

# 16S rRNA sequencing and Targeted Metabolomics Analyses Reveal Rhizosphere Microbial Diversity and Flavonoids Dynamics in *Scutellaria baicalensis* Georgi

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## Research Article

**Keywords:** *Scutellaria baicalensis* Georgi, metabonomics, 16s rRNA, correlation Analysis, physicochemical properties

**Posted Date:** June 26th, 2024

**DOI:** <https://doi.org/10.21203/rs.3.rs-4533559/v1>

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# Abstract

## Aims

*Scutellaria baicalensis* Georgi is a commonly used bulk Chinese medicinal material in China. Clinically, it is often divided into Ziqin and Kuqin respectively into medicine, and herbal research and pharmacological studies have shown that Ziqin and Kuqin have different effects. Therefore, we collected the HuangQin and its rhizosphere soils at different growth periods to explore the influence of soil microorganisms on flavonoid synthesis in HuangQin.

## Methods

In this study, high-throughput sequencing and the UPLC-MS/MS-based metabolomics method were used to sequencing 16S rRNA sequencing and metabolic analysis of HuangQin rhizosphere soil samples and HuangQin, respectively.

## Results

The results showed that the number of OTUs for the four years were 7594, 10227, 10280, and 9796, respectively. And with prolonged cultivation, the abundance of *Pseudarthrobacter*, *Achromobacter* and other beneficial bacteria gradually decreased. A total of 552 secondary metabolites were identified in the metabolome. Correlation analysis of soil physicochemical properties and differential microorganisms showed that *Pseudarthrobacter* was significantly correlated with TN, AN, AK, and  $\text{NH}_4^+\text{-N}$ , *Nocardioides* exhibited a notable correlation with TN and AK, as well as a significant association with AN and pH, and there is a significant correlation between *Haliangium* and AP.

## Conclusion

This study showed that soil physicochemical properties and soil microorganisms would affect the accumulation of flavonoids in *S. baicalensis*, which provided an important basis for improving the quality of *S. baicalensis* through reasonable fertilization and precise microbiota management.

## Introduction

*Scutellaria baicalensis* Georgi (*S. baicalensis*) is a perennial herb commonly known as 'Huang Qin', valued for its dried roots (Chinese Pharmacopoeia Commission, 2020). Modern pharmacological investigations have revealed diverse therapeutic properties associated with Huang Qin, including anti-inflammatory, anti-tumor, anti-viral, and anti-microbial effects (Yoon et al. 2009; Huang et al. 2024; Wang et al. 2018). Within traditional Chinese medicine, the roots of HuangQin aged two to three years are

referred to as 'ZiQin', while those exceeding three years are termed 'KuQin' (Tao 1994; Li 2008). Remarkably, classical medical texts note substantial disparities between ZiQin and KuQin, not only in physical appearance but also in clinical efficacy (Sun et al. 2023; Ren et al. 2020). ZiQin typically exhibits no hollow decay, whereas KuQin manifests an escalating decay area and darkening of the decayed portion over time (Xiao et al. 2023).

The principal bioactive constituents of *S. baicalensis* are flavones, notably baicalin, wogonoside, baicalein, and wogonin (Gao et al. 1999; Zhao et al. 2016; Min 2009). Variations in the types and ratios of these constituents, primarily flavones, between ZiQin and KuQin in vivo suggest potential disparities in therapeutic efficacy (Yang et al. 2017). Given that divergent pharmacological effects of HuangQin stem solely from differing growth periods, we undertook the collection of *S. baicalensis* roots and their rhizosphere soil across various growth years to explore the influence of soil microorganisms on the synthesis of medicinal compounds.

The biosynthetic pathway of flavones in HuangQin has been extensively elucidated, predominantly through the root-specific flavone pathway (Fang et al. 2022). The Gente isoflavonoid synthesis pathway originates from cinnamic acid (Fig. 1), synthesized from the amino acid phenylalanine as a biosynthetic precursor under the action of SbPAL. Cinnamic acid is subsequently converted to cinnamoyl CoA by cinnamoyl CoA ligase. Pine chalcone synthetase catalyzes cinnamoyl CoA to produce pinocembrin chalcone, which undergoes isomerization by chalcone isomerase to yield pinocembrin. Flavonoid synthetase then converts pinocembrin to chrysin, which is further hydroxylated by 6-hydroxylase to baicalein (Liu et al. 2021). Baicalein is glucuronidated to baicalin by baicalin-7-O-glucuronosyltransferase, while chrysin is converted to norwogonin by F8H. Norwogonin undergoes O-methylation at position 8 by O-methyl transferase (OMT) to yield wogonin, which is finally glucuronidated to wogonoside by baicalin-7-O-glucuronosyltransferase (Pei et al. 2023).

Increasingly, investigations have elucidated the significance of soil factors in shaping the growth and quality attributes of medicinal plants (Chen et al. 2022). Notably, distinct nitrogen utilization patterns have been linked to the modulation of secondary metabolism networks, particularly flavonoid biosynthesis in *Glycyrrhiza uralensis* (Sun et al. 2023). Moreover, the microbial diversity within the rhizosphere has been shown to exert beneficial effects on plant growth, development, and the accumulation of bioactive compounds (Pant et al. 2021; Xiao et al. 2022). In *Artemisia annua* L, the interplay between soil microorganisms and roots significantly impacts the emission and concentration of terpenes (Pivetta et al. 2023). Tomato root metabolites have been observed to recruit advantageous rhizosphere components, enhancing plant resilience (Chaouqi et al. 2023). Concomitantly, in *Angelica sinensis*, the composition of rhizosphere microorganisms correlates strongly with the levels of active constituents such as organic acids, flavonoids, and fatty acids over cultivation years (Rodés-Bachs and Vander Fels-Klerx 2023). Furthermore, root exudates play a pivotal role in regulating the yield and quality of *Panax ginseng* by influencing rhizome biomass (Yao et al. 2019).

Isolates of *Bacillus* from *A. sinensis* rhizosphere soil have been demonstrated to promote plant growth and biomass accumulation, while modulating the accumulation of specific compounds such as butylphthalide and ligustolide (Feng et al. 2021). Additionally, rhizosphere microorganisms in citrus soils have been found to activate terpene synthesis and enhance monoterpene accumulation through interactions with the host immune system (Su et al. 2023). Notably, significant enrichment of specific genera, including *Rhizobacter*, *Variovorax*, *Polaromonas*, and *Mycobacterium*, in *Anisodus tanguticus* rhizospheres has been linked to variations in anisodine contents, aboveground biomass, and nitrogen levels (Wang et al. 2023). The integration of microbiome and metabolome analyses facilitates the identification of beneficial bacteria with high correlation to essential active ingredients, thereby enhancing medicinal plant quality. For instance, in *G. uralensis*, joint transcriptome, microbiome, and metabolome analyses have unveiled critical environmental factors influencing the accumulation of liquiritin and glycyrrhizic acid (Chen et al. 2022).

Consequently, in this investigation, rhizosphere soil and corresponding roots were collected from one-year, two-year-old, three-year-old, and four-year-old *S. baicalensis* for 16S rRNA gene sequencing and metabolomic profiling. Results indicate the highest microbial species richness in three-year-old *S. baicalensis* soil, declining from three to four years, with the lowest diversity observed in one-year-old soil. Metabolomic analyses revealed a total of 552 secondary metabolites, comprising 537 flavonoids and 15 tannins across all samples. Further scrutiny unveiled similar metabolite compositions among the four groups, albeit with considerable variation in individual metabolite levels within each group.

## Materials and methods

### Plant roots and Rhizosphere soil sample collection

Rhizosphere soil and roots of *S. baicalensis* were collected from Fengxiang District, Baoji City, Shaanxi Province, utilizing a five-point sampling method (Li et al. 2021). Rhizosphere soil samples were gathered from within a 3 cm cubic meter surrounding the roots of plants aged one, two, three, and four years. The collected soil samples were sieved using a 20-mesh sieve and stored at -80°C. Fibrous roots and residual branches were carefully removed post-sampling and rinsed thrice with PBS buffer to eliminate surface soil residues. Subsequently, the samples were dried, sectioned into 2 cm lengths, and stored at -80°C. Rhizosphere soil samples from one to four years old *S. baicalensis* were designated as Soil\_HQ\_1Y, Soil\_HQ\_2Y, Soil\_HQ\_3Y, and Soil\_HQ\_4Y, respectively. Similarly, roots from one to four years old *S. baicalensis* were denoted as HQ\_1Y, HQ\_2Y, HQ\_3Y, and HQ\_4Y, correspondingly. Each sample was represented by three biological replicates.

### Soil physicochemical properties testing

Soil physical and chemical properties were analyzed by Yangling Xinhua Ecological Technology Co., Ltd. Soil pH was measured using a Sardolus PB-10 pH meter. Total Nitrogen (TN) in the soil was determined employing the sulfuric acid-catalyst digestion method (Bran-LuBBé). Total Potassium (TK) content in the soil was assessed via the flame photometric method (Bao 2000). Total Phosphorus (TP) and Available

Phosphorus (AP) in the soil was determined using the NaOH melting method coupled with the molybdenum-antimony anti-colorimetric method (Bao 2000; Bao 2000). Organic carbon and organic matter were quantified using the potassium dichromate-concentrated sulfuric acid external heating method (Bao 2000). Ammonium nitrogen ( $\text{NH}_4^+\text{-N}$ ), Alkali-hydrolyzable nitrogen (AN) and Nitrate nitrogen ( $\text{NO}_3\text{-N}$ ) was analyzed utilizing the potassium chloride extraction method (Bran-LuBBé). Flame photometry was used to determine Available Potassium (AK) and slowly available potassium (SAK). The drying method was used to determine the Soil Moisture Content (SMC).

#### Soil DNA extraction and sequencing

Soil samples were collected from each of the four different years of HuangQin, and genomic DNA was extracted from the samples utilizing the CTAB method. The purity and concentration of DNA were assessed via agarose gel electrophoresis, and samples were subsequently diluted to a concentration of 1 ng/ $\mu\text{L}$  using sterile water. Specific primers were employed to amplify the 16S rDNA gene sequence of microorganisms, and PCR amplification was conducted using 15  $\mu\text{L}$  of Phusion High-Fidelity PCR Master Mix (New England Biolabs). The resulting PCR products were pooled, purified, and subjected to library preparation, followed by sequencing on an Illumina NovaSeq platform to generate 250 bp paired-end reads.

#### Rhizosphere microbiome analysis

The 16S sequencing data obtained from Illumina NovaSeq sequencing underwent quality control and were subsequently trimmed to generate Clean Tags. Chimera filtering was implemented to retain valid data suitable for subsequent analysis. Each sample's valid data were clustered into Operational Taxonomic Units (OTUs) based on the 97% sequence similarity principle, followed by species annotation of the OTU sequences.

#### Metabolite profiling analysis

In this study, a total of 12 samples were selected and categorized into 4 groups for metabolic analysis. Sample preparation and extraction procedures were conducted as follows: Biological samples were subjected to vacuum freeze-drying using a lyophilizer (Scientz-100F) until completely dehydrated. Subsequently, the dried samples were ground to a fine powder using a grinder (MM 400, Retsch) operating at 30 Hz for 1.5 minutes. A precise amount of 50 mg of sample powder was weighed using an electronic balance (MS105DM). To this, 70% methanol water internal standard extract pre-cooled to  $-20^\circ\text{C}$  at a volume of 1200  $\mu\text{L}$  per 50 mg of sample was added (less than 50 mg of sample required a proportionate adjustment of the extraction agent volume to maintain the 1200  $\mu\text{L}$  ratio). The mixture was vortexed once every 30 minutes for a total of 6 vortex cycles, each lasting 30 seconds. Following centrifugation at 12000 rpm for 3 minutes, the supernatant was aspirated, and the sample was filtered through a microporous membrane with a pore size of 0.22  $\mu\text{m}$ . The filtrate was then transferred to a vial for subsequent analysis using UPLC-MS/MS.

The mass spectrometry conditions were configured as follows: Electrospray ionization (ESI) temperature was maintained at 550°C. The ion spray voltage (IS) was set to 5500 V for positive ion mode and – 4500 V for negative ion mode. Gas settings included ion source gas I (GSI), gas II (GSII), and curtain gas (CUR), which were adjusted to 50, 60, and 25 psi, respectively. Collision-induced ionization parameters were set to high, and the collision gas (nitrogen) was maintained at medium pressure during QQQ scan operation. Further optimization involved the adjustment of declustering potential (DP) and collision energy (CE) for each multiple reaction monitoring (MRM) ion pair. Specific MRM transitions were monitored in each epoch based on the elution profile of metabolites within that epoch.

PCA (Principal Component Analysis) was conducted using R ([www.r-project.org](http://www.r-project.org)) to analyze the metabolomic data. For two-group comparisons, differential metabolites were identified based on Variable Importance in Projection (VIP) scores ( $VIP > 1$ ) and absolute Log2 Fold Change ( $|\text{Log2FC}| \geq 1.0$ ). In multi-group analyses, differential metabolites were determined using VIP scores ( $VIP > 1$ ) and P-values ( $P\text{-value} < 0.05$ ). The identified metabolites were annotated using the KEGG compound database (<http://www.kegg.jp/kegg/compound/>), and subsequently mapped to the KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>).

## Results

### Determining the growth years of HuangQin

The root's thickness visibly increased with advancing growth years (Fig. 2). Observation of HuangQin slices revealed that from the third year onwards, the central hollow expanded with increasing growth years. To ascertain the growth years of HuangQin, we employed phloroglucinol-hcl staining, which selectively stains wooden walls red. Staining results indicated that one-year-old HuangQin slices displayed a single circle of red xylem, whereas two-year-old slices exhibited two distinct rounds of red xylem. Additionally, the central regions of one- and two-year-old HuangQin slices remained intact. However, starting from the third year, a black cavity emerged at the center of the slice, accompanied by the presence of three rounds of red xylem. Slices from the third and fourth years depicted three and four rounds of red xylem, respectively. Notably, the proportion of decay increased progressively from the third to fourth year, with a color transition from brown to brownish-black. Collectively, phloroglucinol staining results provided confirmation of HuangQin's growth age.

### Soil physical and chemical properties in different years

To gain a comprehensive understanding of the rhizosphere soil, we conducted analyses of its physicochemical properties. Results revealed that with increasing planting duration, the pH of the soil is always slightly alkaline and the pH value has slightly increased. The levels of SAK exhibited a gradual upward trend from the first year to the fourth year. TN, TK, and AK displayed an upward trajectory during the initial two years, followed by a gradual decline from the second to fourth years (Table 1). Conversely, TP,  $\text{NH}_4^+\text{-N}$ , AP, and SMC exhibited a declining trend from the first to second years, followed by an

increase from the second to third years, and subsequently a decrease from the third to fourth years. AN, NO<sub>3</sub>-N, and acid-soluble potassium content increased from the first to second year, decreased from the second to third year, and then increased again from the third to fourth year. Overall, Soil\_HQ\_2Y exhibited the highest levels of TN, TK, AN, NO<sub>3</sub>-N and AK. Soil\_HQ\_3Y recorded the highest levels of TP and AP. Conversely, Soil\_HQ\_4Y displayed the highest levels of acid-soluble potassium, SAK, and pH value. Only the NH<sub>4</sub><sup>+</sup>-N content was highest in Soil\_HQ\_1Y.

Table 1  
Mineral element content in rhizosphere soil of HuangQin at different years

|                                     | HQ-1Y             | HQ-2Y              | HQ-3Y             | HQ-4Y              |
|-------------------------------------|-------------------|--------------------|-------------------|--------------------|
| Total Nitrogen(g/kg)                | 0.7767 ± 0.0153   | 0.9700 ± 0.0200    | 0.8700 ± 0.0100   | 0.7833 ± 0.0231    |
| Total Phosphorus(g/kg)              | 0.5767 ± 0.0153   | 0.5100 ± 0.0100    | 0.6500 ± 0.0100   | 0.4633 ± 0.0058    |
| Total Potassium(g/kg)               | 20.2633 ± 0.1650  | 21.1600 ± 0.1758   | 21.1533 ± 0.4292  | 20.7600 ± 0.2722   |
| Acid soluble potassium(g/kg)        | 645.3333 ± 4.6188 | 888.0000 ± 24.0000 | 866.6667 ± 4.6188 | 925.3333 ± 23.0940 |
| slowly available potassium(g/kg)    | 548.0000 ± 4.3589 | 748.0000 ± 24.5153 | 750.0000 ± 3.6056 | 823.0000 ± 22.5389 |
| Alkali-hydrolyzable nitrogen(mg/kg) | 35.9100 ± 0.6750  | 44.7200 ± 1.1778   | 37.9467 ± 1.1720  | 39.0767 ± 1.0333   |
| Ammonium nitrogen(mg/kg)            | 0.7767 ± 0.3215   | 0.5600 ± 0.0100    | 0.6633 ± 0.0252   | 0.6233 ± 0.0058    |
| Nitrate nitrogen(mg/kg)             | 4.3233 ± 0.1274   | 4.1667 ± 0.0058    | 1.8533 ± 0.1234   | 2.9667 ± 0.1102    |
| Available Phosphorus(mg/kg)         | 8.9133 ± 0.0513   | 5.8100 ± 0.1852    | 10.5633 ± 0.1528  | 3.2100 ± 0.2307    |
| Available Potassium(mg/kg)          | 97.3333 ± 0.5774  | 140.0000 ± 1.0000  | 116.6667 ± 1.5275 | 102.3333 ± 1.1547  |
| pH                                  | 8.0267 ± 0.03215  | 8.0400 ± 0.0361    | 8.0567 ± 0.0252   | 8.1500 ± 0.0200    |
| Soil Moisture Content(%)            | 16.1067 ± 0.0777  | 14.5067 ± 0.1629   | 16.0800 ± 0.1353  | 14.3033 ± 0.2540   |

Microbial community structure of HuangQin planted in different years

In rhizosphere soil samples from the first year to the fourth year, the number of operational taxonomic units (OTUs) observed were 7594, 10227, 10280, and 9796, respectively (Fig. 3). Notably, Soil-HQ-3Y exhibited the highest number of OTUs, whereas Soil-HQ-1Y displayed the lowest count. The total OTU

count increased from Soil-HQ-1Y to Soil-HQ-3Y, followed by a decrease from Soil-HQ-3Y to Soil-HQ-4Y. This pattern suggests an initial increase followed by a decrease in OTU count with increasing planting years of *S. baicalensis*, with a peak observed at 3 years. To delineate common and unique characteristics of soil microorganisms across different years, a Venn plot analysis was performed. The total number of common OTUs across all four groups was 2432, 32.03% (Soil-HQ-1Y), 23.78% (Soil-HQ-2Y), 23.66% (Soil-HQ-3Y), and 24.83% (Soil-HQ-4Y) of the total OTU in each group, respectively. And a total of 4636 OTUs were shared between Soil-HQ-2Y and Soil-HQ-3Y, whereas Soil-HQ-1Y and Soil-HQ-2Y exhibited the fewest shared OTUs, totaling 3430. Additionally, Unique OTU counts for Soil-HQ-1Y to Soil-HQ-4Y were 2835, 4474, 4275, and 4269, respectively. The results showed that the total number of bacterial OTU and the number of endemic bacteria in HuangQin soil at different growth years decreased with the increase of years. The soil bacterial species of Soil-HQ-1Y and Soil-HQ-2Y were the most different, and the soil bacterial species of Soil-HQ-2Y and Soil-HQ-3Y were the smallest, and nearly half of the bacterial species were the same. The above results indicated that planting years had a great effect on the amount of OTU in HuangQin soil.

A total of 41 phyla, 78 classes, 159 orders, 298 families, 619 genera, and 380 species were detected in 4 groups of soil samples. Analysis of soil microorganism composition and structural changes across different years revealed the top 10 microflora at the phylum level (Fig. 4a), with varying abundance percentages. These included *Myxococcota* (2.20%-3.15%), *Bacteroidota* (1.75%-4.06%), *Planctomycetes* (3.91%-4.95%), *Gemmatimonadota* (3.74%-5.93%), *unidentified\_Bacteria* (5.26%-7.36%), *Crenarchaeota* (3.13%-8.59%), *Actinobacteria* (7.45%-21.35%), *Acidobacteriota* (8.75%-15.14%), *Actinobacteriota* (11.47%-13.33%), and *Proteobacteria* (15.57%-32.13%). Notably, *Proteobacteria*, *Actinobacteriota*, *Acidobacteriota*, *Actinobacteria* and *Crenarchaeota* emerged as dominant bacterial phyla. *Proteobacteria* was the most dominant microbial group in the soil, with a relative abundance of 20.83%, followed by *Actinobacteriota*, *Acidobacteriota*, *Actinobacteria* and *Crenarchaeota*, all of which accounted for about 12%. These five dominant groups together accounted for 58.07% of the total bacterial abundance. Moreover, the abundance of the five phyla *Myxococcota*, *Planctomycetes*, *Crenarchaeota* and *Acidobacteriota* increased year by year, reaching the highest value in the fourth year. The abundance of *Bacteroidota*, *unidentified\_Bacteria* and *Proteobacteria* showed a decreasing and then increasing trend, among them, *Bacteroidota* and *Proteobacteria* both had only small increases, and they were still the most abundant in the first year. Finally, the abundance of *Actinobacteria* and *Actinobacteriota* showed a trend of first increasing and then decreasing.

At the genus level, *Achromobacter* was the dominant genus in the Soil\_HQ\_1Y bacterial community composition, with a relative abundance of 12.30% (Fig. 4b), followed by *unidentified\_Burkholderiaceae* (4.08%), *Pseudarthrobacter* (2.68%), *Gaiella* (1.68%), *Blastococcus* (0.82%), *Solirubrobacter* (0.86%), *Sphingomonas* (0.70%), *Nocardioides* (0.66%), *Haliangium* (0.63%) and *unidentified\_Methyloirabilota* (0.54%). Among the Soil\_HQ\_2Y bacterial community composition, *Pseudarthrobacter* was the dominant genus with a relative abundance of 13.25%, followed by *Gaiella* (1.81%), *Blastococcus* (1.5%), *Nocardioides* (1.31%), *Haliangium* (0.84%), *Solirubrobacter* (0.83%), *unidentified\_Methyloirabilota* (0.67%), *Sphingomonas* (0.56%) and *Achromobacter* (0.01%). Among the Soil\_HQ\_3Y bacterial

community composition, *Pseudarthrobacter* was the dominant genus with a relative abundance of 2.61%, followed by *Gaiella* (2.03%), *Blastococcus* (1.44%), *Solirubrobacter* (0.95%), *unidentified\_Methyloirabilota* (0.92%), *Nocardioides* (0.84%), *Haliangium* (0.81%), *Sphingomonas* (0.67%) and *unidentified\_Burkholderiaceae* (0.01%). Lastly, in the Soil\_HQ\_4Y of the bacterial community composition, *Pseudarthrobacter* was the dominant genus with a relative abundance of 2.34%, followed by *Gaiella* (1.79%), *unidentified\_Methyloirabilota* (1.15%), *Haliangium* (1.03%), *Solirubrobacter* (0.93%), *Blastococcus* (0.71%), *Sphingomonas* (0.68%), *Nocardioides* (0.56%), *Achromobacter* (0.04%) and *unidentified\_Burkholderiaceae* (0.02%). Among them, the abundance of *Pseudarthrobacter* in the four groups of samples has been greater than 2%, and the overall trend is to increase first and then decrease, reaching a peak in the second year. *Blastococcus* and *Nocardioides* are in perfect tune with its trend. *Gaiella* has a similar trend, peaking only in its third year. The abundance of *Sphingomonas*, *unidentified\_Burkholderiaceae* and *Achromobacter* decreased first and then increased, while the abundance of *unidentified\_Methyloirabilota* and *Haliangium* increased overall. The abundance of *Solirubrobacter* changes in a trend similar to N.

Based on the linear discriminant analysis effect size (LEfSe), a linear discriminant analysis (LDA) distribution histogram with a score cutoff of  $\pm 4$  was employed to illustrate taxa with significant differences in abundance between different groups (Fig. 4c). LEfSe analysis showed that the bulk soil indicator bacteria in Soil\_HQ\_1Y were *Burkholderia\_pseudomallei* (5.04), *Achromobacter* (5.02) and *Proteobacteria* (4.98), among the Soil\_HQ\_2Y were *Pseudarthrobacter* (5.14), *Micrococcaceae* (4.92) and *Micrococcales* (4.88), and in the Soil\_HQ\_3Y were *Nitrososphaerales* (4.56), *Nitrososphaeria* (4.51) and *Crenarchaeota* (4.46), and finally in the Soil\_HQ\_4Y are *Nitrosomonadaceae* (4.3), *Metagenome* (4.16) and *Pyrinomonadaceae* (4.15). These results also showed that there were significant differences in bacterial abundance in rhizosphere soils of HuangQin in different planting years.

#### Flavonoid metabolomics analysis and differential metabolite screening

To gain insights into the changes occurring in the main active ingredients of HuangQin during the transition from Ziqin to Kuqin, flavonoids in the four groups of samples were identified using UPLC-MS platform with broadly targeted metabolome technology. For root samples from HQ-1Y to HQ-4Y, we detected 550, 547, 547, and 547 metabolites, respectively. Flavonoids constituted 97.28% of the metabolite composition, while tannins accounted for 2.7%. Among the identified flavonoids, a total of 537 were classified into secondary structures (Fig. 5a), comprising 282 flavones (52.5%), 105 flavonols (19.6%), 49 flavonones (9.1%), 37 isoflavones (6.9%), 25 other flavonoids (4.7%), 18 chalcones (3.4%), 15 flavanonols (2.8%), and 6 flavanols (1.0%).

The principal component analysis (PCA) results demonstrated good repeatability of biological samples, with distinct separation among different samples (Fig. 5b). The contribution rates of the first two principal components were 32.42% and 21.68%, respectively. The cumulative rate reached 54.10%, which effectively discriminates HuangQin samples for different years, indicating that there were certain differences in the chemical composition of HuangQin across different years. Orthogonal partial least

squares discriminant analysis (OPLS-DA), a supervised pattern recognition method, effectively excluded non-study-related effects to identify differential metabolites. The model's Q<sup>2</sup> value of 0.937 indicated its appropriateness (Fig. 5c). The OPLS-DA score plot (Fig. 5d) revealed significant segregation among the different comparison groups. Pairwise comparisons were conducted among the radix samples of the four HuangQin accessions to identify metabolites responsible for observed differences. Through correlation analysis, PCA, cluster heat map analysis, and OPLS-DA model analysis, high biological sample repeatability was confirmed.

### Differential Metabolite Analysis

552 compounds were screened for differential metabolites, resulting in the identification of 222 differential metabolites (121 upregulated and 102 downregulated) in HQ-2Y compared to HQ-1Y (Fig. 6a), 140 (106 upregulated and 34 downregulated) in HQ-3Y compared to HQ-2Y, 178 (61 upregulated and 117 downregulated) in HQ-4Y compared to HQ-3Y, 206 (142 upregulated and 64 downregulated) in HQ-3Y compared to HQ-1Y, 210 (109 upregulated and 101 downregulated) in HQ-4Y compared to HQ-1Y, and 151 (75 upregulated and 76 downregulated) in HQ-4Y compared to HQ-2Y. The types of differential metabolites in HQ-1Y and HQ-2Y encompass 45.9% flavones (Fig. 6b), 20.2% flavonols, 9.9% flavanones, 7.6% isoflavones, 5.8% chalcones, 3.1% flavanonols, 3.6% other flavonoids, 3.1% tannin, and 0.4% flavanols. In HQ-2Y and HQ-3Y, the differential metabolite types included 53.5% flavones, 18.5% flavonols, 9.2% flavanones, 8.5% isoflavones, 4.2% other flavonoids, 3.5% chalcones, and 2.1% flavanonols. The increased proportion of flavones by 8% was the main reason for the change, while the proportions of other types did not show significant changes, and no significant differences were observed in tannin and flavanol metabolites. Over the course of 2 to 3 years, the contents of chrysin, baicalein, norwogonin, and baicalin increased, albeit not significantly, indicating ongoing accumulation of active ingredients in HuangQin during this period. In HQ-3Y and HQ-4Y, the differential metabolite types comprised 48.3% flavones, 21.3% flavonols, 9.5% flavanones, 8.4% isoflavones, 4.4% tannin, 3.9% other flavonoids, 1.6% flavanonols, 1.1% chalcones, and 1.1% flavanols. Notably, baicalin, a principal component in HuangQin, exhibited a significant down-regulation by 0.44 times over the course of three to four years, with its content halved, while chrysin, baicalein, and norwogonin all showed up-regulation, although not significantly. Furthermore, the content of chrysin in HQ-4Y reached its highest value, and Oroxylin A content in HuangQin was significantly up-regulated at each stage, reaching its peak in the fourth year. In HQ-2Y and HQ-4Y, the differential metabolite types comprised 48.3% flavones, 19.8% flavonols, 7.9% flavanones, 6.6% isoflavones, 5.9% other flavonoids, 3.9% chalcones, 3.3% tannin, 3.3% flavanonols, and 0.6% flavanols. At this stage, significant changes occurred in the main components of HuangQin, with chrysin, baicalein, norwogonin, and Oroxylin A being significantly up-regulated, while baicalin was significantly down-regulated. This suggests continuous accumulation of norwogonin, chrysin, Oroxylin A, and baicalein over the 2 to 4-year period, while baicalin underwent significant reduction. Therefore, it is speculated that the 3-year growth period of HuangQin may be the time node to distinguish ZiQin and KuQin.

Effects of soil physicochemical properties on bacteria at different planting years of HuangQin

By integrating the prominent microorganisms in soil physicochemical properties and the abundance at the genus level, a notable correlation emerged with *Pseudarthrobacter* exhibiting a significant association with TN, AN, AK, and  $\text{NH}_4^+\text{-N}$  (Fig. 7a). Specifically, as TN, AN, and AK increased, the abundance of *Pseudarthrobacter* demonstrated a corresponding rise, whereas with a decrease in  $\text{NH}_4^+\text{-N}$ , the abundance of *Pseudarthrobacter* increased, peaking at two years. *Pseudarthrobacter* has been recognized for its role in promoting plant growth, supported by studies revealing that *Pseudarthrobacter* sp. NIBRBAC000502770 can enhance the growth of *Geum aleppicum* and facilitate the accumulation of flavonoids.

When  $\text{NH}_4^+\text{-N}$  decreases during the 1–2 year period, there is a corresponding decrease in the abundance of *Sphingomonas*.  $\text{NH}_4^+\text{-N}$  reaches its peak at 3 years, coinciding with the highest abundance of *Sphingomonas* during the same period, while AN exhibits an opposite trend. *Sphingomonas* species have demonstrated significant agricultural applications, particularly in promoting plant growth and enhancing plant stress resistance. Certain strains of *Sphingomonas* have been identified as potential promoters of crop growth, particularly under stress conditions such as drought, salinity, and heavy metal exposure in agricultural soils, attributed to their capacity to produce plant growth hormones like gibberellin and indoleacetic acid. Furthermore, a significant correlation was observed between *Haliangium* and AP, with both showing a decrease in abundance from 3 to 4 years, coinciding with the lowest AP levels at year 4. *Haliangium* species may play a crucial role in the degradation of polycyclic aromatic hydrocarbons (PAHs) in biochar-enhanced rhizosphere soils.

*Nocardioides* exhibited a notable correlation with TN and AK, as well as a significant association with AN and pH. The abundance of *Nocardioides* increased during the 1–2 year period but decreased from 2 to 4 years, mirroring the trends observed in TN and AK. AN followed a similar trajectory as *Nocardioides*, with a slight increase observed during 3–4 years, whereas pH remained relatively stable during 1–3 years before increasing during 3–4 years. *Achromobacter* demonstrated a significant correlation with TP, SK, SAP, and  $\text{NH}_4^+\text{-N}$ , with its highest abundance observed at 1 year and gradually decreasing over subsequent years. The general trend for TP, SK, SAP, and  $\text{NH}_4^+\text{-N}$  was an increase during 1–3 years followed by a decrease in 3–4 years. Moreover, a significant correlation was noted between *Blastococcus* and AP, with *Blastococcus* being most abundant at 3 years, consistent with the trend observed in AP.

RDA analysis was used to further explore the relationship between rhizosphere soil microbial community and soil physicochemical factors in HuangQin. The results showed that the contents of pH, TN, TK,  $\text{NO}_3\text{-N}$  and AP had an effect on the distribution of soil bacterial communities (Fig. 7b). The influence of PH, TN and TK was greater, and the influence of  $\text{NO}_3\text{-N}$  and AP was relatively small. *Pseudarthrobacter* was positively correlated with TN, TK and  $\text{NO}_3\text{-N}$  in soil. and it was negatively correlated with the content of AP and PH. *Achromobacter* and *unidentified\_Burkholderiaceae* were positively correlated with the content of AP in soil. There was a negative correlation with the contents of TN, TK,  $\text{NO}_3\text{-N}$  and PH, and

the rest of the bacteria were all gathered at the origin, which was not significantly correlated with soil physical and chemical factors.

#### Correlation analysis of differential metabolites with differential microorganisms

Spearman correlation hierarchical cluster analysis (Fig. 8a) of differential microorganisms and metabolites over the 3 to 4 year period revealed a significant reduction in the content of baicalin. Rhizosphere bacteria were found to exhibit both negative and positive correlations with baicalin levels. On one hand, rhizosphere bacteria negatively correlated with baicalin included *Polycyclovorans*, *Pseudomonas*, *Aquicella*, *Phaselicystis*, *Puia*, *Novosphingobium*, *unidentified\_Sphingobacteriales*, *Pseudoxanthomonas*, *Sphaerisorangium*, *Methylothera*, *Rickettsiella*, *unidentified\_Azospirillaceae*, and *unidentified\_Rickettsiales*. On the other hand, rhizosphere bacteria that positively correlated with baicalin included *Nocardioides*, *Iamia*, *Gemmatimonas*, *unidentified\_Nitrosomonadaceae*, *Geodermatophilus*, *Parviterribacter*, *Caenimonas*, *unidentified\_Holophagae*, *Promicromonospora*, *Cohnella*, *Longimicrobium*, *Sporichthya*, *Smaragdicoccus*, *Rhodocytophaga*, *Qipengyuania*, *Herbidospora*, and *Fictibacillus*. Therefore, we speculate that Bacteria such as *Polyceclovolens*, *Psadormonas*, *Aquitella*, etc. may inhibit the synthesis of baicalin, while bacteria such as *Nocatiodes*, *Yamia*, and *Germatimonas* promote the accumulation of baicalin.

At two to four years (Fig. 8b), the content of baicalein significantly increased, showing an inverse correlation with *Pirellula*, *Polycyclovorans*, *Nitrosospira*, *Puia*, *Methylothera*, *Paludibaculum*, *Quadrishpaera*, *Sphaerisorangium*, *Rickettsiella*, *unidentified\_Bacteroidia*, *unidentified\_Azospirillaceae*, and *Mucilaginibacter*. Conversely, baicalein exhibited positive correlations with *Blastococcus*, *Skermanella*, *unidentified\_Acidimicrobiia*, *unidentified\_Nitrosomonadaceae*, *Phaselicystis*, *Marmoricola*, *Parviterribacter*, *Cellulomonas*, *Microbacterium*, *Amaricoccus*, *Actinoplanes*, *Actinomycetospora*, *Adhaeribacter*, *unidentified\_Illumatobacteraceae*, *Dactylosporangium*, *Polaromonas*, *unidentified\_Solirubrobacterales*, *Lysinibacillus*, *Paenarthrobacter*, *unidentified\_Rhodospirillaceae*, *Parafrigoribacterium*, *Brevundimonas*, and *Williamsia*. The content of chrysin significantly increased, showing positive correlations with *Pirellula*, *unidentified\_Chloroflexi*, *Polycyclovorans*, *Nitrosospira*, *unidentified\_Planctomycetota*, *Quadrishpaera*, *Rickettsiella*, *unidentified\_Bacteroidia*, *unidentified\_Azospirillaceae*, while it was inversely correlated with *Blastococcus*, *Skermanella*, and *unidentified\_Acidimicrobiia*. Additionally, *unidentified\_Nitrosomonadaceae*, *Phaselicystis*, *Marmoricola*, *Cellulomonas*, *Microbacterium*, *Actinoplanes*, *Actinomycetospora*, *Adhaeribacter*, *Pseudolabrys*, *Promicromonospora*, *Polaromonas*, *unidentified\_Solirubrobacterales*, *Lysinibacillus*, *unidentified\_Actinobacteriota*, *unidentified\_Rhodospirillaceae*, *Parafrigoribacterium*, and *Bdellovibrio* were negatively correlated. Norwogonin content significantly increased and positively correlated with *Pirellula*, *Polycyclovorans*, *Nitrosospira*, *Niastella*, *Puia*, *unidentified\_Pseudonocardiaceae*, *Pseudoxanthomonas*, *Methylothera*, *Pseudoflavitalea*, *Quadrishpaera*, *Sphaerisorangium*, *Rickettsiella*, *unidentified\_Bacteroidia*, *unidentified\_Azospirillaceae*, and *Mucilaginibacter*. Conversely, it was negatively correlated with *Pseudarthrobacter*, *Cellulomonas*, *Microbacterium*, *Actinoplanes*, *Actinomycetospora*, *unidentified\_Sandaracinaceae*, *Adhaeribacter*, *unidentified\_Illumatobacteraceae*,

*Promicromonospora*, *unidentified\_Vicinamibacteraceae*, *unidentified\_Solirubrobacterales*, *Lysinibacillus*, *Coxiella*, *unidentified\_Actinobacteriota*, *Paenarthrobacter*, *unidentified\_Rhodanobacteraceae*, *Sporichthya*, *Isoptericola*, *Brevundimonas*, *unidentified\_Simkaniaceae*, *Williamsia*, *Peredibacter*, and *unidentified\_Vicinamibacteria*. Baicalein content significantly increased and was positively correlated with *Pirellula*, *unidentified\_Chloroflexi*, *Polycyclovorans*, *Nitrosospira*, *unidentified\_Pseudonocardiaceae*, *Pseudoxanthomonas*, *Pseudoflavitalea*, *Quadrisphaera*, *Sphaerisporangium*, *Rickettsiella*, *unidentified\_Bacteroidia*, and *unidentified\_Azospirillaceae*. Conversely, it was negatively correlated with *unidentified\_Acidimicrobiia*, *Cellulomonas*, *Actinomycetospora*, *Adhaeribacter*, *Promicromonospora*, *unidentified\_Vicinamibacteraceae*, *Lysinibacillus*, *unidentified\_Actinobacteriota*, *unidentified\_Rhodospirillaceae*, *unidentified\_Rhodanobacteraceae*, *Isoptericola*, *Parafrigoribacterium* and *Bdellovibrio*.

## Discussion

Soil bacteria exhibit robust metabolic activity, rapid proliferation, diverse species composition, and substantial population size, thereby constituting a pivotal component of the soil microecological environment. Concurrently, they exert a significant influence on the growth dynamics of medicinal plants (Zharkova et al. 2023; Xu et al. 2023). The present investigation revealed a close association between alterations in bacterial communities and the duration of plant cultivation. Specifically, the diversity of soil bacterial communities exhibited an initial increase followed by a subsequent decline after four years of continuous HuangQin cultivation. Subsequent analysis of bacterial abundance at the microbial genus level identified *Sphingomonas*, *unidentified\_Burkholderiaceae*, and *Achromobacter* as the predominant taxa in the first year, while *Nocardioideis*, *Blastococcus*, and *Pseudarthrobacter* dominated in the second year. *Solirubrobacter* and *Gaiella* were prevalent in the third year, with *Haliangium* being predominant in the fourth year. Notably, *Achromobacter* exhibited a growth-promoting effect on plants, with its abundance peaking in the initial two years of HuangQin cultivation before declining. The gram-negative bacillus *Achromobacter* sp. 5B1, recognized as a probiotic, modulates plant physiological traits, including root development via auxin signaling pathways (Jiménez-Vázquez et al. 2020). Similarly, *unidentified\_Burkholderiaceae*, known for its beneficial plant functions such as colonization, nitrogen fixation, and pollutant degradation, displayed a substantial impact on plant growth, exemplified by *Burkholderia* phytofirmans PsJN. The multifaceted functions of *Sphingomonas* genus species encompass the production of beneficial plant hormones, enhancement of plant growth under stress conditions, and remediation of environmental pollutants (Zúñiga et al. 2013). Consequently, the dominance of *Achromobacter* during the initial HuangQin cultivation period implies its pivotal role in root development, while the modest abundance of *Burkholderiaceae* may contribute to the accumulation of active ingredients in HuangQin. *Pseudarthrobacter*, exhibiting effects on growth enhancement and flavonoid content augmentation in *Geum aleppicum*, evidenced its peak abundance in the second year, coinciding with the rapid growth phase of HuangQin and the accumulation of active ingredients (Fan et al. 2023; Asimakoula et al. 2023). However, with prolonged HuangQin cultivation, beneficial bacteria abundance declined gradually, as evidenced by the reduction in *Pseudarthrobacter* and

*unidentified\_Burkholderiaceae* from the second to fourth years. Soil microorganisms, serving as drivers of organic matter transformation and nutrient cycling, significantly influence soil nutrient availability. Alterations in soil microorganism diversity and structure may consequently exert an influence on plant composition.

The physicochemical properties of soil play a crucial role in shaping the composition of soil microbial communities (Philippot et al. 2024). Soil pH is an important chemical property of soil, exerting significant influence on the activity of soil microorganisms, the decomposition of minerals and organic matter, as well as the release, fixation and migration of soil nutrients, thus affecting the bioavailability and toxicity of the elements. Furthermore, it has been established that optimal pH level plays a pivotal role in the accumulation of organic matter, and alteration in soil pH will also exert an influence on the carbon cycling process of microorganisms. For example, soil pH is a major factor influencing the microbial community of the massive soil and rhizosphere soil of *Ageratina adenophora* (Li et al. 2022). Several studies have demonstrated that alterations in soil pH exert significant influence on the composition and structure of soil microbial communities, thereby giving rise to persistent challenges in continuous cropping obstacles (Tian et al. 2021; Malik et al. 2018). The soil pH consistently exhibited weak alkalinity throughout the duration of this study, with only a slightly observed over the years of cultivation. This finding aligns with the optimal growth conditions for HuangQin, which thrive under neutral to weakly alkaline environments. The soil microbial community structure can be directly or indirectly influenced by factors such as nitrogen and phosphorus content in the soil, as well as soil moisture levels, which ultimately impact crop yields (Wang et al. 2021). Nitrogen is a large nutrient that is necessary for plant growth and development. The element actively participates in numerous vital physiological and biochemical reactions as well as material metabolism processes, thereby serving as the primary limiting factor in plant growth and yield. Phosphorus and potassium are also indispensable nutrients for plant growth and development (Lyzenga et al. 2023; Pogrzeba et al. 2017). The three elements of fertilizer, namely nitrogen, phosphorus and potassium, collectively play an indispensable role in plant growth. Their effects on plants are not isolated but rather exhibit a coordinated influence on each other (Sun et al. 2022). The latest findings indicate that the judicious application of N, P and K fertilizers can increase the yield of yam, establishing a significantly correlation between yam yield and the appropriate utilization of N, P and K fertilizers (Chen et al. 2023). The composition of the microbiome in the core area of *Citrus reticulata* 'Chachi' was related to factors such as pH, organic matter, AN, K, and P (Su et al. 2023). The diversity of bacterial communities in Mulberry and Alfalfa intercropping soils was correlated with variations in soil total carbon, available phosphate, and available potassium (Zhang et al. 2018). Therefore, we hypothesized that pH, TN, TK, NO<sub>3</sub>-N and AP might exert significant influence on the composition and diversity of microbial communities in the rhizosphere soil of HuangQin.

In this investigation, 12 samples were systematically selected and grouped for metabolic profiling. Leveraging the UPLC-MS/MS detection platform and a bespoke database, a comprehensive analysis identified a total of 552 metabolites, predominantly flavonoids (537) with a minor presence of tannins (15). The subsequent analysis revealed a consistent metabolite composition across the four vintages of HuangQin, indicating minimal variation likely attributed to their shared origin. Employing OPLS-DA

analysis with stringent criteria, 222, 140, and 178 differential metabolites were delineated for the transitions from the first to second year, second to third year, and third to fourth year, respectively. Integrating this with the biosynthesis pathway of main flavonoids in HuangQin revealed stable levels of Chrysin, Baicalin, Baicalein, and Norwogonin during the initial 1 to 2 years, with Chrysin and Baicalin showing accumulation trends while Baicalein and Norwogonin exhibited decreased levels (Sfig1).

**SFig. 1** The content variation trends of Chrysin, Baicalein, Norwogonin, and Baicalin in HuangQin across four years.

Notably, the subsequent 2 to 3-year period witnessed a notable accumulation phase, particularly marked by a significant surge in Chrysin content, reaching a four-fold increase compared to the previous biennial period. Additionally, although the levels of Baicalin, Baicalein, and Norwogonin displayed modest changes, all demonstrated a consistent trend towards accumulation. This phase coincided with a speculated enhancement in flavonoid synthase (SbFNSII-2) activity, consequently elevating the catalytic formation of these compounds. In the subsequent 3 to 4-year period, while Baicalin levels exhibited a substantial decline, the levels of Chrysin, Baicalein, and Norwogonin continued to rise steadily, indicating persistent accumulation. This trend could be attributed to the enhanced activities of flavonoid synthase (SbFNSII-2), F6H, and F8H enzymes, facilitating increased catalysis. Remarkably, over the extended 2 to 4-year duration, there were significant elevations in the levels of Chrysin, Baicalein, and Norwogonin, highlighting an almost six-fold increase in Chrysin content and a twofold rise in both Baicalein and Norwogonin levels. Conversely, Baicalin levels exhibited a pronounced reduction by half. These findings underscore the distinct developmental stages of HuangQin and its active constituents, indicating that the third year as the optimal harvest period due to the peak Baicalin content. However, the fourth year represents a favorable harvesting window for elevated levelsof Chrysin, Baicalein, and Norwogonin. Notably, differences in Baicalin, Baicalein, and Norwogonin content between ZiQin and KuQin variants indicate potential clinical implications, necessitating further investigation for tailored therapeutic applications.

## Declarations

### Acknowledgements

We express our gratitude to Mengmeng Liu from Hebei University for the valuable input in reviewing this manuscript, Yuxing Liu from Baoji City Fengxiang County Chinese herbal medicine planting professional cooperative for providing the field for sample collection.

### Funding

Xianyang young and middle-aged scientific and technological innovation leading talent project (S2022-QXNL-RC-1334); Shaanxi University of Traditional Chinese Medicine Discipline Innovation Team Project (2019-QN01).

## Competing Interests

Conflict of interest the authors declare no conflict of interest regarding the publication of this article.

## Author contributions

Yimin Li conceived the idea, planned the research and designed the experiments. Xiaochen Hu and Jing Tang conducted the experiment in the field, Ying Chen, Yonggang Yan and Jing Gao collected the root samples and processed the root samples. Yan Wang and Quan Guo analyzed the data and wrote the article. Gang Zhang and Feng Yan contributed to data interpretation, writing and editing of the article. All authors read and approved the manuscript.

## Data availability

The datasets generated and analyzed during the current study are available upon reasonable request from the corresponding author.

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## Figures

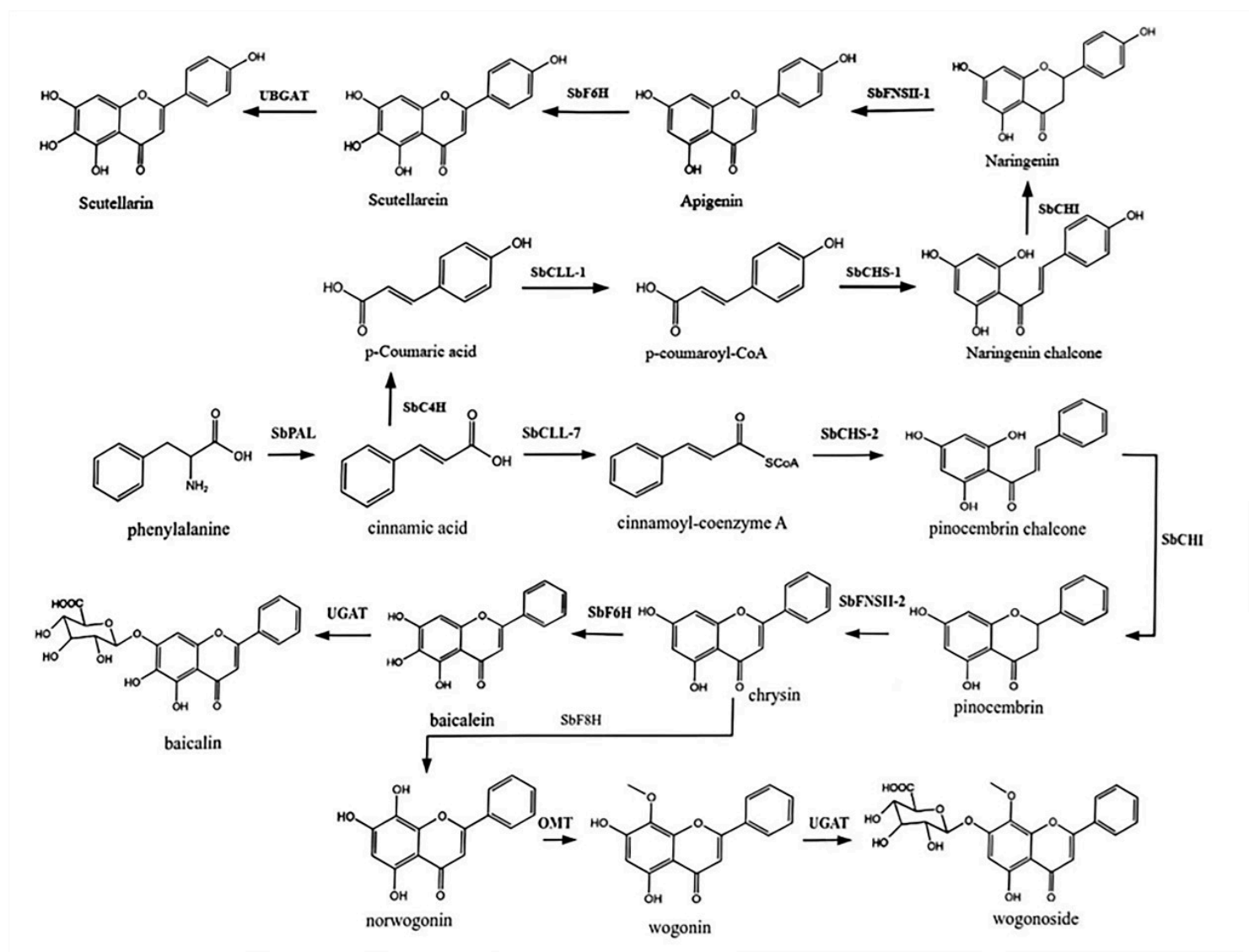


**Figure 1**

Two flavonoid biosynthesis pathways in HuangQin



**Figure 2**

Photos of HuangQin samples and corresponding staining photos. a1, b1: HQ-1Y; a2, b2: HQ-2Y; a3, b3: HQ-3Y; a4, b4: HQ-4Y.

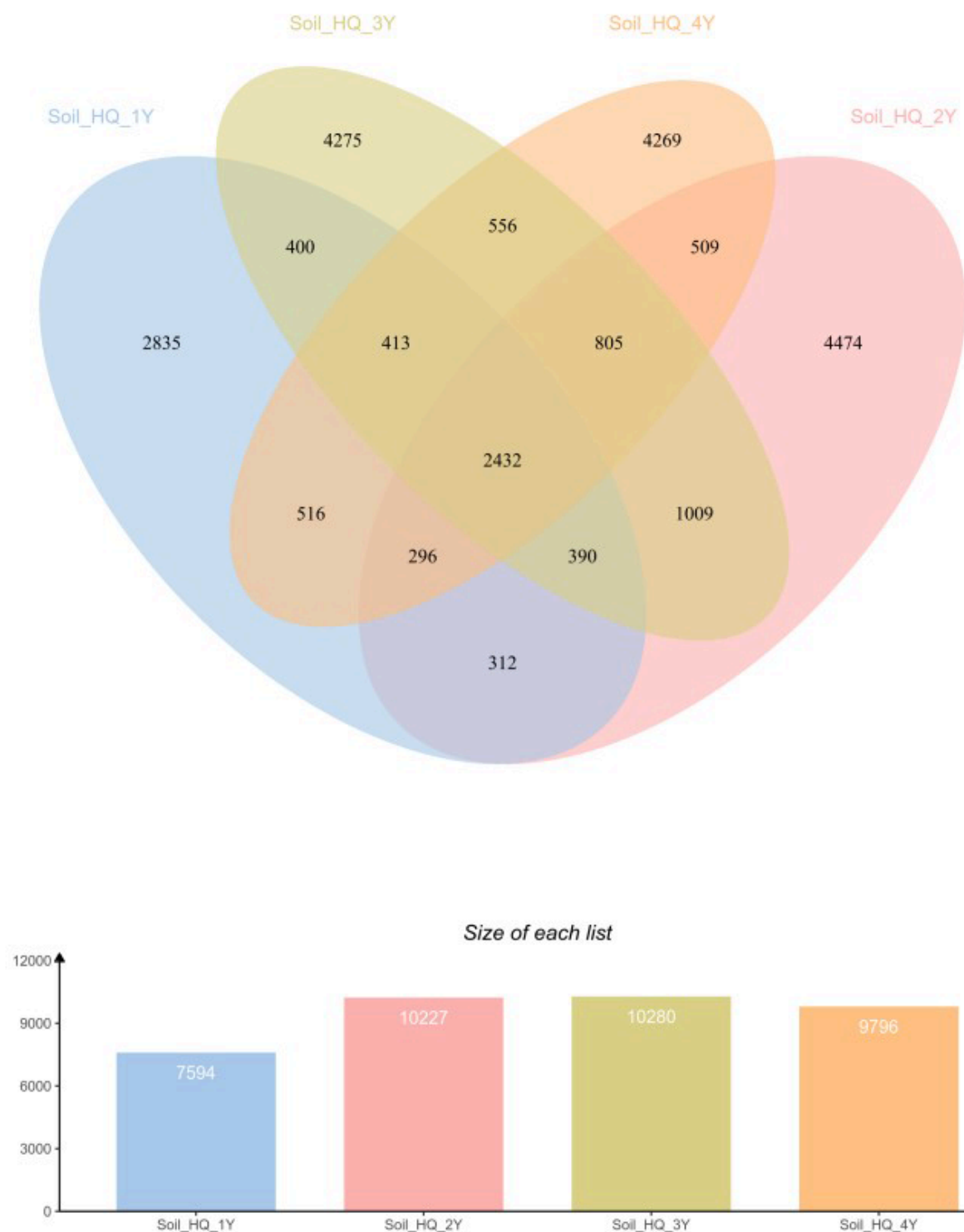


**Figure 3**

OTUs in rhizosphere soil microbial of Huangqin varies over a span of four years. Venn diagram illustrating the number of shared and unique OTUs in the rhizosphere soil of Huangqin across different years. Temporal variations in soil microorganism's populations as depicted by Histograms across multiple years. The abscissa represents the different groupings, and the ordinate represents the number of OTUs.

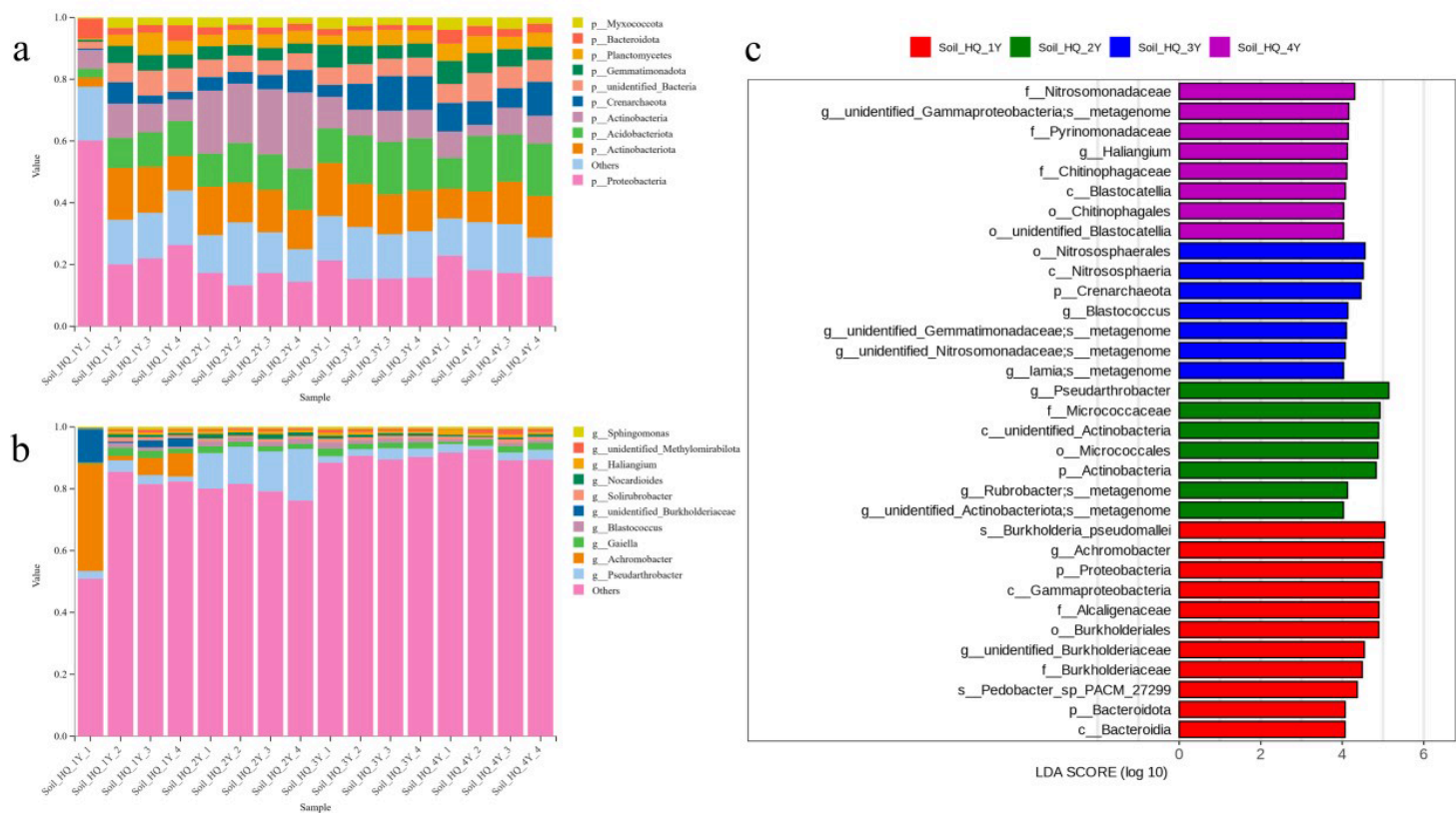


**Figure 4**

Species composition and abundance of bacterial communities in the rhizosphere soil of Skullcap with varying cultivation durations (a: door level b: genus level) c: ASV-based histogram of LDA value distribution. The displayed species exhibit an LDA score exceeding 4.

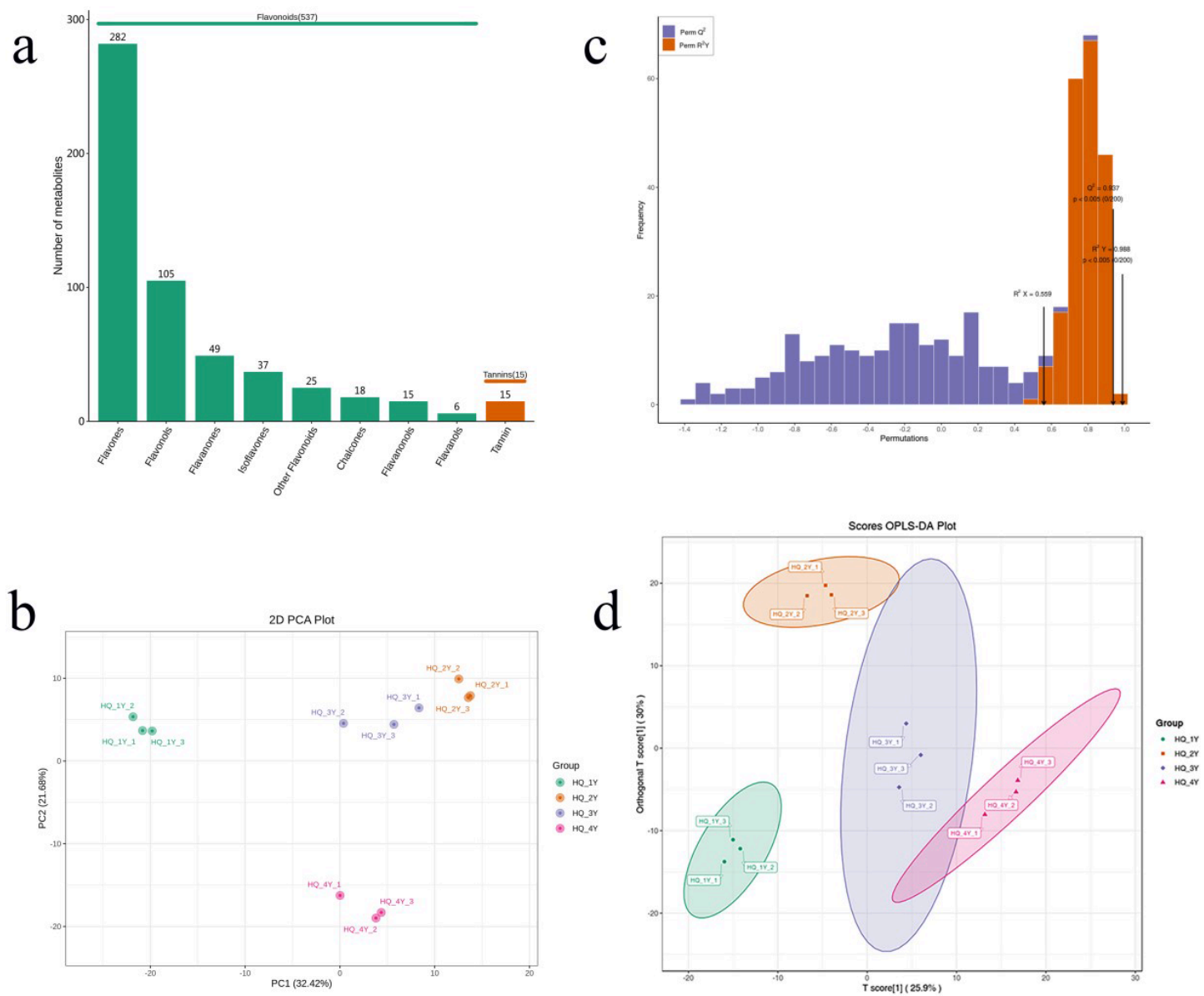


**Figure 5**

Composition of metabolites categories in Huangqin. a: Histogram depicting the secondary classification of all identified metabolites. b: PCA grouping analysis. c: OPLS-DA verification Diagram. d: OPLS-DA score graph

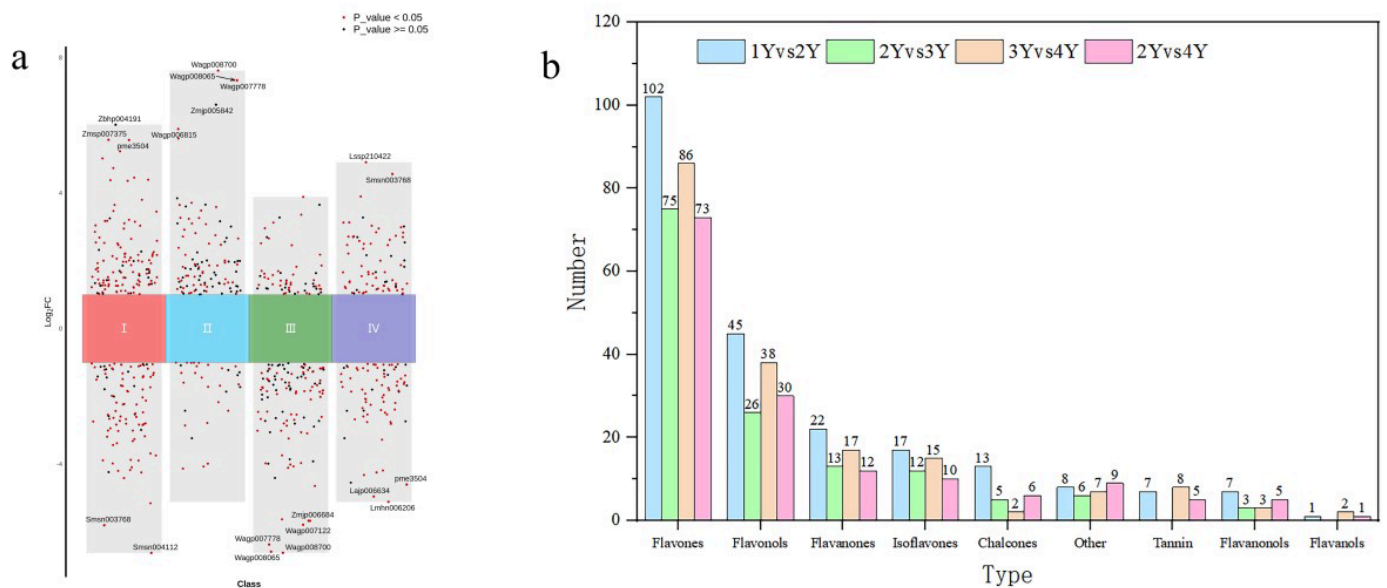


**Figure 6**

Metabolic profiling of differential metabolites. **a**: Differential metabolite volcano plot. The horizontal axis numbers represent different difference groups, □: HQ-1Y vs HQ-2Y; □: HQ-2Y vs HQ-3Y; □: HQ-3Y vs HQ-4Y; □: HQ-2Y vs HQ-4Y. The vertical axis represents the log<sub>2</sub>FC value of difference substances in each difference group, the red dot represents the difference species (p value<0.05), and the black color represents the difference species (p value≥0.05), **b**: Differential metabolite composition map. The horizontal axis represents the substances classified by the secondary classification of HuangQin substances, and the vertical axis represents the number of metabolites

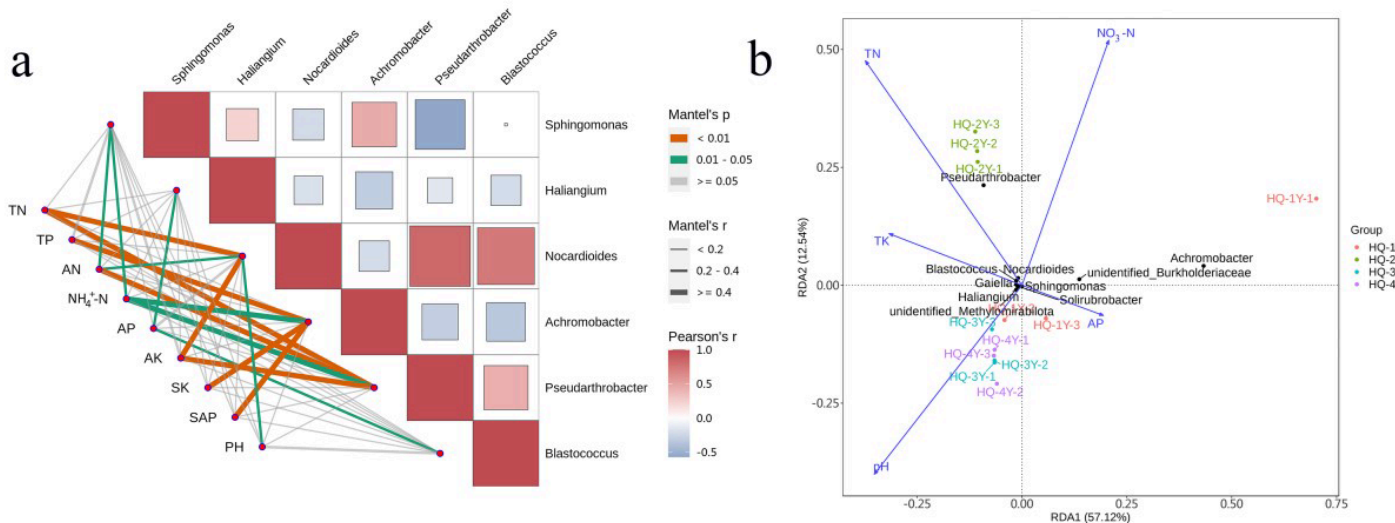


**Figure 7**

Integrated analysis of soil physicochemistry and soil microbial communities. a: Pairwise comparisons of physicochemical factors and differential microorganisms are shown, with a colour gradient denoting Pearson's correlation coefficient. Edge width corresponds to Mantel's R statistic for the corresponding distance correlations, Blue and red are the anchor colors for gradients with negative to positive correlations, respectively. The orange color on the lower left side is the very significant line color (Mantel's  $p < 0.01$ ), the green color is the prominent line color ( $0.01 \leq \text{Mantel's } p < 0.05$ ), and the gray color is the inconspicuous line color (Mantel's  $p > 0.05$ ). b: RDA analysis of the soil bacterial community in relation to soil physicochemical properties. Black labels indicate different microorganisms, blue labels and arrows indicate different physicochemical factors, and colored dots indicate samples belonging to different groups. The length of the arrow indicates the intensity of the influence of environmental factors on microbial changes, and the longer the arrow, the greater the influence of environmental factors on microbial changes. The vertical distance from the sample point to the environmental factor line segment and its extension line indicates the intensity of microbial influence on the sample, and the closer the distance, the greater the influence of environmental factors on the sample. Taking the central origin as the starting point, the microorganisms are in the same direction as the arrows, indicating that the environmental factors are positively correlated with the changes of microorganisms, and vice versa indicates a negative correlation.

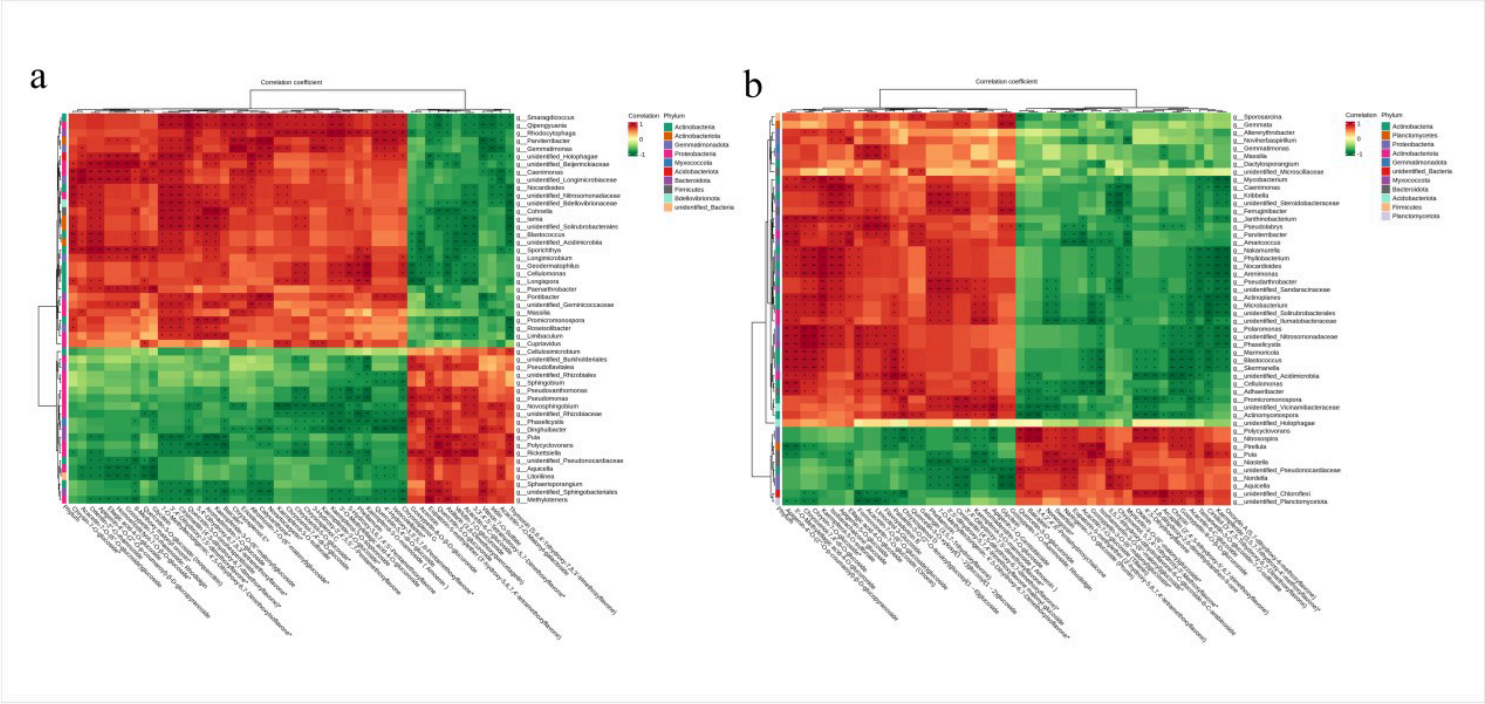


Figure 8

Hierarchical cluster heat map of spearman correlation between differential microorganisms and differential metabolites. a: Un\_4Y\_vs\_Un\_3Y; b: Un\_4Y\_vs\_Un\_2Y

Supplementary Files

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- [SFig.1.docx](#)