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Effects of continuous cropping on growth and rhizosphere soil microbial community structure of Salvia miltiorrhiza Bge

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Article

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Abstract

Continuous cropping has restricted the development of high-quality and high-yield *Salvia miltiorrhiza*, which has become an urgent problem to be addressed. The evolution of microbial rhizosphere communities is closely related to plant growth, which may be a key factor that is aggravating obstacles to continuous cropping. Therefore, this study explored the effects of continuous cropping on the microbial rhizosphere community of *S. miltiorrhiza*. Methods: The effects of continuous cropping on the physiological and morphological indices of *S. miltiorrhiza* were evaluated by pot experiments. The V4 region of the rhizosphere bacterial 16S rDNA gene and ITS1 region of the fungal gene were sequenced by NovaSeq platform high-throughput sequencing technology to explore the effects of continuous cropping on the bacterial and fungal community structure in the rhizosphere of *S. miltiorrhiza*. Results: After continuous cropping, the biomass of *S. miltiorrhiza* decreased, the plant wilted and dwarfed, and the effective leaf area, main root length, and diameter significantly decreased. The accumulation of total chlorophyll, carbohydrates, and effective components was significantly reduced, and photosynthesis was reduced. The bacterial and fungal community composition and function in the rhizosphere soil altered significantly. The bacterial diversity in continuous cropping soil decreased, while the fungal community diversity increased, along with the emergence of pathogenic fungi such as *Fusarium solani*. Conclusion: Continuous cropping led to morphological changes, weak physiological activity, and reduced bioaccumulation of *S. miltiorrhiza*, and affected the composition of the microbial rhizosphere community. This may be a key factor that aggravates obstacles in continuous cropping.

1 Introduction

The dried roots and rhizomes of *Salvia miltiorrhiza* Bge. have various pharmacological effects, such as decreasing inflammation, reducing oxidative stress, promoting blood circulation, and removing blood stasis, and they are the first choice for the prevention and treatment of cardiovascular and cerebrovascular diseases [1]. Consequently, *S. miltiorrhiza* Bge. has broad prospects in the fields of drug production, food health care, skin care, and cosmetics. Owing to the constant increase in the demand for *S. miltiorrhiza*, continuous cropping of *S. miltiorrhiza* has been developed. However, continuous cropping can lead to various complications such as deterioration of soil texture, aggravation of pests and diseases, and increase in physiologically dead seedlings, emerging as one of the main factors limiting the increase in yield, quality, and market expansion of *S. miltiorrhiza* [2].

Assessment of the quality of medicinal plants includes an evaluation of the appearance quality and internal quality. Appearance quality is assessed by the morphology of roots and effective leaf area, while internal quality includes an assessment of the accumulation of carbohydrates and active ingredients. Green plants accomplish morphological construction via photosynthesis, and use organic matter such as carbohydrates as energy raw materials for growth and development. In addition, the synthesis and accumulation of active ingredients in medicinal plants are sometimes closely associated with photosynthetic products. However, most medicinal rhizomatous plants, such as *Pseudostellaria heterophylla* [3], *Lilium L* [4], *Atractylodes macrocephala* [5], and *Panax ginseng* [6], suffer from quality

deterioration and even crop failure. Similarly, the impact of continuous cropping on the *S. miltiorrhiza* industry is also directly reflected on changes in yield and quality. Therefore, a systematic study of continuous cropping using a series of indicators such as aboveground and underground morphology, biological accumulation, and growth physiology of *S. miltiorrhiza* plants is crucial to elucidate the underlying mechanism of continuous cropping and formulate corresponding countermeasures.

Soil microorganisms are widely involved in the transformation of plant nutrients and degradation of harmful substances, and are known as the second genome of plants, closely related to plant growth and metabolism. Plants, soil, and microorganisms perform functions such as material exchange and signal transmission in the rhizosphere environment, and their interaction network along with the environment can effectively resist various biotic and environmental stresses [7]. However, related studies have shown that continuous cropping can affect the evolution of microbial rhizosphere community structure and aggravate soil-borne diseases [8–10], making microorganisms a key factor that limits productivity in continuous cropping. With the extension of planting years, some plant pathogens get enriched in the rhizosphere of continuous cropping plants, while some functional beneficial bacteria such as *Pseudomonas* and *Bacillus* show negative feedback effects [11–13]. Increasing studies have indicated that continuous cropping obstacles are mainly caused by an imbalance in the microbial rhizosphere community structure, allelopathic autotoxicity, and deterioration of soil physical and chemical properties [14–17]. However, to date, knowledge about the effects of *S. miltiorrhiza* continuous cropping system on microbial rhizosphere community structure is limited.

With the development of molecular biology techniques, high-throughput nucleic acid sequence analysis has been widely used for the classification and identification of microorganisms. In particular, the high-throughput, high-depth, and high-precision NovaSeq sequencing platform provides the possibility to elucidate the interaction between soil microorganisms and continuous cropping systems [18]. Therefore, in this study, potted *S. miltiorrhiza* was used to explore the degree of damage of continuous cropping of *S. miltiorrhiza* on plant growth and physiological indices. The bacterial and fungal community structure and diversity in the rhizosphere soil of *S. miltiorrhiza* under continuous cropping were analyzed by using a new-generation high-throughput sequencing technology. The objective of this study was to explore the physiological mechanism of yield reduction and quality decline after continuous cropping of *S. miltiorrhiza* and its correlation with rhizosphere microflora changes. The obtained results provide an important theoretical and practical foundation for elucidating the mechanism of continuous cropping obstacles of medicinal plants and improving the cultivation soil for the production of Chinese medicinal materials.

2 Materials and methods

2.1 *S. miltiorrhiza* seedling and sampling

High-quality *Salvia miltiorrhiza* seeds were screened from a *S. miltiorrhiza* planting base at Ziguang Ecological Park in Miaoshan Town, Laiwu City, Shandong Province, China, and used in pot experiments. In brief, *S. miltiorrhiza* was cultivated in non-continuous cropping and continuous cropping soils (Table 1) in a light culture room at Shandong Provincial Analysis and Testing Center (Shandong, China). When the *S. miltiorrhiza* plants in the continuous cropping soil showed disease symptoms, the plants from each group were collected for morphological and physiological activity assays. Fresh soil samples of groups F (Noncontinuous cropping) and L (Continuous cropping) (5 replicates in each group) were obtained by a "shaking root method [31]," sieved using 40 mesh, stored in dry ice, and transported to Nuohe Science and Technology Co., Ltd., (Beijing, China) for high-throughput sequencing of 16S rDNA and fungal ITS amplicons based on the Illumina NovaSeq platform.

Table 1
Soil composition of non-continuous cropping and continuous cropping

Treatment	Sterilized soil	Planting soil	Continuous cropping soil
F group	50%	50%	0
L group	50%	0	50%

2.2 Determination of morphological indices

The *S. miltiorrhiza* plants in groups F and L were taken out from a ceramic flowerpots, and the soil was shaken off. After cleaning, the fresh weights (g) of the aboveground and underground plant parts were measured. Then, the samples were heated in an oven at 85°C for 20 min, cooled to 70°C, and dried to a constant weight. The dry weight data were recorded and the drying rate was calculated. The number of complete leaves, number of dry or diseased leaves, and total number of leaves in each group were recorded. The maximum leaf length and maximum leaf width of each leaf were measured, and the leaf length:leaf width ratio and leaf area (in cm^2) were calculated. The length and cross-sectional diameter of the main root of *S. miltiorrhiza* from the reed head to the root tip were determined, which were recorded as the longest root length and the main root diameter, respectively (in cm). Furthermore, the number of roots (strip) with a diameter > 0.2 cm was recorded.

2.3 Evaluation of physiological indices

2.3.1 Determination of chlorophyll and sugar contents

The fresh leaves of groups F and L were collected and their veins were removed, and cut into fragments of 1–2-mm width. Then, 1.0 g of the leaves fragments were immersed in 50 mL of 95% ethanol for extraction (dark) until the leaf tissue became completely white. The absorbance (A) of the extract was determined using UV-2700 ultraviolet spectrophotometer at the wavelengths of 665, 649, and 470 nm, and the contents of total chlorophyll, chlorophyll a (C-a), chlorophyll b (C-b), and carotenoids (C-carotenoid) were calculated as follows:

C-a = 13.95 × A665-6.88 × A649

C-b = 24.96 × A649-7.32 × A665

C-carotenoid = (1000 × A470-2.05 × C-a-114.8 × C-b) / 24

Chlorophyll content (mg/g) = (C chlorophyll × V extract × dilution) / sample fresh weight

To determine the soluble sugar and sucrose contents, 3.0 g of the leaves and roots of *S. miltiorrhiza* were extracted with ethanol (80%) in a water bath (80°C) for 30 min. After cooling to room temperature, the extracts were centrifuged (4000 rpm for 1.5 min) and the supernatants were collected. The soluble sugar content was measured using anthrone colorimetry, and the sucrose content was determined by resorcinol method. For the calculation of glucose and fructose contents, the leaves and roots of *S. miltiorrhiza* were dried and crushed, and 0.1 g was of the crushed samples was mixed with 5 mL of distilled water and ground in a motor. Then, the ground samples were centrifuged (3000 rpm for 1.5 min) and the supernatant was collected and subjected to anthrone colorimetry to ascertain the contents of glucose and fructose.

2.3.2 Determination of active ingredients content

The root tissue of *S. miltiorrhiza* from each group was crushed and passed through a 50-mesh sieve. Then, 0.5 g of the crushed sample was mixed with equal amount of methanol (70%) and stirred ultrasonically for 30 min, and the filtrate was passed through a 0.45-μm microporous membrane for subsequent high-performance liquid chromatography (HPLC). The HPLC analysis conditions were as follows: Compass chromatographic column C18 (4.6 mm × 250 mm, 5 μm); mobile phase: ultrapure water (with 0.2% acetic acid) as aqueous phase and acetonitrile as organic phase; gradient program: 0–25 min, 5–35% B; 25–30 min, 35–55% B; 30–40 min, 55–75% B; 40–50 min, 75–95% B; flow rate: 1.0 mL/min; column temperature: 25°C; and injection volume: 10 μL.

2.4 Rhizosphere soil microbial amplicon sequencing

2.4.1 DNA extraction and PCR amplification

Genomic DNA from the rhizosphere soil of each group was extracted by the Cetyltrimethylammonium Bromide (CTAB), and the purity and concentration of the DNA was assessed by agarose gel electrophoresis. Then, appropriate amount of DNA was diluted to 1 ng/μL using sterile water, and employed as a template for PCR amplification of the bacterial 16S V4 and fungal ITS1-5F regions to assess the microbial diversity in the rhizosphere soil. The primer sequences used for PCR are shown in Table 2. A TruSeq®DNA PCR-Free Sample Preparation Kit was employed to construct a DNA library. The constructed library was quantified using Qubit and Q-PCR, and NovaSeq 6000 was used for sequencing.

Table 2
PCR amplification region and primer sequence

Group	Amplification region	Primer sequence
Bacteria	16Sv4	F:GTGCCAGCMGCCGCGGTAA
		R:GGACTACHVGGGTWTCTAAT
Fungi	ITS1-5F	F:GGAAGTAAAAGTCGTAACAAGG
		R:GCTGCGTTCTTCATCGATGC

2.4.2 Sequence analysis

The offline data obtained from NovaSeq high-throughput sequencing were split into sample data, and the final effective data were obtained and comprehensively analyzed by splicing filtering and removal of chimeric sequences. Uparse v7.0.1001 software was employed to perform OTU cluster analysis on the effective sequences with 97% consistency, and species annotation was performed on the representative sequences. Chao1 index, Shannon index, Simpson index, and coverage were calculated using Qiime software (version 1.9.1), and the data were processed and plotted using Excel software.

3. Results

3.1 Effect of continuous cropping on the growth of *S. miltiorrhiza*

As shown in Table 3, the fresh and dry weights of the aboveground and underground plant parts of the continuous cropping group were reduced (aboveground parts: decreased by 27.1% and 30.2%, respectively; underground parts: decreased by 25.3% and 25.2%, respectively), when compared with those of the non-continuous cropping group. Furthermore, when compared with the non-continuous cropping group, continuous cropping group presented lower number of normal leaves and leaf area and higher rate of dead leaves, while the leaf length:leaf width ratio did not exhibit much difference. The underground part of the stubble of *S. miltiorrhiza* under non-continuous cropping was obviously more developed, characterized by thicker main roots, long roots, and multiple roots, when compared with that under continuous cropping. These results indicated that continuous cropping conditions were not conducive to the morphological growth and biomass accumulation of the aboveground and underground parts of *S. miltiorrhiza*, and significantly affected the overall yield of *S. miltiorrhiza*.

Table 3
Effect of continuous cropping on growth index of *Salvia miltiorrhiza* Note: Different lowercase letters in the same column indicate significant differences.

morphological index	F	L
Aboveground fresh weight (g / plant)	4.76 ± 1.17ab	3.47 ± 0.90b
Aboveground dry weight (g / plant)	0.86 ± 0.16bc	0.60 ± 0.07c
Fresh weight of underground part (g / plant)	4.78 ± 1.79ab	3.57 ± 1.25b
Dry weight of underground part (g / plant)	1.27 ± 0.48a	0.95 ± 0.50a
Drying rate %	26.75 ± 4.32a	24.75 ± 5.85a
Number of normal leaves (pieces)	31.0 ± 3.67bc	26.0 ± 7.87c
Number of dead leaves (pieces)	7.5 ± 3.20b	12.5 ± 4.15ab
Total number of leaves (pieces)	38.5 ± 1.66b	38.5 ± 4.50b
Leaf area (cm^2)	217.60 ± 37.94b	183.76 ± 47.86b
Leaf length / leaf width	1.33 ~ 1.93	1.22 ~ 1.77
The longest root length (cm)	16.00 ± 2.45a	14.75 ± 2.59b
Main root diameter (cm)	0.63 ± 0.13a	0.50 ± 0.07a
Number of root (article)	5.75 ± 0.43b	4.25 ± 1.09b

3.2 Differences in the physiological indices between continuous cropping and non-continuous cropping groups

3.2.1 Differences in the chlorophyll, soluble sugar, and sucrose contents

The contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in the continuous cropping group decreased by 42.4%, 41.5%, 42.1%, and 37.0%, when compared with those in the non-continuous cropping group, respectively (Fig. 1). Furthermore, the contents of soluble sugar, sucrose, glucose, and fructose in the leaves and roots of *S. miltiorrhiza* under continuous cropping were lower than those under non-continuous cropping, and the sugar components in the leaves of group L were reduced by 65.4%, 45.0%, 35.4%, and 44.6%, when compared with those in the leaves of group F, respectively (Table 4). Besides, the contents of soluble sugar, sucrose, and fructose in the roots of group L decreased by 59.9%, 26.3%, and 31.8%, when compared with those in the roots of group F, respectively.

Table 4
Table of content of sugar components in *Salvia miltiorrhiza* Note: different lowercase letters in the same column indicate significant differences; * P < 0.05.

group	soluble sugar(mg/g)	sucrose(mg/g)	glucose(mg/g)	fructose(mg/g)
F- leaf	1.641 ± 0.09ab	0.768 ± 0.06ab	0.754 ± 0.11	0.623 ± 0.25ab
L- leaf	0.567 ± 0.04c	0.318 ± 0.04c	0.487 ± 0.15*	0.345 ± 0.08b
F- root	2.463 ± 0.16a	0.994 ± 0.15a	——	0.825 ± 0.22a
L- root	0.987 ± 0.15bc	0.733 ± 0.17b	——	0.458 ± 0.11ab

3.2.2 Difference in the active ingredients content

It can be seen from Table 5 that the effect of continuous cropping on the active ingredients content of *S. miltiorrhiza* was specific. When compared with non-continuous cropping, continuous cropping increased the content of salvianolic acid B by 28.8% and decreased the content of rosmarinic acid by 51.4%. The contents of tanshinone I, tanshinone IIA, and dihydrotanshinone I decreased by 55.4%, 4.4%, and 100%, respectively, while that of cryptotanshinone increased by 50.0% after continuous cropping, when compared with those following non-continuous cropping. In particular, the content of dihydrotanshinone I was difficult to detect in the roots of *S. miltiorrhiza* after continuous cropping. These results revealed that the content of active ingredients in *S. miltiorrhiza* significantly decreased after continuous cropping, reducing the overall quality of *S. miltiorrhiza*.

Table 5
The content of effective components in *Salvia miltiorrhiza* (mg / g) Note: The content of dihydrotanshinone in group L was extremely low and did not reach the lowest value of detection.* is significant at 0.05 level, * * is 0.01 level, * * * is 0.001 level.

active principle	F	L
Tanshinone Ⅱ	0.148 ± 0.002	0.066 ± 0.0001***
Tanshinone $Ⅱ_A$	1.137 ± 0.005	1.087 ± 0.005**
Cryptotanshinone	0.008 ± 0.0004	0.012 ± 0.0002**
Dihydrotanshinone Ⅰ	0.075 ± 0.002	0***
Salvianolic acid B	12.5 ± 0.082	16.1 ± 0.170***
Rosmarinic acid	5.383 ± 0.045	2.617 ± 0.012***

3.3 Analysis of amplicon sequencing results

3.3.1 Sequencing data statistics and OTU analysis

The original high-throughput sequences of rhizosphere soil genomic DNA of groups F and L were spliced to obtain spliced sequences, and the effective sequences were obtained after filtering the chimeras (Table 6). After continuous cropping of *S. miltiorrhiza*, the number of raw tags and effective tags decreased, while those of bacterial and fungal OTUs increased by 3.45% and 3.78%, respectively. Subsequently, cluster analysis was performed on the representative OTU sequences obtained from groups F and L, and the sequences were subjected to species annotation at six different classification levels of phylum, class, order, family, genus, and species. As shown in Table 7, following continuous cropping, the OTU number of the soil bacterial community decreased by 0.3%, 1.67%, and 2.48% at the order, family, and genus levels, respectively, while that of the fungal community increased by 15.38%, 5.45%, and 1.67% at the phylum, order, and family levels, respectively. In addition, the fungi:bacteria ratio in continuous cropping soil showed an increasing trend, when compared with that in non-continuous cropping soil.

Table 6
Sequencing data statistics of bacteria and fungi in rhizosphere soil of continuous and non-continuous cropping of *Salvia miltiorrhiza* Note: Original tags = tag sequences obtained by splicing; effective label = the label sequence finally used for subsequent analysis after filtering the chimera; base = the base of the final valid data; effectiveness (%) = the percentage of the number of valid labels to the number of original PEs; oTUs = the number of operational taxonomic units.

index	Bacteria		Fungi	
	F group	L group	F group	L group
Raw Tags	83256	82089	75428	74409
Effective Tags	77993	76756	74600	73511
Base(nt)	19741183	19424898	17312674	16595521
Effective (%)	92.52	92.32	89.00	86.85
OTUs	3942	4078	980	1017

Table 7
OTU statistics of bacterial and fungal communities in rhizosphere soil of continuous and non-continuous cropping of *Salvia miltiorrhiza*

Microbial type	Treatment	Phylum	Class	Order	Family	Genus	Species
Bacteria	F group	66	150	307	420	644	288
	L group	70	154	306	413	628	278
Fungi	F group	13	48	110	235	381	524
	L group	15	50	116	242	385	528

3.3.2 Differences in the influences of α-diversity index

The PD-whole-tree of the soil bacterial and fungal communities in groups F and L varied by 5.89% and 4.32%, respectively, indicating that the diversity of rhizosphere microbial communities significantly changed after continuous cropping (Table 8). After continuous cropping, the Shannon index decreased by 9%, whereas the Chao1 index increased by 1.80%, implying that the rhizosphere soil bacterial diversity decreased after continuous cropping. In contrast, the Shannon and Simpson indices of the rhizosphere soil fungal community increased by 8.6% and 3.6%, respectively, after continuous cropping, when compared with those noted after non-continuous cropping, suggesting that the diversity of the soil fungal community increased and the species distribution was more uniform after continuous cropping.

Table 8
Effects of continuous cropping on the alfa diversity index of bacteria and fungi in rhizosphere soil of *Salvia miltiorrhiza*

Microbial type	Treatment	Shannon	Simpson	Chao1	Goods-coverage	PD-whole-tree
Bacteria	F group	9.94	0.997	4298.82	0.9904	241.0228
	L group	9.85	0.993	4376.41	0.9912	255.2186
Fungi	F group	5.12	0.896	1237.13	0.9936	507.4566
	L group	5.56	0.932	1254.23	0.9940	485.54

Note: Shannon: The total number of categories in the sample and their proportions. The higher the community diversity, the more uniform the species distribution, and the larger the Shannon index. Chao1: Estimation of the total number of species in community samples. Coverage of goods: Coverage. The higher the coverage of the sort, the larger the index. PD whole tree: the genetic relationship of species in the community. Simpson: Diversity and uniformity of species distribution in a community. The analysis used the Simpson diversity index (1-D). The better the species evenness, the greater the Simpson index.

3.3.3 Differences in the effects of β-diversity index

The bacterial composition significantly varied between continuous and non-continuous cropping soils. Principal coordinate analysis showed the differences in the soil bacterial communities between groups F and L (Fig. 2A). The first and second principal component axes (contribution: 22.67% and 12.71%, respectively) distinguished the soil bacterial communities between continuous cropping and non-continuous cropping groups. In addition, the first and second principal component axes (contribution: 44.01% and 28.54%, respectively) also exhibited differences in the soil fungal community composition between groups F and L (Fig. 2B).

3.3.4 Analysis of the rhizosphere soil bacterial community structure after continuous cropping

The soil bacterial community OTUs derived from the non-continuous cropping and continuous cropping groups were analyzed (Fig. 3A). The Venn diagram showed that the number of OTUs shared by the two

groups was 5747. The number of unique OTUs in the non-continuous cropping and continuous cropping groups were 1099 and 1297, respectively. With the increase in the duration of continuous cropping, some specific bacterial strains disappeared and some new strains were enriched, indicating change in the structure and composition of the soil bacterial community. There was no significant difference in the relative abundance of each dominant phylum, class, and order of the soil bacterial community in groups F and L. The dominant phyla in groups F and L were Firmicutes (17.2% and 18.1%, respectively), Proteobacteria (24.1% and 24.3%, respectively), Acinetobacter (7.2% and 7.5%, respectively), Actinobacteria (4.8% and 4.4%, respectively), and Bacteroidetes (4.9% and 4.8%, respectively) (Fig. 3B). The dominant classes in groups F and L were Clostridium (10.2% and 8.0%, respectively), Bacillus (6.3% and 9.2%, respectively), γ-Proteobacteria (13.8% and 14.2%, respectively), α-Proteobacteria (10.3% and 10.1%, respectively), and Bacteroidia (4.8% and 4.8%, respectively) (Fig. 4A). The dominant orders in groups F and L were Leptospirales (5.3% and 3.3%, respectively), Oscillospirales (3.1% and 1.8%, respectively), Lactobacillus (2.2% and 2.3%, respectively), and Rhizopus (4.2% and 4.2%, respectively) (Fig. 4B).

Simper (Similarity percentage) was used to break down the Bray-Curtis difference index to quantify the contribution of each species to the difference between the continuous cropping and non-continuous cropping groups. At the family level (Fig. 5A), the relative abundance of Lachnospiracea and Nitrosopharacea, both of which exhibit soil nitrogen cycle function, decreased by 38.32% and 22.75%, respectively, in the rhizosphere soil of the continuous cropping group. Furthermore, the relative abundance of Erysipelotrichaceae and Ruminococcaceae decreased by 42.49% and 40.82%, respectively, while those of Staphylococcaceae, Peptostreptococcaceae, Lactobacillaceae, and Acidithiobacillaceae decreased at varying degrees in the rhizosphere soil of the continuous cropping group. At the genus level (Fig. 5B), the relative abundance of the dominant genera of the rhizosphere soil bacterial community in groups F and L showed significant variation after continuous cropping. In particular, the relative abundances of *Staphylococcus* (0.3% and 2.2%, respectively), *Romboutsia* (0.6% and 2.1%, respectively), *Acidithiobacillus* (0.006% and 1.1%, respectively), *Leptospirillum* (0.0006% and 1.0%, respectively), *Allobaculum* (0.9% and 0.5%, respectively), *Blautia* (1.3% and 0.8%, respectively), *Lactobacillus* (0.6% and 0.8%, respectively), *Pseudomonas* (1.4% and 1.0%, respectively), *Ruminococcus* (0.9% and 0.4%, respectively), and *Bacillus* (0.8% and 1.6%, respectively) significantly differed between groups F and L.

3.3.5 Analysis of the rhizosphere soil fungal community structure after continuous cropping

The soil fungal community OTUs (Fig. 6A) derived from the non-continuous cropping and continuous cropping groups were analyzed. The Venn diagram showed that the number of OTUs shared between groups F and L was 1356. The number of unique OTUs in the non-continuous cropping and continuous cropping groups were 968 and 1007, respectively. Furthermore, significant differences in the soil fungal community at the phylum level were observed between the groups F and L (Fig. 6B), and the relative abundances of the top 10 dominant phyla, including Mortiellomycota, Chytridillomycota, Zoopagomycota, Aphelidiomycota, Mucoromycota, Glomeromycota, and Blastocladiomycota, increased

by 127.5%, 325.0%, 32.4%, 253.1%, 265.7%, 14.0%, and 1230.8%, respectively, while those of Ascomycota, Basidiomycota, and Rozellomycota decreased by 19.42%, 90.6%, and 30.9%, respectively, after continuous cropping.

As shown in Fig. 7A, the dominant fungal classes in groups F and L were Sordariomycetes (37.2% and 38.7%, respectively), Mortierellomycetes (9.1% and 20.7%, respectively), Agaricomycetes (5.8% and 4.4%, respectively), Orbiliomycetes (10.4% and 6.1%, respectively), Dothideomycetes (10.2% and 4.0%, respectively), Eurotiomycetes (7.4% and 4.9%, respectively), Leotiomycetes (2.3% and 1.2%, respectively), and Tremellomycetes (1.7% and 2.6%, respectively). Furthermore, the dominant fungal orders in groups F and L were Pyrrophyta (23.7% and 16.6%, respectively), Mortierela (9.1% and 20.7%, respectively), Thesephorales (4.6% and 0.08%, respectively), Orbiliales (10.4% and 6.1%, respectively), Microscales (3.0% and 9.2%, respectively), Capnodiales (8.0% and 1.1%, respectively), Eurotiales (8.0% and 1.1%, respectively), Pleospores (7.0% and 4.6%, respectively), and Sordariales (2.0% and 2.7%, respectively) (Fig. 7B). The cluster heatmap of species abundance (Fig. 7C) revealed significant differences in species richness and composition at the family level between groups F and L. The proportion of *Coniochaetacaeae* and *Coniochaetacaeae* in the rhizosphere fungal community of continuous cropping *S. miltiorrhiza* increased, while the proportion of *Coniochaetacaeae* and *Coniochaetacaeae* decreased.

With the increase in the duration of continuous cropping, the structure and composition of the soil fungal community changed, and some common soil-borne pathogenic fungi, such as *Leptosphaeria turcica*, some *Cladosporium* spp., some subspecies of *Alternaria*, *Fusarium solani*, and other subspecies of *Fusarium*, were enriched. Based on LEfSe (LDA Effect Size) analysis, the relationship between continuous cropping and non-continuous cropping soils was further explored. The results revealed that the abundance of the microbial species in the *S. miltiorrhiza* soil was significantly different before and after continuous cropping. A total of 30 biomarkers with LDA score > 4 were enriched in the two groups (Fig. 8A), with the abundance of *Mortierella*, *Lophotrichus*, *Conocybe*, *Aspergillus*, *Arthrobotrys*, and *Cladosporium* being statistically different in the two groups (Fig. 8B). *Mortierella polycephala*, *Lophotrichus* sp., *Mortierella stylospora*, and *Conocybe pubescens* were the biomarkers in the continuous cropping soil, while *Mortierella alpina*, *Arthrobotrys amerospora*, and *Cladosporium* sp. were the biomarkers in the non-continuous cropping soil.

3.3.6 Function prediction analysis

Based on the FunGuild tool, the corresponding categories of microbial ecological functions were annotated, and the statistical results are shown in Fig. 9A and B. In the soil bacterial community, plant and animal pathological nutrition functions were relatively predominant. The abundance of saprophytic bacteria was relatively low and presented only slight variation between groups F and L. These results suggested that the function of bacteria in the rhizosphere soil of *S. miltiorrhiza* before and after continuous cropping was similar. The abundance of internal parasitic nutritional fungi in the soil fungal community decreased, while that of saprophytic nutritional fungi significantly increased after continuous cropping. Subsequently, principal component analysis was performed on the abundance statistics results based on the database functional annotations (Fig. 9C). The soil fungal functional abundance

showed good separation. The first and second principal component axes (contribution: 24.3% and 18.76%, respectively) distinguished the soil fungal composition between groups F and L. In contrast, the soil bacterial functional abundance between the two groups did not exhibit good separation (Fig. 9D). Therefore, it can be concluded that continuous cropping changed the function of the rhizosphere soil fungi, and that long-term continuous cropping caused the enrichment of some pathogenic fungi that gradually became the dominant groups.

3.3.7 Correlation analysis between soil microbial species abundance and *S. miltiorrhiza* growth index

The *S. miltiorrhiza* root morphology and root bioaccumulation were significantly positively correlated with the relative abundances of *Lecythophora*, *Aspergillus*, *Alternaria*, *Eurotium*, and other fungi, and the bacterium *Pseudomonas*, and were negatively correlated with *Rhizophlycti* and *Papulaspora*. The contents of chlorophyll a, chlorophyll b, and carotenoids in *S. miltiorrhiza* were significantly positively correlated with the relative abundances of *Eurotium*, *Macroventuria*, *Gibberella*, *Geomyces*, *Aspergillus*, *Cladosporium*, *Arthrobotrys*, *Solirubrobacter*, *Clostridium innocuum*, *Lysobacter*, and *Gaiella*, but significantly negatively correlated with the relative abundances of fungi such as *Papulaspora*, *Fusicolla*, *Chaetomium*, *Solicoccozyma*, and *Mortierella*, and some bacteria such as *Ohtaekwangia*, *Streptomyces*, *Bacillus*, and *Leptospirillum* (Fig. 10A and 10B). In general, the microbial species abundance and community structure were significantly correlated with the root morphological accumulation and photosynthetic intensity of *S. miltiorrhiza*.

4.Discussion

Continuous cropping can significantly influence the morphological and physiological indices of *S. miltiorrhiza*. The development degree of roots and leaves, carbohydrate content, and active ingredients content are important indices to detect the growth and development ability of plants. The number of leaves and leaf area are related to the photosynthesis of plants and the yield of photosynthetic crops. The morphology and vitality of roots directly affect the absorption of nutrients by host plants and transport efficiency of photosynthetic products, thus affecting the growth and development of *S. miltiorrhiza*. Accordingly, in the present study, pot experiment with *S. miltiorrhiza* was performed, and the results showed that the biomass of *S. miltiorrhiza* decreased, aboveground leaves dried up, effective leaf area decreased, and underground main roots became shorter with lower diameter after continuous cropping. The effects of continuous cropping on the physiological indices of *S. miltiorrhiza* included decrease in the total chlorophyll content, significant decrease in the accumulation of soluble sugar, sucrose, glucose, and fructose, increase in the content of salvianolic acid B in the water-soluble components of *S. miltiorrhiza*, and significant decrease in the content of rosmarinic acid. Furthermore, except cryptotanshinone, the contents of tanshinone I, tanshinone IIA, and dihydrotanshinone I decreased after continuous cropping. Furthermore, the pot experiment demonstrated that the rhizosphere soil bacterial and fungal community compositions altered after continuous cropping. The diversity of the rhizosphere soil bacterial community decreased, while that of the rhizosphere soil fungal

community increased following continuous cropping. Besides, continuous cropping led to a downward trend in the soil bacteria:fungi ratio, enrichment of strong pathogenic fungi such as *Alternaria* subspecies and *F. solani*, and an obvious transformation of the soil into “fungal type.” The results of Spearman analysis showed a significant correlation between the rhizosphere microbial population structure and root development degree and photosynthesis intensity of *S. miltiorrhiza* under continuous cropping, implying that the change in the microbial population could significantly alter the nutrients transport, growth, and development of *S. miltiorrhiza*.

After continuous cropping of *S. miltiorrhiza*, the relative abundance of Actinobacteria and Bacteroidetes decreased to varying degrees, while that of Firmicutes and other oligotrophic bacteria increased, indicating that the continuous cropping system altered the rhizosphere soil environment, causing some soil microorganisms to modify their survival strategies to adapt to this change. Previous studies have shown that *Mortierella* can infect plants and cause large-scale crop infections. In the present study, at the family level, the abundance of Nitrosophaeraceae involved in soil nitrogen cycle decreased, while that of pathogenic Mortierellaceae and Cystaceae increased. These results further confirmed that the abundance and structure of soil bacterial community were affected by the continuous cropping system. The change in the soil bacterial community diversity after continuous cropping could be attributed to the decrease in the abundance of environment-friendly bacteria and increase in the abundance of pathogenic bacteria, leading to the dysfunction of rhizosphere soil microflora and more serious problem of continuous cropping obstacle. The results of the present study on the growth and physiology of *S. miltiorrhiza* before and after continuous cropping also confirmed this viewpoint. Furthermore, the changes in the soil fungal community composition and structure before and after continuous cropping were analyzed by ITS sequencing, and the findings showed that the continuous cropping system had significant influence on the relative abundance of the rhizosphere soil fungi at the phylum, class, order, and genus levels. The diversity index analysis showed that the soil fungal community structure significantly changed and the fungal richness and diversity significantly increased after continuous cropping. The results of cluster analysis at the genus level revealed that the abundances of *Alternaria* (an important plant pathogenic fungus) and *Fusarium* significantly increased, and pathogenic fungi such as *F. solani* were enriched in the rhizosphere soil of *S. miltiorrhiza* under continuous cropping, which may be related to the aggravation of disease after continuous cropping of *S. miltiorrhiza*. The increase in the abundance of pathogenic fungi to gradually become the dominant group can destroy the balance of the rhizosphere soil microecology and cause deterioration of its growth environment.

Rhizosphere is a micro-soil area close to plant roots. As an organic combination of plant-soil-microorganisms, the micro-ecosystem formed by rhizosphere is an important part of plant-environment interaction and function [19], which is closely related to the occurrence of continuous cropping obstacles. It is generally believed that the changes in soil nutrients, root allelochemicals, and microbial community evolution in the rhizosphere soil micro-ecological system are the main causes of continuous cropping obstacles [20]. In particular, the richness and diversity of soil microorganisms can comprehensively reflect the overall changes in the soil microflora and indicate the health status of soil, especially the

bacteria:fungi ratio, which is the most important feature of soil function and can be used as a key indicator to assess the process of rhizosphere micro-ecosystem [21]. Many studies have reported that the soil bacteria:fungi ratio is significantly affected by the cropping system [22, 23]. For instance, Wu [24] found that the rhizosphere microflora of *Pseudostellaria heterophylla* was prone to imbalance and structural disorder under continuous monoculture conditions, which may be caused by the intervention of phenolic acids. Similarly, Yu [25] also demonstrated that the abundance of harmful microorganisms in the rhizosphere soil increased after continuous cropping of *Asarum*, disturbing the balance between beneficial and harmful bacteria. In general, the microbial community stability and resistance to environmental interference enhance with the increasing richness of the soil microbial diversity. Therefore, the decrease in rhizosphere bacterial richness and diversity and the increase in harmful fungal abundance may be one of the factors responsible for the poor growth of *S. miltiorrhiza*, and the results of the present study also confirmed this notion.

At present, studies on continuous cropping obstacles of medicinal plants mainly focus on the deterioration of soil environment, allelopathic autotoxicity, rhizosphere microecological imbalance, and frequent soil-borne diseases. The dynamic changes and functional analysis of rhizosphere microbial communities of medicinal plants have become crucial for understanding the phenomenon of continuous cropping obstacles of medicinal plants. On the one hand, functional microorganisms and pathogenic fungi aggressively compete for rhizosphere ecological sites under the mediation of root exudates, and their abundance ratio can negatively affect the growth and metabolism of medicinal plants after breaking a balance point [26]. On the other hand, long-term single cultivation of medicinal plants can cause continuous accumulation of the acid root exudates. Chemokines such as phenolic acids and coumarins can attract pathogenic fungi to the rhizosphere. It has been confirmed that components such as coumaric acid, benzoic acid, and saponins secreted by the roots of *Panax notoginseng* and *Panax ginseng* exhibit strong chemotaxis to pathogens, resulting in an increase in pathogen abundance in the rhizosphere soil with the increase in the duration of continuous cropping [27–28]. Moreover, the acidified soil environment is more conducive to the colonization and development of the soil fungal community. Consequently, continuous cropping soil gradually transforms from the bacterial type to fungal type, and this constant imbalance of the microflora negatively affects the host plant, causing abnormal growth and development [29–30]. However, current research, including the present study, on the challenges related to continuous cropping of medicinal plants has mainly focused on the unilateral changes in plants and rhizosphere microenvironment after continuous cropping, ignoring the interaction among plants, root exudates, and rhizosphere microorganisms and the subsequent dynamic correlation. Hence, future studies should explore the material transformation between plant and soil rhizosphere microorganisms and clarify the factors driving the formation of rhizosphere soil microbial communities.

5. Conclusion

In summary, the continuous cropping system significantly affected the appearance, physiological activity, and biological accumulation of *S. miltiorrhiza*, causing changes in the bacterial and fungal

community structure and metabolic function in the rhizosphere soil. When compared with non-continuous cropping soil, the bacterial diversity decreased and the fungal diversity increased in continuous cropping soil. At the same time, the composition of bacterial and fungal communities in the rhizosphere soil under continuous cropping of *S. miltiorrhiza* also changed, and pathogenic fungi such as *F. solani* were enriched. These changes might lead to further deterioration of the quality of *S. miltiorrhiza*. In summary, the present study systematically elucidated the effects of continuous cropping system on the growth and development of *S. miltiorrhiza* and rhizosphere microflora, and discussed the mechanism of continuous cropping obstacles and quality decline of Chinese medicinal materials based on rhizosphere microorganisms. The results obtained are crucial for regulating rhizosphere soil microecology and improving the cultivation of Chinese medicinal materials.

Declarations

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contribution

J.J. and W.L. designed the experiments. J.J.□B.Z.□Y.M. and X.F. carried out sample collection, data analysis, and wrote the manuscript. X.W. and L.G. analyzed data. J.J., B.Z., G.Y. and W.L .assisted the experiments. All authors approved the final manuscript.

Data Availability

Sequence data that support the findings of this study have been deposited in the NCBI ShortRead Archive database (accession number: PRJNA1129673, PRJNA1129675).

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Figures

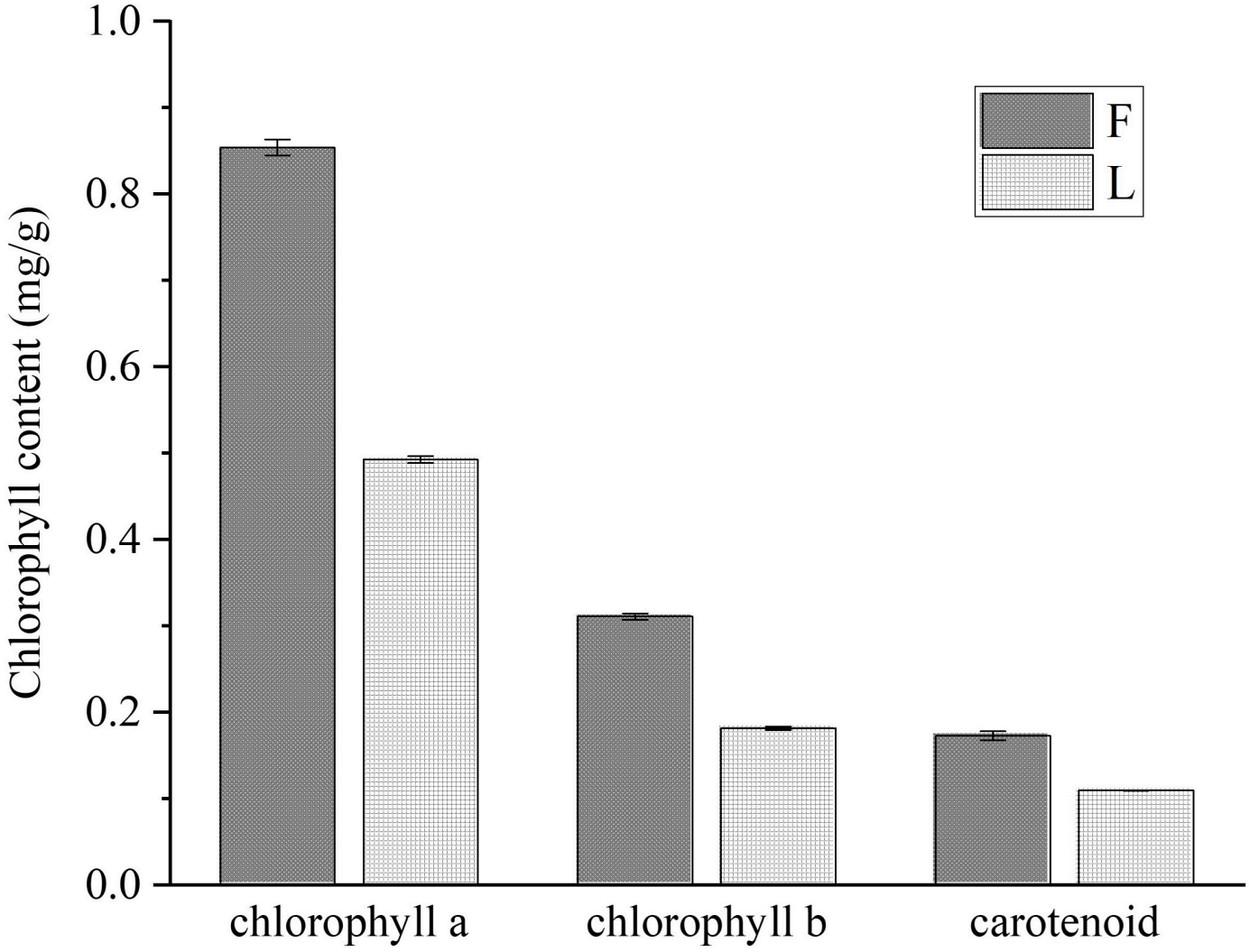


Figure 1

Difference of chlorophyll content between continuous cropping and non-continuous cropping *Salvia miltiorrhiza*

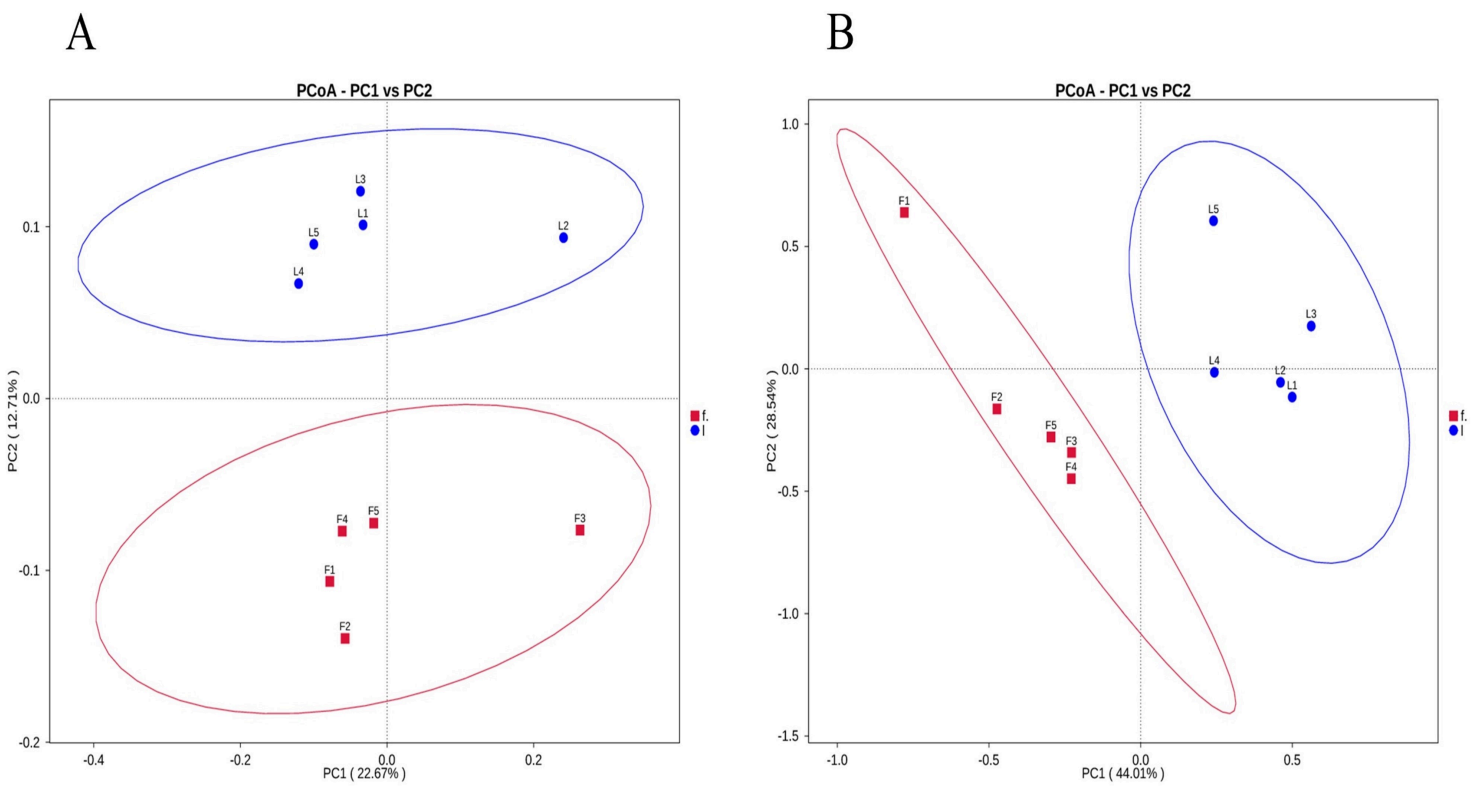


Figure 2

Differences in microbial community structure based on principal coordinate analysis (PCoA). A: Bacteria, B: Fungi

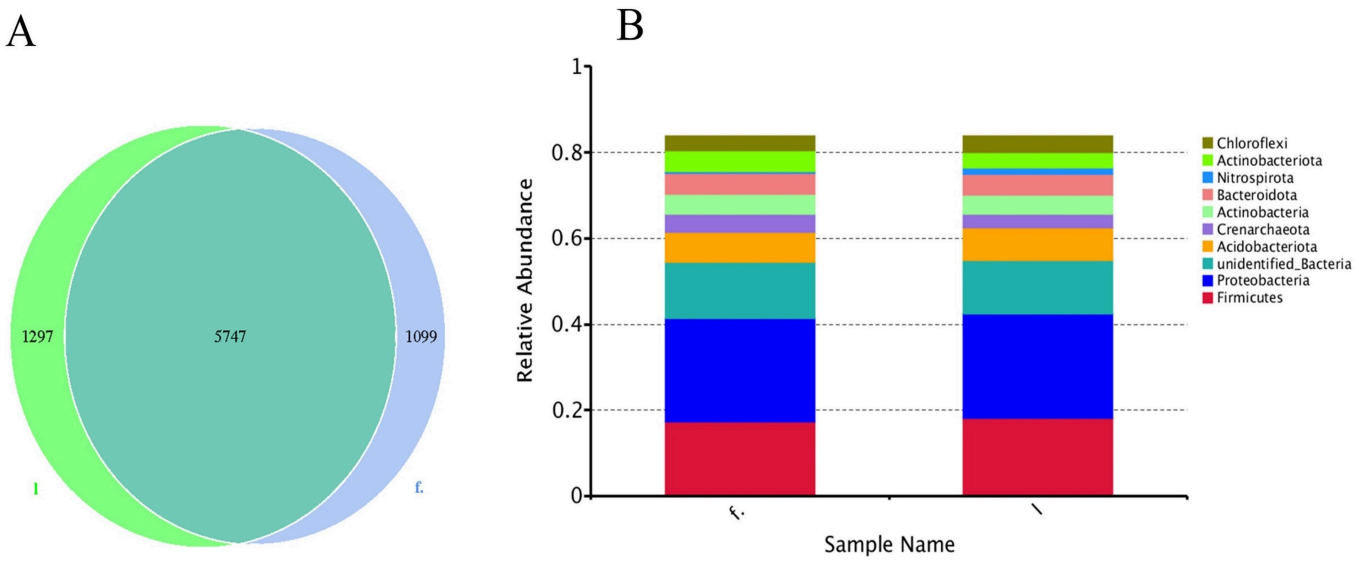


Figure 3

Structure and distribution of bacterial community in the rhizosphere of continuous cropping Salvia miltiorrhiza A: OUT Venn diagram, B: relative abundance of bacteria at the phylum level.

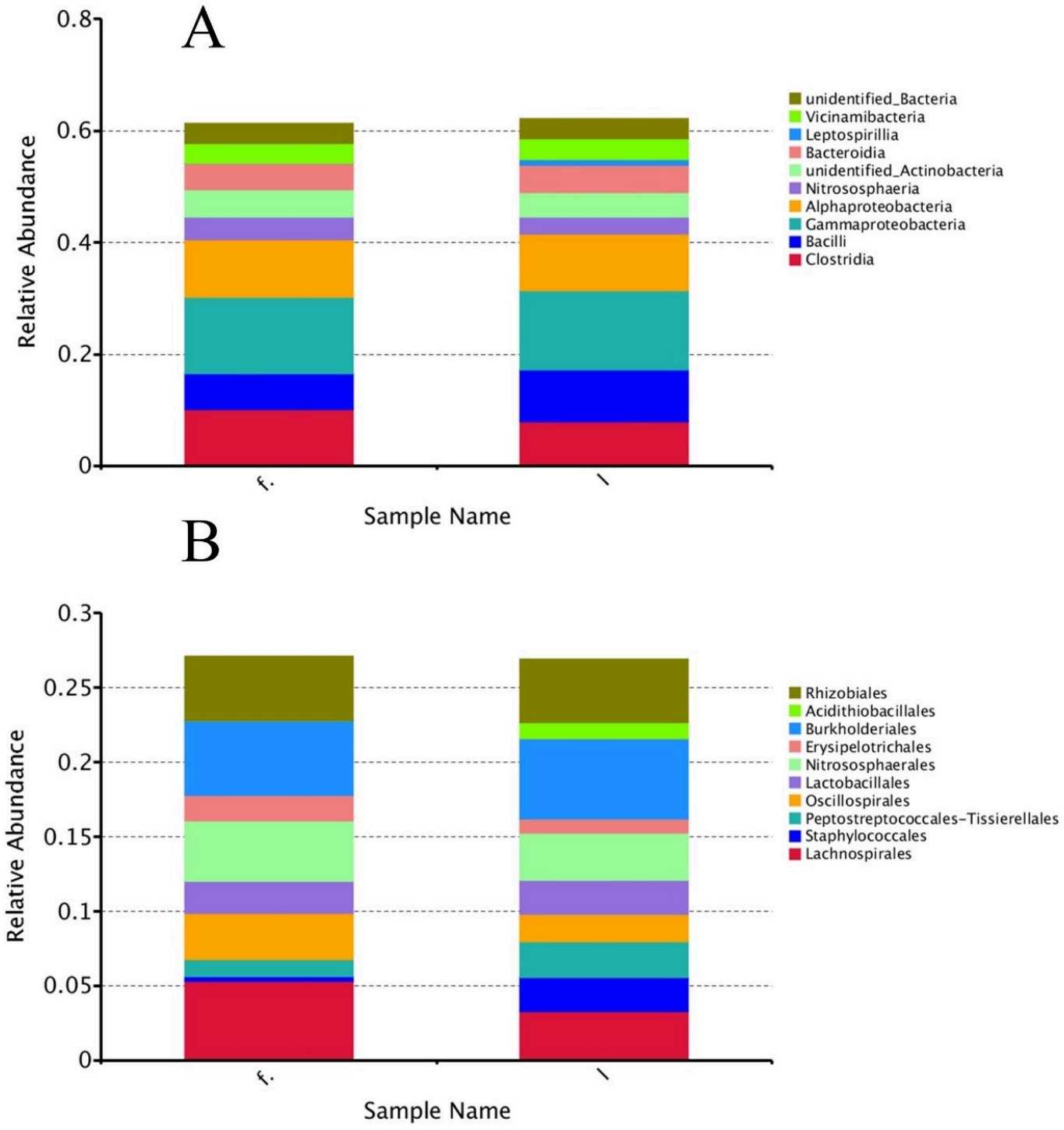


Figure 4

The relative abundance of soil bacteria at the class and order level in the continuous cropping and non-continuous cropping of Salvia miltiorrhizaA: class level, B: order level

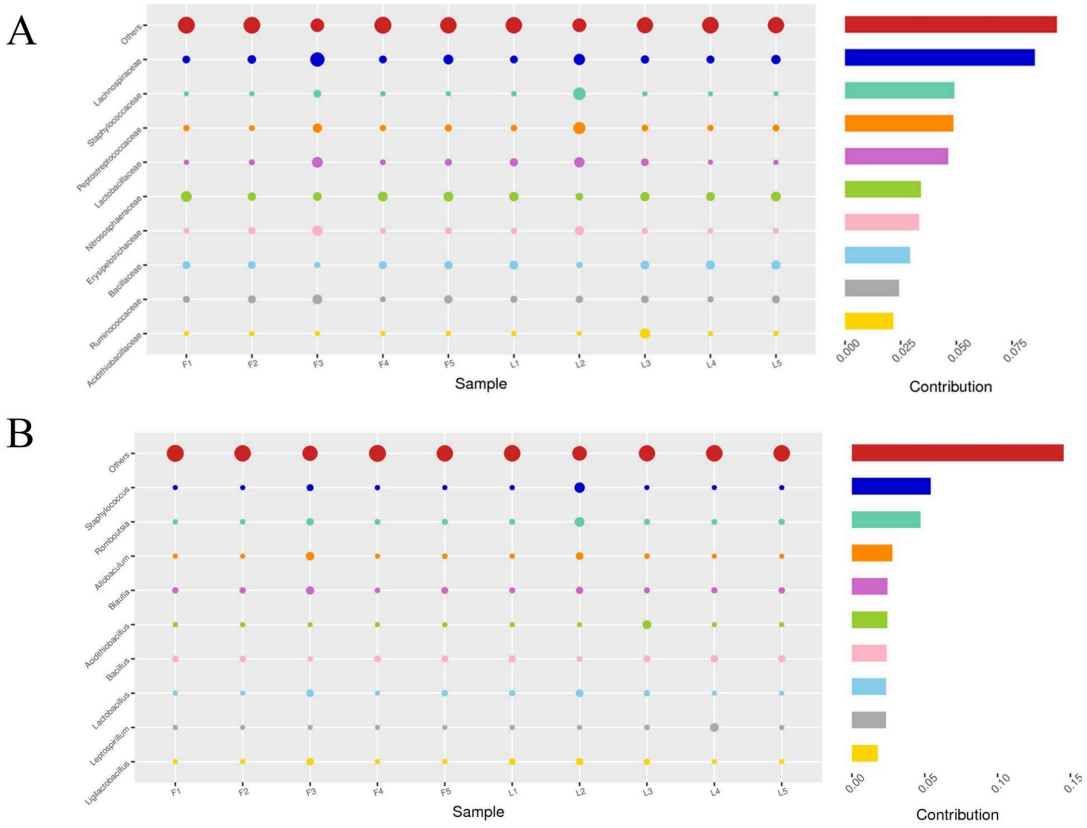


Figure 5

Contribution of bacterial community differences based on Simper analysis A: family level, B: genus level

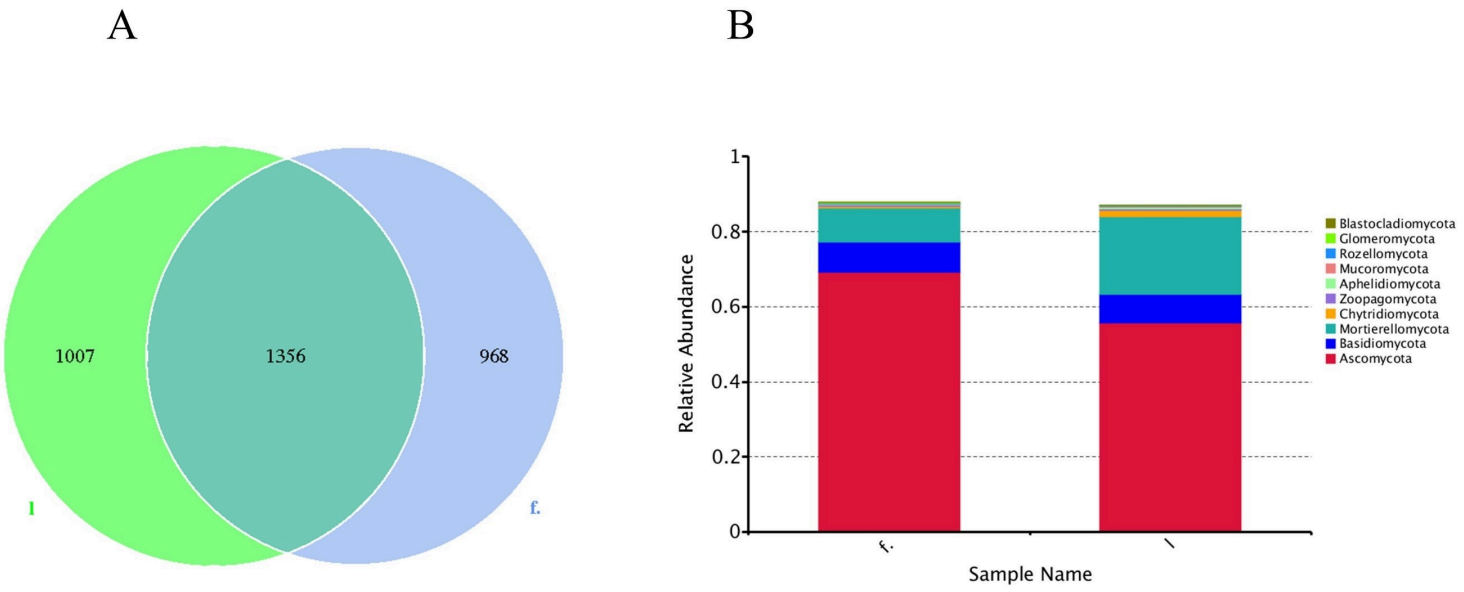


Figure 6

Structure and distribution of rhizosphere fungal community in continuous cropping *Salvia miltiorrhiza* A: OUT Wayne diagram, B: the relative abundance of fungi at the phylum classification level

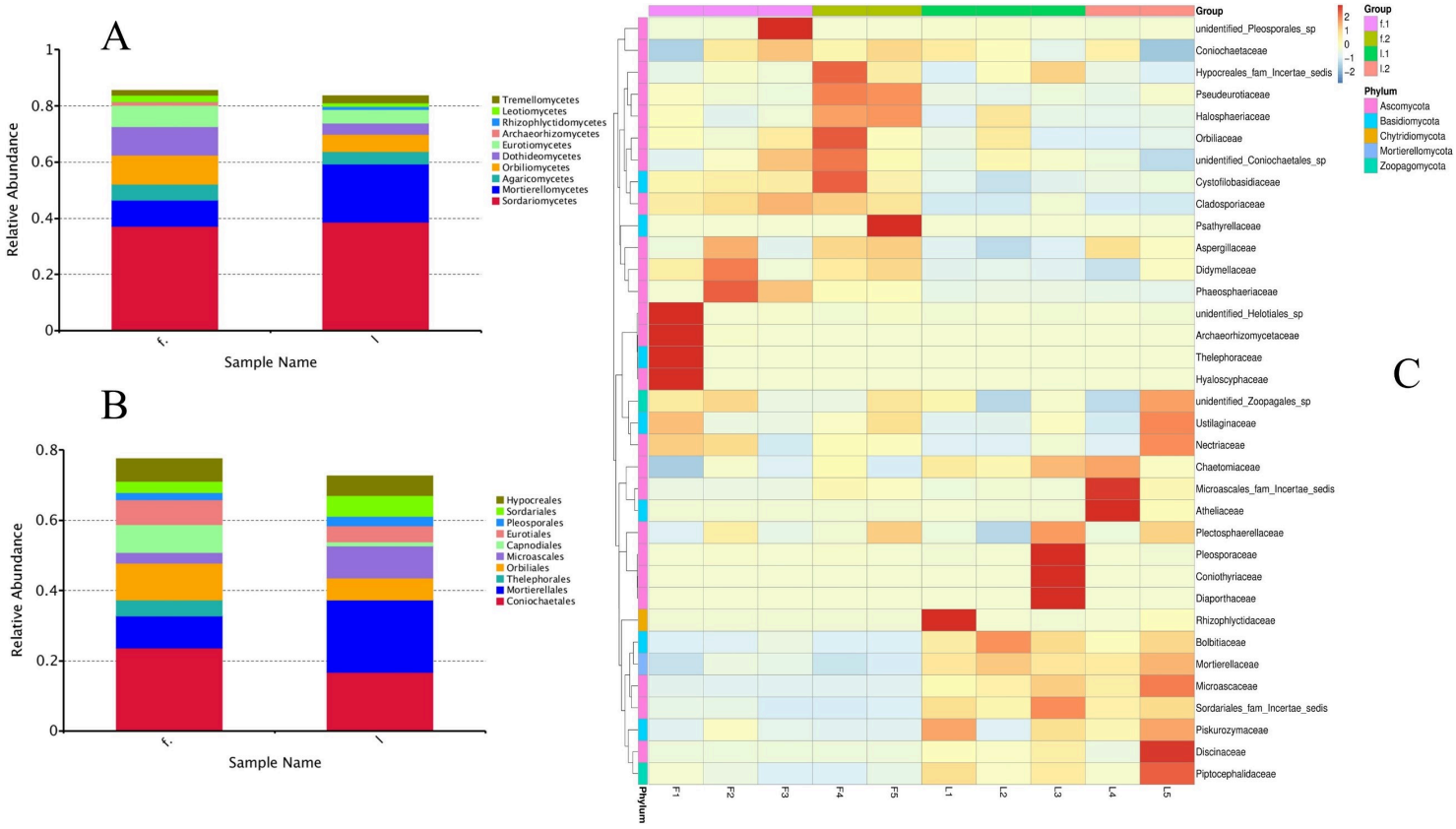


Figure 7

The relative abundance of soil fungi at class and order classification level in continuous cropping and non-continuous cropping *Salvia miltiorrhiza* at class and order classification level A: class level, B: order level, C: family level of species abundance clustering heat map

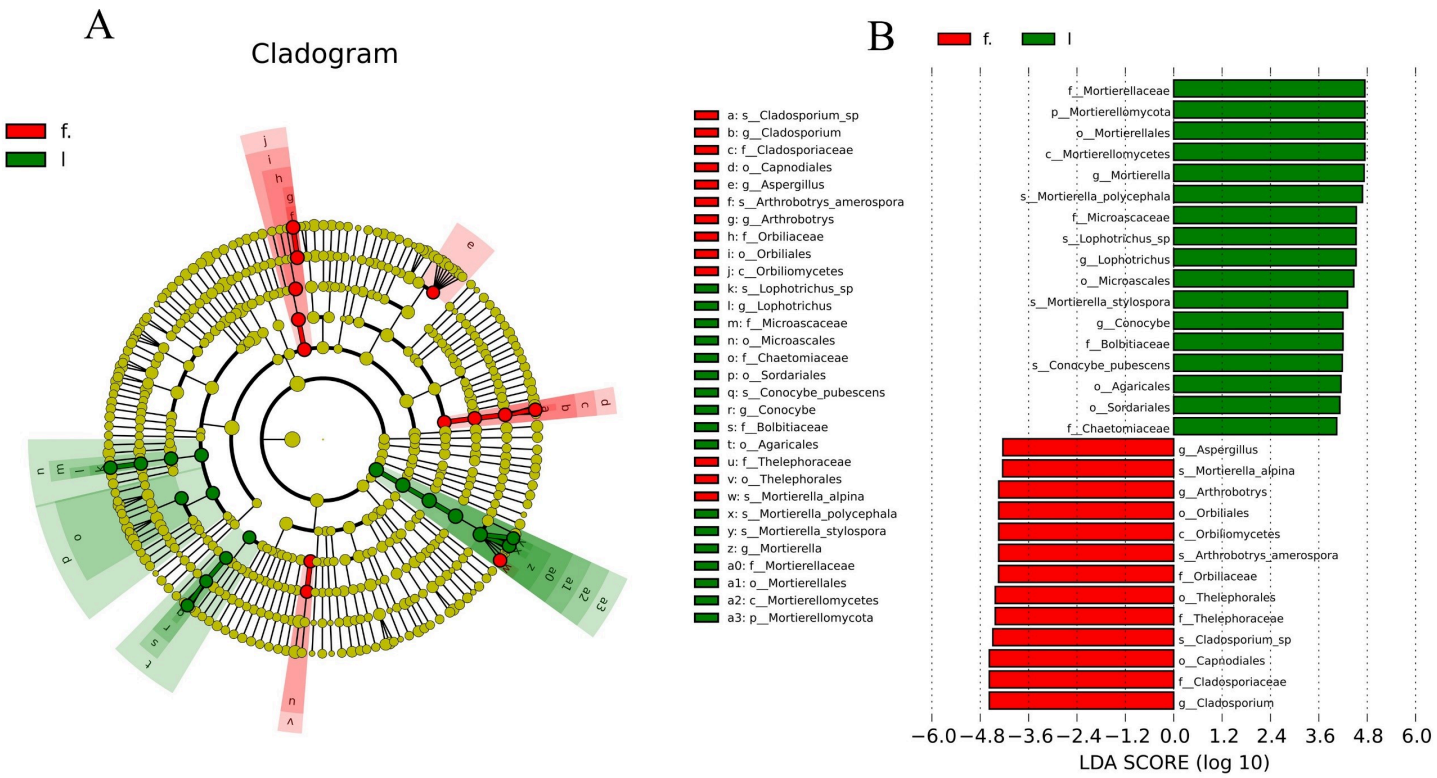


Figure 8

LEfSe analysis of rhizosphere soil microorganisms in continuous cropping and non-continuous cropping *Salvia miltiorrhiza* A: LDA value distribution histogram, B: evolutionary branch diagram

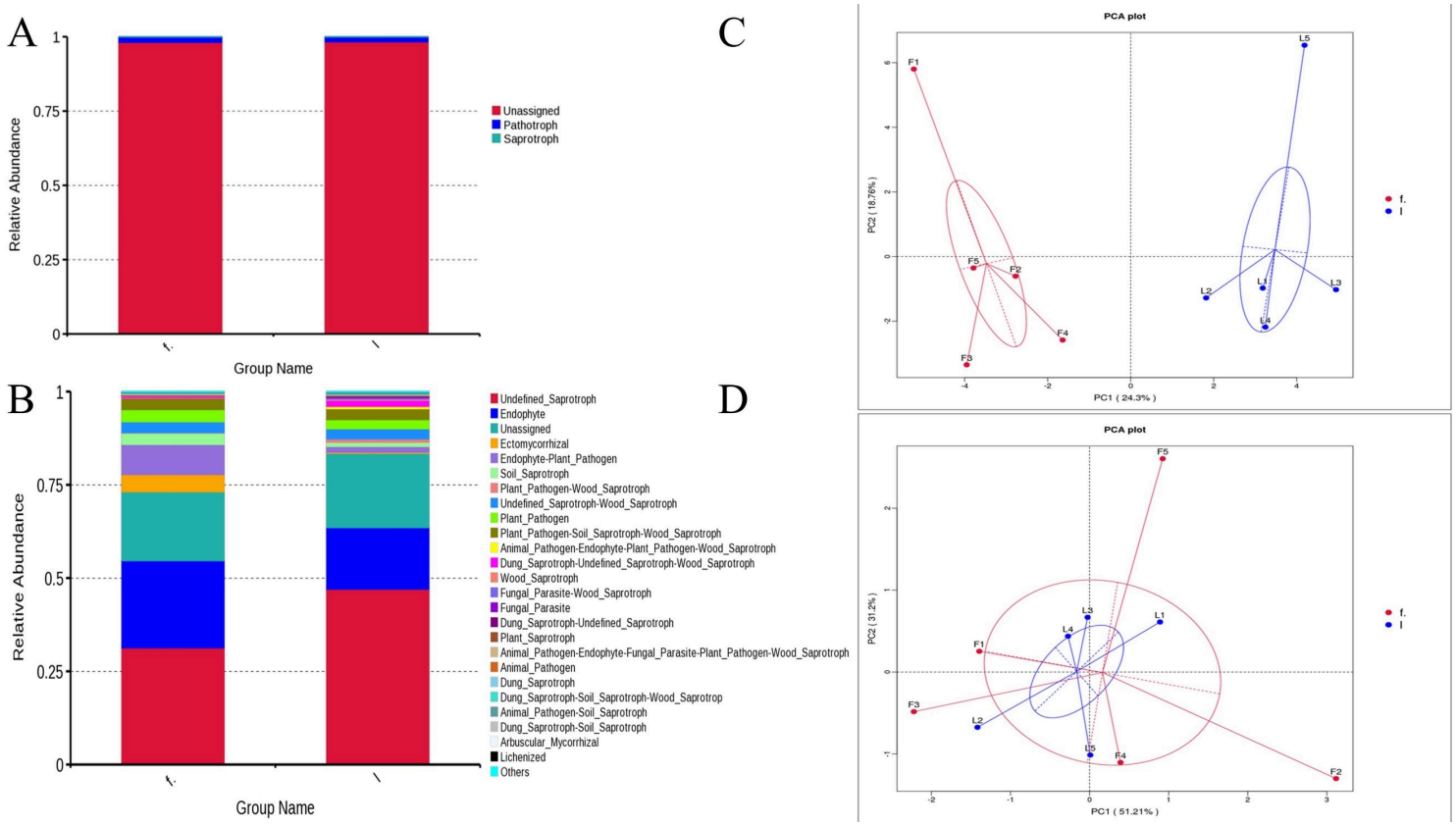


Figure 9

F group and L group function annotation relative abundance display A: bacterial FunGuild function annotation relative abundance histogram, B: fungal FunGuild function annotation relative abundance histogram, C: fungal FunGuild function annotation PCA result display, D: bacterial FunGuild function annotation PCA result display

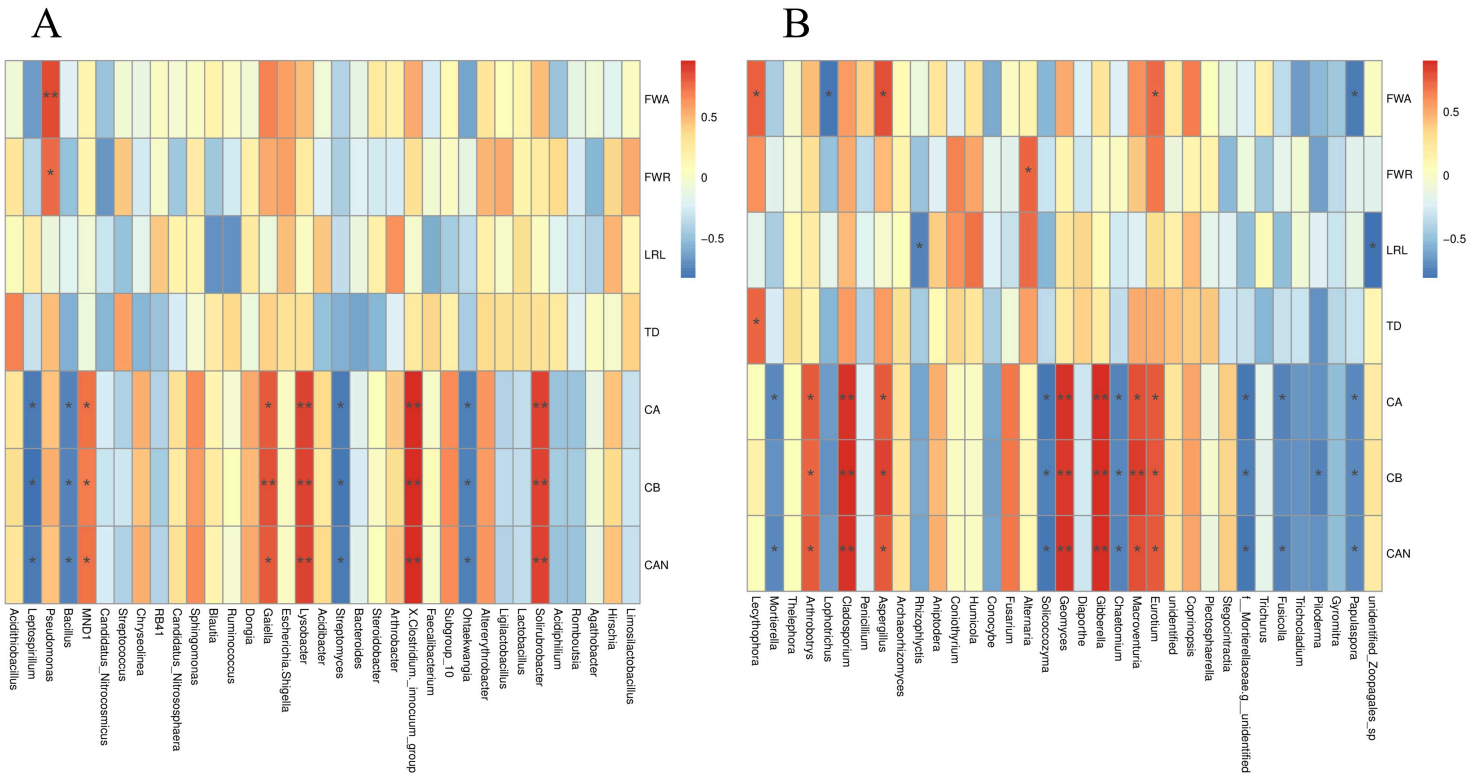


Figure 10

Spearman analysis of bacterial and fungal species abundance and physiological indexes of *Salvia miltiorrhiza*

Supplementary Files

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