sustained supply of potassium is required for the growth of *P. indusiatus*, and high-efficiency replenishment in the later stages particularly serves as a crucial guarantee for supporting fruiting body development. Correlation analysis revealed that the bacterial genus *Bryobacter* and the fungal genus *Schizophyllum* were positively associated with TK content. Notably, *Bryobacter* may possess functional attributes characteristic of potassium-solubilizing microorganisms (KSMs) [51]. Previous studies have confirmed that KSMs can mediate the conversion of fixed soil potassium into bioavailable forms through organic acid-induced acidolysis, thereby promoting potassium uptake and ultimately enhancing crop yields [52]. Thus, reasonably optimizing the relative abundance of *Bryobacter* theoretically represents a promising strategy to improve *P. indusiatus* productivity. Conversely, the growth of *Schizophyllum* may exert an antagonistic effect on the growth of *P. indusiatus* by competing for nutrients and space [53]. Therefore, the dynamics of weed molds such as *Schizophyllum* necessitate close attention during cultivation, and maintaining the substrate micro-ecological balance is required to effectively suppress their further proliferation and expansion.

Soil pH serves as a critical environmental factor influencing the growth and development of *P. indusiatus*, as it directly governs nutrient bioavailability and the structure of microbial communities. In this study, a progressive decrease in soil acidity was observed during the cultivation process, likely resulting from mycelial metabolism and the decomposition of organic matter, which collectively modify the soil acid–base environment [10]. Our findings indicate that the genus *Schizophyllum* exhibits higher abundance under more alkaline conditions, whereas *Candida* is better adapted to acidic environments. Given that *P. indusiatus* thrives in slightly acidic soil, deviations in pH can significantly disrupt ecological balance. Excessive alkalinity may enable competitors such as *Schizophyllum* to gain a competitive advantage, thereby inhibiting mycelial expansion. Conversely, excessively low pH can suppress beneficial microorganisms and promote the over-proliferation of *Candida*, increasing the risk of pathogenicity [54]. Maintaining a slightly acidic soil profile is therefore essential for healthy *P. indusiatus* cultivation. Consequently, since soil pH tended to rise and approach neutrality or alkalinity in this study, the application of humic acid theoretically represents a viable strategy to modulate and sustain a slightly acidic soil environment [55]. Conversely, if the initial substrate environment becomes excessively acidic, appropriate adjustments can be implemented by applying physiologically alkaline soil conditioners, such as lime water, to neutralize the acidity [56]. These targeted management strategies are crucial for satisfying the nutritional and growth requirements of *P. indusiatus*.

This study systematically analyzed the dynamic patterns of soil nutrients, pH, and microbial communities throughout the developmental cycle of *P. indusiatus*. The results reveal a close and dynamic relationship between soil physicochemical properties and microbial community structure across different growth stages. Our findings indicate that imbalances in soil nutrient composition, fluctuations in pH, and microbial dysbiosis are likely key factors contributing to suboptimal quality and reduced yields in the casing soil cultivation of *P. indusiatus*. Therefore, maintaining a stable and optimized soil environment is essential for the sustainable and high-yield production of this mushroom species.

## 5. Conclusions

This study indicates that the cultivation of *P. indusiatus* is accompanied by stage-specific micro-ecological regulation of the soil. During cultivation, with organic materials such as decomposed bamboo chips, spent mushroom substrate (SMS), and crop residues serving as the baseline, the requirements for nutrients like nitrogen, phosphorus, and potassium in the soil appear to change dynamically based on different stages; meanwhile, it also implies a potential link to the dynamic shifts in soil pH. Furthermore, the potential value of functional microbial taxa, such as *Massilia*, *Acidobacteriaceae*, and *Terriglobales\_incertae\_sedis*, in promoting soil organic matter decomposition and improving soil structure is suggested, providing a preliminary theoretical basis for future choices of *Bacillus subtilis* to regulate the over-proliferation of *Candida*, as well as for suppressing contamination by weed molds like *Schizophyllum*. Therefore, by deeply analyzing the intrinsic successional mechanisms of scientific fertilization and microbial community regulation in preventing soil-borne diseases and maintaining soil health, this work can provide strong theoretical support for the sustainable development of the mushroom industry. Further experimental studies and productivity trials will be carried out to validate these prescriptive management recommendations and their direct impacts on yield.

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