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Root-associated endophytic bacterial community composition and structure of three medicinal licorices and their changes with the growing year

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Research article

Keywords: Endophytic bacteria, High-throughput sequencing, Growing year, Liquorice variety, Soil physicochemical, Plant secondary metabolites

Posted Date: June 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-36992/v1

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Version of Record: A version of this preprint was published on September 21st, 2020. See the published version at https://doi.org/10.1186/s12866-020-01977-3.

Abstract

Background

The dried roots and rhizomes of medicinal licorices are widely used worldwide as a traditional medicinal herb, the activities of which are attributed to a variety of effective components that can be extracted from licorice root. Endophytes and plants form a symbiotic relationship, which is an important source of host secondary metabolites.

Results

In this study, we used high-throughput sequencing technology and high-performance liquid chromatography to explore the composition and structure of the endophytic bacterial community and the content of effective components (glycyrrhizic acid, liquiritin and total flavonoids) in different varieties of medicinal licorices (*Glycyrrhiza uralensis Fisch*., *Glycyrrhiza glabra L*.and *Glycyrrhiza inflata Batal*.) and in different planting years (1–3 years). Our results showed that the contents of the effective components in the roots of medicinal licorices were not affected by the variety, but were significantly affected by the growing year (1–3) ($P < 0.05$), and with a trend of stable increase in the contents observed with each growing year. In 27 samples, 73,316 effective sequences with an average length of 372 nucleotides (nt) were obtained after quality control, and 2,432 effective operational taxonomic units (OTUs) were obtained at 97% identity. The phylum Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes, and the genera unified-*Rhizobiaceae*, *Pseudomonas*, *Novosphingobium*, and *Pantoea* were significantly dominant in the 27 samples. Distance-based redundancy analysis (db-RDA) showed that the content of total flavonoids explained the differences in composition and distribution of endophytic bacterial communities in roots of cultivated medicinal liquorices to the greatest extent. Total soil salt was the most important factor that significantly affected the endophytic bacterial community in soil factors, followed by ammonium nitrogen and nitrate nitrogen. Among the leaf nutrition factors, leaf water content had the most significant effect on the endophytic bacterial community, followed by total phosphorus and total potassium.

Conclusions

This study not only provides information on the composition and distribution of endophytic bacteria in the roots of medicinal licorices, but also reveals the influence of abiotic factors on the community structure of endophytic bacteria and effective components, which provides a reference for improving the quality of licorice.

Background

Endophytes are microorganisms that colonize the internal tissues of plants during the whole, or part of their life cycle and do not cause damage or disease to the host plants [1]. These endophytes include bacteria, fungi, archaea and unicellular eukaryotes [2]. The internal niches of plants provide a protective barrier from environmental influences that allow bacteria to live and reproduce [3]. In return, endophytic bacteria also benefit plants. First, endophytic bacteria can promote the growth and development of host plants by producing a range of nutrients and promoting primary and secondary nutrient uptake through atmospheric nitrogen fixation [4]. Second, endophytic bacteria can participate in aspects of the plant's phosphate solubilization activity [5], such as osmotic adjustment [6] and stomatal regulation [7], to increase the plant's ecological adaptability. Furthermore, endophytic bacteria may overcome environmental stress conditions, such as drought and soil salinity stress [8, 9], and improve plant growth. Last, but not least, as biological control agents, endophytic bacteria can produce or promote the production of secondary metabolites by host plants to reduce or prevent damage caused by certain pathogens [10, 11]. Numerous studies have shown that an in-depth understanding of the diversity of endophytic bacteria will elucidate the function of interaction between microorganisms and plants, which will be conducive to the development of strategies for ecological environment restoration and sustainable agricultural, such as remediation of the soil environment [12] and increasing crop production [13].

Endophytic bacteria have been isolated from both monocotyledonous and dicotyledonous plants [3], ranging from woody plants [14], such as walnut [15] and poplar [16], to herbaceous crop plants, such as rice [17] and wheat [18]. Modern molecular technology, especially high-throughput sequencing technology, have facilitated characterization of the composition and structure of microbial communities in different plants and has substantially increased our understanding of the composition and diversity of endophytic bacterial communities in the plants[6, 19]. These techniques provide information more rapidly and accurately than conventional culturing methods, which are time-consuming and ignore uncultivable microorganisms may be overlooked [11, 20]. High-throughput sequencing technology has been used to quickly and accurately analyze the diversity of endogenous bacterial communities in a variety of medicinal plant tissues, such as *Panax notoginseng* [21]and *Astragali radix*[22], which makes it a practical approach to the isolation and identification of the community composition and diversity of endogenous bacteria in medicinal licorices.

The dried roots and rhizomes of three medicinal licorices (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra*) are widely used worldwide as a traditional medicinal herb [23]. Many clinical and experimental studies have demonstrated that the root of medicinal licorices has a variety of pharmacological properties [24], such as anti-inflammatory [25], antiviral and antitumor [26], immunomodulatory [27], hepatoprotective activities[28], which are attributed to a variety of bioactive constituents extracted from licorice root, mainly including triterpene saponins and flavonoids [29]. Glycyrrhizic acid, which is present at the highest levels among the triterpene saponins, is an important pharmacologically bioactive constituent with anti-inflammatory [30], antiviral [31], immune regulation [32] and other biological properties. Recent studies have shown that licorice flavonoids have a variety of pharmacological activities and have gradually become a hot spot in pharmacological research [33, 34].

Effective ingredients contained within medicinal plants are the material basis for clinical efficacy and an important index of the quality of medicinal materials. Furthermore, these materials are the evolutionary result of the interaction between plants and their natural environment [35, 36]. Studies have shown that the accumulation of effective ingredients in licorice roots is affected by both the ecological environment and regulation of nutrient elements, such as water [37] and nitrogen sources and phosphate concentrations in the soil[38], to patterns of distribution in the root distribution pattern that are affected by factors such as year [39] and biomass [40].

Currently, there is little information about the composition of endophytic bacterial communities in the roots of medicinal licorices at different times in the planting year, and the factors influencing the community structure are not clear. Furthermore, the factors influencing the content of effective components of medicinal licorices also remain to be elucidated. Therefore, in this study, we conducted a three-year licorice planting experiment in the field and used Illumina high-throughput sequencing technology to detect the composition and diversity of the endophytic bacterial community of medicinal licorice roots to explore the factors affecting the accumulation of effective ingredients and the structure of the endophytic bacterial community.

1. Results

1.1 The effect of the growing year on the content of effective components in the roots of medicinal licorices

The results of one-way ANOVA showed that the contents of the effective components (glycyrrhizic acid (GIA), liquiritin (LI) and total flavonoid (GTF)) in the roots of medicinal licorices (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra*) were not affected by the variety, but were significantly affected by the growing year (1–3) ($P < 0.05$), with a trend of stable increase in the contents observed with each growing year (Table 1). Specifically, the contents of GIA, LI and GTF in the year 3 were significantly higher than those in year 1, the contents of GIA and GTF in year 2 were significantly higher than those in year 1, and the contents of GIA and LI in year 3 were significantly higher than those in year 2.

Table 1
Effect of variety and year on the effective components of licorice root

		GIA (%)	GTF (%)	LI (%)
year	E	0.3242 ± 0.1874a	3.2218 ± 0.1201a	0.1391 ± 0.0180a
	R	0.8980 ± 0.2390b	4.4406 ± 0.3264b	0.4930 ± 0.3088a
	S	1.6777 ± 0.6720c	4.6161 ± 0.1900b	1.2327 ± 0.5549b
Variety	W	0.9400 ± 0.6510a	3.9734 ± 0.7061a	0.6873 ± 0.4858a
	D	1.0909 ± 0.8121a	4.0989 ± 0.6765a	0.7039 ± 0.6562a
	G	0.8690 ± 0.6855a	4.2061 ± 0.6841a	0.4736 ± 0.6328a

Description: values are means followed by standard deviation. Different lowercase letters indicate significant differences in year and Variety ($p < 0.05$) (E, R and S: years 1, 2, and 3, respectively; W, G and D: *Glycyrrhiza uralensis Fisch.*, *Glycyrrhiza glabra L.*and *Glycyrrhiza inflata Batal.*, respectively).

In addition, Spearman correlation analysis showed that the content of effective components was significantly correlated with soil physicochemical properties and nutritional components of leaves (Table 2). In terms of soil physicochemical factors, GIA and LI had a very significant positive correlation with soil ammonium nitrogen (SAN) ($R^2 > 0$; $P < 0.01$), but had a very significant negative correlation with total salt (TS) ($R^2 < 0$; $P < 0.01$); GTF had a very significant positive correlation with SAN ($R^2 > 0$; $P < 0.01$), but had a very significant negative correlation with nitrate nitrogen (SNN) ($R^2 < 0$; $P < 0.01$), and had a significant negative correlation with TS ($R^2 < 0.01$; $P < 0.05$). In terms of leaf nutrition, GIA, LI and GTF were very negatively correlated with water content (PWC), total potassium (PTK) and crude fiber (CF). In addition, GTF also showed a very significant very negative correlation with total phosphorus (PTP). There was a significant relationship between leaf nutrients and a small number of variables. Specifically, PWC was positively correlated with SNN, but negatively correlated with SAN; organic carbon (POC) was positively correlated with soil total potassium (STK); total nitrogen (PTN) was positively correlated with POC; total phosphorus (PTP) was very positively correlated with SNN, TS and PTN; PTK was very positively correlated with SNN, TS, PWC, PTN and PTP, but negatively correlated with SAN; CF was very positively correlated with PWC and PTK.

Table 2
Spearman correlation coefficient of the content of effective components with soil physicochemical properties and nutritional components of

	GIA	GTF	LI	SOM	STN	STP	STK	SNN	SAN	TS	PWC	POC	PTN	PTP
GIA	1.000													
GTF	0.774**	1.000												
LI	0.964**	0.716**	1.000											
SOM	0.173	0.156	0.173	1.000										
STN	0.262	0.060	0.233	0.578**	1.000									
STP	0.230	0.032	0.218	0.438**	0.713**	1.000								
STK	-0.283	-0.025	-0.283	0.147	-0.051	-0.131	1.000							
SNN	-0.363	-0.490**	-0.359	0.057	-0.179	-0.216	-0.234	1.000						
SAN	0.600**	0.535**	0.593**	0.396*	0.323	0.433*	0.027	-0.233	1.000					
TS	-0.511**	-0.449*	-0.499**	-0.294	-0.474*	-0.452*	-0.259	0.760**	-0.490**	1.000				
PWC	-731**	-0.807**	-0.737**	0.077	0.019	-0.017	0.073	0.484*	-0.405*	0.343	1.000			
POC	-0.331	-0.114	-0.311	0.321	0.013	-0.142	0.430*	0.321	0.077	0.324	0.111	1.000		
PTN	-0.261	-0.297	-0.192	0.098	0.101	0.136	0.028	0.243	0.059	0.206	0.245	0.444*	1.000	
PTP	-0.298	-0.537**	-0.223	-0.073	-0.027	-0.095	-0.357	0.654**	-0.264	0.656**	0.368	0.259	0.608**	1.00
PTK	-0.670**	-0.707**	-0.634**	-0.074	-0.013	0.048	-0.123	0.528**	-0.421*	0.623**	0.644**	0.336	0.587**	0.79
CF	-0.684**	-0.662**	-0.699**	-0.053	0.070	0.259	0.062	0.207	-0.349	0.173	0.806**	-0.024	0.378	0.22

Description: the values are the correlation coefficients. ** means $P < 0.01$; * means $P < 0.05$.

1.2 Composition of bacterial community in the root of medicinal liquorices

In 27 samples, 73,316 effective sequences with an average length of 372 nucleotides (nt) were obtained after quality control. The sequencing results for each sample are listed in Supplementary table S1. The effective sequences were clustered into operational taxonomic units (OTUs) with 97% identity, and a total of 2,432 OTUs were obtained. The rarefaction curves different colors represent different samples, showed that the number of OTU in each sample increased gradually with quantity of sequence, thus confirming that the amount of sequencing data is adequate (Fig. 1). According to the OTUs, 28 phyla, 45 classes, 106 orders, 216 families, 508 genera and 313 species were annotated. Figure 2a shows the 10 bacterial phyla with the greatest abundance in the bacterial community in the roots of medicinal licorices. Proteobacteria (76.28%) dominated the observed sequences at the phylum level, and Actinobacteria (10.62%), Bacteroidetes (6.92%) and Firmicutes (3.42%) were found to be the predominant phyla in each group, this was followed by unidentified_bacteria (0.80%), Chlamydiae (0.42%), Tenericutes (0.30%), Acidobacteria (0.21%), Verrucomicrobia (0.20%), Planctomycetes (0.13%) and others (0.70%).

In terms of genus (Fig. 2b), unified-*Rhizobiaceae* (17.62%) was more abundant than other genera, and *Pseudomonas* (7.49%), *Novosphingobium* (4.52%), *Pantoea* (2.23%), *Bacteroides* (1.43%), and *Halomonas* (1.14%) were found to be the main genera in all groups, followed by *Nocardiopsis* (0.88%), *Sinomicrobium* (0.55%), unidentified-*bacteria* (0.77%), *Methylophilus* (0.90%) and others (62.46%). Details of the composition information of top 10 dominant bacteria at other classification levels are listed in Supplementary table S2. Specifically speaking, Alphaproteobacteria (46.20%), Gammaproteobacteria (28.54%), unidentified-Actinobacteria (9.86%) dominate at the class taxonomic level; the dominant species at the order taxonomic level are Rhizobiales (26.63%), Sphingomonadales (10.90%), Gammaproteobacteria (8.58%); the dominant species at the family taxonomic level are Rhizobiaceae (19.16%), Pseudomonadaceae (7.49%), Sphingomonadaceae (10.90%); the dominant species at the species taxonomic level are *Pantoea-brenneri* (1.97%), *Neorhizobium-huautlense* (2.73%), and *Pseudomonas-psychrotolerans* (1.08%).

1.3 Effects of years and varieties on alpha diversity and beta diversity in the bacterial community of roots.

The alpha diversity index of each group is shown in Supplementary table S3. One-way ANOVA showed that there was no significant difference between the alpha diversity indexes (Shannon, Simpson, Chao1, ACE) among the groups, reflecting the diversity and richness of bacterial communities. The results showed that the planting year and varieties did not affect the diversity and richness of endophytic bacterial communities in the root of medicinal licorices (Table 3). However, beta diversity analysis showed significant differences in the endophytic bacterial community structures among the different groups. Specifically, we used the weighted-UniFrac and unweighted-UniFrac distances to construct a beta diversity distance heat map to measure the coefficient of the difference between the two groups (Fig. 3). The distances among the nine groups based on weighted-UniFrac ranged from 0.2 to 0.674, which indicated the existence of differences in endophytic bacterial community of roots of medicinal licorices between different varieties and different planting years. More specifically, the samples collected from E.D showed a high degree of difference compared with other sampling groups: 0.481 in E.W, 0.570 in E.G, 0.631 in R.D, and 0.526 in S.D.

Table 3
Endophytic bacteria community richness estimators and diversity index in licorice root with different growth year and variety.

		Shannon	Simpson	Chao1	ACE
year	E	5.8688 ± 1.3289a	0.9347 ± 0.0611a	626.172 ± 152.8335a	623.8419 ± 148.8065a
	R	6.2889 ± 0.7270a	0.9572 ± 0.0349a	689.9542 ± 100.8890a	690.1123 ± 100.0530a
	S	5.4513 ± 1.2254a	0.9132 ± 0.0687a	633.2774 ± 159.4251a	637.8597 ± 145.1567a
variety	W	6.0936 ± 1.0528a	0.9424 ± 0.0642a	634.6612 ± 165.1966a	632.2988 ± 154.0842a
	D	5.2879 ± 1.1309a	0.9146 ± 0.0643a	611.0410 ± 106.3678a	620.3057 ± 92.8245a
	G	6.2276 ± 1.1055a	0.9481 ± 0.0421a	703.7014 ± 133.7225a	699.2094 ± 139.7966a

Description: the mean is followed by the standard deviation. Different letters indicated statistically significant difference ($P < 0.05$) (E, R and S: years 1, 2, and 3, respectively; W, G and D: *Glycyrrhiza uralensis Fisch.*, *Glycyrrhiza glabra L.*and *Glycyrrhiza inflata Batal.*, respectively).

We used linear discriminant analysis (LDA) effect size (LEfSe) to identify discriminative taxa among different varieties of medicinal licorices and different planting years. As shown in Fig. 4, the results of LEfSe analysis of all years and varieties based on the rank sum test revealed a total of 16 biomarkers with significant differences that were contained in the E.D (5 taxa), E.W (7 taxa), R.G (2 taxa) and R.W (2 taxa) groups. No discriminative taxa were observed in year 3. These biomarkers included Variovorax, Nocardiopsis, Methylophaga, Pelagibacterium, Halomonas and Sinomicrobium at the genus taxonomic level.

1.4 The relationship between the dominant phylum and genus of endophytic bacteria and the effective components, soil physicochemical properties and leaf nutrition.

Spearman correlation analysis showed that there was a significant relationship between dominant bacteria phylum and effective ingredients, soil physicochemical properties and leaf nutrition (Table 4). Specifically, Proteobacteria showed a very significant negative correlation with PTN; Actinobacteria showed a significant negative correlation with SAN, GIA and LI ($R^2 < 0$; $P < 0.05$); Firmicutes showed a significant positive correlation with PTN and CF; and Acidobacteria showed a significant positive correlation with POC ($R^2 > 0$; $P < 0.05$).

Table 4
Spearman correlation analyses testing the relationship between the relative abundance of dominant bacterial phyla in the root of licorice and effective in physicochemical properties and leaf nutrition.

	Proteobacteria	Actinobacteria	Bacteroidetes	Firmicutes	Unidentified-Bacteria	Chlamydiae	Tenericutes	Acidobacteria	Verrucomicrobia
SOM	0.098	-0.219	-0.096	-0.190	-0.253	-0.167	-0.305	-0.246	0.092
STN	0.242	-0.358	-0.004	-0.178	-0.236	-0.207	-0.131	-0.252	-0.020
STP	-0.024	-0.236	0.209	0.099	-0.129	0.125	0.165	-0.319	-0.070
STK	-0.220	0.087	0.216	0.091	-0.127	0.060	-0.225	0.111	0.256
SNN	0.075	0.154	-0.137	-0.005	-0.128	-0.241	0.292	0.128	-0.355
SAN	0.073	-0.455*	0.250	0.138	0.223	0.107	0.067	-0.068	0.208
TS	0.034	0.274	-0.228	0.045	-0.195	-0.276	0.311	0.214	-0.289
PWC	-0.088	0.111	0.020	0.173	-0.270	-0.094	0.047	0.038	-0.204
POC	-0.178	0.016	0.181	0.354	-0.290	-0.225	0.074	0.434*	0.077
PTN	-0.499**	0.291	0.187	0.430*	0.236	0.008	0.339	0.263	0.066
PTP	0.022	0.122	-0.134	0.090	-0.162	-0.177	0.238	0.130	-0.199
PTK	-0.202	0.218	-0.010	0.267	-0.339	-0.172	0.279	0.117	-0.186
CF	-0.330	0.206	0.095	0.408*	-0.054	0.170	0.343	0.121	-0.022
GIA	0.358	-0.463*	0.049	-0.274	0.185	0.040	-0.097	-0.204	-0.012
GTF	0.168	-0.354	0.031	-0.241	0.248	0.083	-0.038	-0.165	0.221
LI	0.331	-0.458*	0.079	-0.242	0.189	0.074	-0.111	-0.218	0.037

Description: the values are the correlation coefficients. ** means $P < 0.01$; * means $P < 0.05$.

As shown in Fig. 5, there was a significant relationship between the dominant bacteria genus and the effective components of roots, soil physicochemical factors and leaf nutrition. Specifically, unidentified-Rhizobiaceae had a significant positive correlation with SAN, GIA, GTF and LI, but a very significant negative correlation with CF; Pantoea had a significant positive correlation with PTK; Pseudomonas had a significant positive correlation with PTP; Halomonas had a significant positive correlation with SNN, TS, PWC, PTK and CF, but a very significant negative correlation with SAN, GIA, GTF and LI; Nocardiopsis had a significant positive correlation with TS, PWC, PTP, PTK and CF, but a very significant negative correlation with SAN, GIA, GTF and LI;

Novosphingobium had a significant negative correlation with PWC, but a very significant positive correlation with GTF; and Sinomicrobium had significant positive correlation with SNN, PWC, PTK and CF, but a significant negative correlation with GIA, GTF and LI. It is worth noting that we found that the biomarkers with significant differences at the genus taxonomic level of the dominant bacterial community (including Nocardiopsis, Halomonas and Sinomicrobium) had a significant negative correlation with GIA, GTF, and LI ($R^2 < 0$; $P < 0.05$). Details of the interrelationships between the biomarkers with significant differences in the class, orders, families and genera of dominant bacteria and effective components, soil physicochemistry and leaf nutrients are shown in Supplementary table S4. These results showed that the biomarkers with significant differences (except Methylophaga) at each classification level were significantly negatively correlated with GIA, GTF, and LI, while all showed a significant positive correlation with PTK.

1.5 Total flavonoids explained the difference in composition and distribution of endophytic bacteria community in the roots of planting licorices to the greatest extent.

Distance-based redundancy analysis (db-RDA) based on the Bray–Curtis distance showed that the effective components, soil physicochemical and leaf nutrient components had significant effects on the endophytic bacterial community in the roots of medicinal licorices (Fig. 6, Table 5). Specifically, the contents of GIA, GTF, and LI all had a significant effect on the endophytic bacterial community in the roots ($P < 0.05$). Among them, the content of GTF explained the difference in the composition and distribution of endophytic bacterial communities in the roots of cultivated medicinal liquorices to the greatest extent ($R^2 = 0.638$, $P < 0.01$). Among the soil environment factors, TS of soil was identified as the factor that most significantly affects the endophytic bacterial community, followed by SAN and SNN. Among the leaf nutrients factors, PWC was identified as the factor that most significantly affects the endophytic bacterial composition, followed by PTK and PTP. In addition, Mantel tests revealed that the combination of medicinal components is the environmental factor with the greatest correlation with the endophytic bacterial community, indicating that the combination has the greatest impact on the microbial community ($r = 0.260$; $P = 0.008$) (Supplementary table S5).

Table 5
Results for db-RDA testing effects of soil physicochemical properties, leaf nutrients and medicinal components on the composition and distribution of bacterial community in licorice root.

	r^2	*P* Value
SOM	0.118	0.213
STN	0.163	0.113
STP	0.165	0.110
STK	0.035	0.666
SNN	0.296	0.010
SAN	0.428	0.002
TS	0.497	0.000
PWC	0.498	0.001
POC	0.014	0.849
PTN	0.044	0.607
PTP	0.260	0.028
PTK	0.442	0.001
CF	0.247	0.033
GIA	0.555	0.000
GTF	0.638	0.000
LI	0.393	0.005
Description: r^2 is the determinant coefficients of the distribution of the bacterial community by environmental factors.		

2. Discussion

In this study, we first explored the effects of planting year (years 1, 2, and 3) and varieties of medicinal licorices (*Glycyrrhiza uralensis*, *Glycyrrhiza inflata*, and *Glycyrrhiza glabra*) on the accumulation of effective components. We found that the effective components were more affected by planting year than variety (Table 1), which is consistent with the results of Fan et al [49]. On the one hand, the effective components of medicinal plants are the products of physiological activities that occur in the process of cell specialization and maturation [35], and their contents increase with the growth and development of plants. On the other hand, most of the active ingredients in medicinal plants are secondary metabolites [50] (including phenolic compounds, total flavonoids and glycyrrhizin), which accumulate during plant growth and development to provide protection against pathogens and neighboring plants [51]. Similarly, the accumulation of effective components in medicinal licorice roots is easily affected by many factors. In this study, we showed that glycyrrhizic acid (GIA), liquiritin (LI) and total flavonoids (GTF) are negatively correlated with TS in soil (Table 2). We speculate that this effect is mainly because the high concentration of salts (mostly ions like Na^+) in the soil can decrease the uptake of water and nutrients by plants, as well as the rate of photosynthesis [52],

resulting in ion and osmotic stress [53], which inhibits the accumulation of secondary metabolites. However, appropriate salt stress can increase the accumulation of bioactive constituents in licorice by upregulating proteins involved in the related biosynthesis pathways to stimulate the production of glycyrrhizic acid, liquiritin and total flavonoids and other effective components [54, 55]. Wan [56] and Yang [57] et al. demonstrated that appropriate salt stress stimulated the glycometabolism of *Glycyrrhiza*, accelerated the decomposition of substances, and promoted the formation and accumulation of glycyrrhizic acid, liquiritin and total flavonoids in medicinal licorices, while high concentration and long-term salt stress inhibited the production of effective components.

In addition, our results also showed that GIA, LI and GTF had a significant positive correlation with SAN, but have no significant correlation with SNN (Table 2). We speculate that this is related to the concentration of ammonium nitrogen and nitrate nitrogen in soil and the efficiency of root absorption [58]. In addition, Pei et al. [59] showed that different forms and ratios of nitrogen can affect the growth and quality of *Glycyrrhiza*, indicating that the nitrogen sources in the soil in the form of ammonium nitrogen and nitrate nitrogen contribute collectively to the accumulation of effective components in medicinal licorices. Clarifying the role of soil factors in the growth of medicinal licorices will provide a theoretical basis for the synthesis of effective ingredients and rational utilization of medicinal plants in production practice.

In this study, we investigated the composition and diversity of endophytic bacterial communities in three medicinal licorices roots using high-throughput sequencing technology, which provides a large amount of data with more accuracy than that obtained in previous studies using traditional technology [60, 61]. Our study showed no significant differences in the alpha diversity index, which is used to describe the diversity and complexity of the bacterial community, between the planting year and species of medicinal licorices (Table 3). We speculate that this finding may be due to the samples being in the same phenological stage [62] following consistent sampling of mature medicinal licorices in August. Plant phenology is highly sensitive to seasonal changes [63] that involve changes in climate, temperature, light and other aspects of the ecological environments. This is supported by studies by Yang et al. [64–66] showing significant seasonal differences in the diversity of endophytic bacterial communities.

Our results showed specific microbiomes in 27 samples of medicinal licorices. Proteobacteria was the dominant phylum in all samples, followed by Actinobacteria and Bacteroidetes (Fig. 2a), which is consistent with the results of Cheng et al. [67] The phylum Proteobacteria, which constitutes the largest and phenotypically most diverse phylogenetic lineage at present [68], is an important group of microorganisms in evolutionary, geological, and environmental terms [69]. Numerous studies have confirmed that Proteobacteria is the main dominant phylum of the endophytic bacterial communities associated with plants ranging from economic crops, such as soybean [70] and tomato [71], to ornamental plants, such as peony [72].

Medicinal liquorice is a perennial herb of *Glycyrrhiza* in the Leguminosae family. Among the 27 samples of medicinal licorices analyzed in this study, unidentified Rhizobiaceae was the dominant genus (Fig. 2b). Unidentified-*Rhizobiaceae*, which may be non-culturable or not studied, may be a bacterium of potential importance in the endophytic bacterial community of licorice, because of the nitrogen fixation function of rhizobia, which has become a research hotspot in legumes [73]. Wei et al. [74] demonstrated high tolerance to NaCl, pH and temperature in a large number of isolated rhizobia from *Glycyrrhiza uralensis*. Liu et al. [68] showed that inoculation with rhizobia improved the growth of Glycyrrhiza uralensis and increased the dry weight of the underground rhizome. These studies all showed that beneficial rhizobia coexist with and promote the growth of *Glycyrrhiza* medicinal licorices. In addition, our results also showed a significant positive correlation between unidentified-*Rhizobiaceae* and GIA, GTF and LI (Fig. 5), indicating that beneficial rhizobia may improve the contents of effective ingredients of medicinal licorices. Although the scope of beneficial and culturable *Rhizobiae* is still unclear at present, this information provides a direction for future research on improving the efficacy of medicinal licorices.

Although many studies the composition and structure of plant endophytic bacterial communities are based on Illumina MiSeq technology [75], few studies have focused on the influence of planting year and variety on the composition and structure of endophytic bacterial communities in *Glycyrrhiza*. Our results show that the community structure of endophytic bacteria is affected by the planting year and variety of *Glycyrrhiza* (Fig. 3 and Fig. 4), which helps to fill the knowledge gap in this field. Endophytic bacteria exist in plant species as part of the root microbiome. The community structure of endophytic bacteria (species diversity: abundance and relative abundance) in plants is dynamic and affected by abiotic and biological factors, such as soil conditions, biogeography and plant species [76]. In this study, in addition to soil physical and chemical factors and leaf nutrients, the content of effective components in roots was shown to be the key driving factor of endophytic bacterial community (Fig. 6, Table 5, Supplementary table S5), with the total flavonoid content in medicinal licorices shown to explain the differences in the composition and distribution of endophytic bacteria to the greatest extent. Flavonoids, which are a variety of polyphenolic compounds produced in plants by secondary metabolism, can act as a signal of interaction between plants and many microorganisms [77, 78]. For example, flavonoids can act as chemoattractants and inducers of nodulation (nod) and other genes in rhizobia [79]. In recent years, a large number of studies including research by Márton Szoboszlay et al. [80] have also shown that flavonoids have an impact on bacterial communities.

In general, the alpha and beta diversity analyses indicated that the presence of many endophytic bacteria in the 27 different samples of roots of medicinal licorices. The endophytic bacterial structure and composition differed among the samples, and our study also indicates that the endophytic bacterial community in the roots of medicinal licorices is determined by the effective components in the roots. In addition, this result is consistent with the hypothesis that the physical and chemical factors in the soil and leaf nutrition dictate the structure and composition of the endophytic bacterial population, although further studies are required to characterize the roles of these endophytic bacteria. Our study provides useful information for the development of strategies to improve the production and quality of medicinal licorices.

3. Conclusions

Clarification of the accumulation and changes in medicinal components in different growth years and development stages is of great significance for the quality control of medicinal materials and the improvement of clinical efficacy of traditional Chinese medicines. In this study, the contents of glycyrrhizic acid, liquiritin and total flavonoids increased significantly with the planting year, and the accumulation of effective components was affected by the physical and chemical properties of the soil and leaf nutrition. In addition, numerous endophytic bacteria were detected in the 27 samples of roots of medicinal licorices

based on high-throughput sequencing technology. The effective components, soil physicochemical and leaf nutritional factors all have a certain impact on endophytic bacteria community in the roots of medicinal licorices, with content of active components identified as the main factor that dictates the structure of the endophytic bacterial community. The roots of medicinal licorices are the sources of materials used in Chinese herbal medicine and the habitats of various microorganisms. This study not only provides information on the composition and distribution of endophytic bacteria in the roots of medicinal licorices, but also characterizes the influence of abiotic factors on the community structure of endophytic bacteria, which makes an important contribution to the research on the diversity of endophytic bacteria in plants.

4. Methods

4.1 Sample collection

In May 2016, the seeds of three varieties of medicinal licorices (*Glycyrrhiza uralensis Fisch.*, *Glycyrrhiza glabra L.*and *Glycyrrhiza inflata Batal.*) were purchased from Xinjiang Beiling licorice Technology Co., Ltd (Xinjiang, China), and were sown in the south of Yanqi County (86°17'33" E, 42°11'34" N), Xinjiang, China. There were nine separate sample plots (4 × 4 m per plot, 3 plots × 3 replicates for each variety of licorices). We watered the seeds three times during the germination period, middle growth period and mature period. Seeds were sown in this way every year for three years under conditions that simulated the natural growth of medicinal licorice. Field sampling was conducted in August 2017, August 2018 and August 2019, when liquorice roots and rhizosphere soil (depth 0–40 cm) were collected. To ensure that the experiment was representative, three well-grown licorice roots were randomly selected and cut out with sterile scissors. Each root was divided into two parts: one part was placed into a ziplocked bag for the determination of the effective components in the root, while the other part was placed into a sterile bag and quickly transported on ice to the laboratory. To eliminate the interference of other microorganisms, the surface of roots was sterilized in the laboratory by first rinsing soil from the roots under running water followed by washing with sterile distilled water. The roots were then soaked in 75% alcohol for 30 s for surface disinfection, then washed five times with sterile distilled water before soaking in 5% sodium hypochlorite for 5 min. Finally the roots were washed five times with sterile distilled water and air-dried under sterile conditions. To confirm that the surface sterilization process was successful, the last rinse solution was inoculated onto a potato dextrose agar (PDA) plate and cultured at 28℃ for 72 h. No bacterial growth confirmed that the surface sterilization was successful. All root samples were immediately placed on ice and then stored at liquid nitrogen prior to total DNA extraction. Soil samples from the rhizosphere were air-dried and sieved through a 2-mm mesh. The following prior to physicochemical characteristics were analyzed according to the methods described by the Beyaert [41]: organic matter (SOM), total nitrogen (STN), total phosphorus (STP), total potassium (STK), total salt (TS), nitrate nitrogen (SNN) and ammonium nitrogen (SAN). In addition, leaf samples, were air-dried to constant weight, ground to a powder using a pestle and mortar and passed through a 60-mesh sieve. The following plant nutritional components were then analyzed [42]: organic carbon (POC), water content (PWC), total nitrogen (PTN), total phosphorus (PTP), total potassium (PTK) and crude fiber (CF). All the samples were labeled by combination with letters and numbers, with the first letter representing the age of the medicinal licorices (E, R and S: years 1, 2, and 3, respectively), the second letter representing the species (W, G and D: *Glycyrrhiza uralensis Fisch.*, *Glycyrrhiza glabra L.*and *Glycyrrhiza inflata Batal.*, respectively), and the third number representing the replicate number. For example, E.W.3 represents the third repetition of *Glycyrrhiza uralensis* in the first year.

4.2 Determination of active components

The root samples were dried at 60 °C for 72 h to constant weight (it has been confirmed that glycyrrhizic acid (GIA) and liquiritin (LI) do not decompose at this temperature [43]). The dried root samples were ground to a powder with a pestle and mortar and passed through a 60 mesh sieve. An aliquot (0.2 g) of powdered root sample was extracted with 71% chromatographic methanol in an ultrasonic bath (250 W, 40 kHz) at room temperature. The GIA and LI contents in the dried root samples (0.2 g) of the medicinal licorices were determined by high-performance liquid chromatography (HPLC, Agilent-1260 Infinity, USA) using an Agilent ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm) with mobile phase (chromatographic methanol: ultra-pure water: 36% glacial acetic acid = 71:28:1) and mobile phase (acetonitrile:0.5% glacial acetic acid = 1:4) respectively, and a gradient elution flow rate of 1.0 mL•min-1. GIA and LI were detected at 254 nm and 276 nm, respectively. The injection volume was 5 μL and the column temperature was 30 °C. The GIA and LI reference materials (CAS#1405-86-3 and CAS#551-15-5, respectively) were purchased from Solarbio and used for calibration purposes. The total flavonoid content (GTF) in medicinal licorices was determined by ultraviolet spectrophotometry at 334 nm with the LI standard (CAS#551-15-5) as the control.

4.3 DNA extraction and library construction

After immersion in liquid nitrogen, genomic DNA was extracted from the samples using the DNA Quick Plant System kit (Tiangen, China) according to the manufacturer's instructions. The purity and concentration of DNA were detected by 1% agarose gel electrophoresis. According to the concentration, each DNA sample was diluted a final concentration to 1 ng/μL with sterile distilled water for use as a DNA template. The 16S rDNA genes of the V4 region were amplified using specific primers (515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R: 5'-GGACTACHVGGGTWTCTAAT-3') with barcodes[44]. PCR analyses were carried out with Phusion® High-Fidelity PCR Master Mix and GC Buffer (New England Biolabs) to ensure amplification efficiency and accuracy. PCR runs started at at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 5 min.

The PCR product was mixed with the same volume of 1 × loading buffer (containing SYBR green) and then was detected by 2% agarose gel electrophoresis. The PCR product was purified from the target strip using a Gel Extraction Kit (Qiagen, Germany). The libraries were constructed using a TruSeq®DNA PCR-Free Sample Preparation Kit (Illumina, USA) according to the manufacturer's instructions, and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, Amplicon sequencing was performed using the Illumina HiSeq2500 platforms at the Beijing Compass Biotechnology Co., Ltd. (Beijing, China).

4.4 Bioinformatics analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. The paired-end reads of each sample were spliced using FLASH (V1.2.7) [45] and then assembled to generate raw tags. The raw tags were subjected to a strict quality filtering

process using QIIME (V1.9.1) [46] to obtain high-quality clean tags [47]. Effective tags were obtained by comparison of the clean tags sequence with the UCHIME Algorithm [48] and Gold database to detect and remove chimeric sequences.

We used UPARSE software (UPARSE v7.0.1001) to cluster the effective tags of all samples into the same operational taxonomic units (OTUs) with ≥ 97% identity, and taking the sequence with the highest frequency as the representative sequence of each OTU. The taxonomic information for each representative sequence was annotated using the SILVA database (threshold value set to 0.8–1), and multiple sequence alignment was performed using MUSCLE (Version 3.8.31) software to study the phylogenetic relationship of the representative sequences of OTUs among the 27 root samples.

4.5 Statistical analysis

R software (Version 2.15.3) was used for alpha diversity index and beta diversity analyses, rarefaction curve generation, Spearman correlation analysis of heat maps, Mantel tests and distance-based redundancy analysis (db-RDA). LEfSe software was used for linear discriminant analysis (LDA) and effect size (LEfSe) analysis with the default filtering value of LDA score set at 4. Statistical analysis (including one-way analysis of variance (ANOVA) and Spearman correlation analysis) were performed with SPSS 19.0 (IBM Inc., Armonk, USA), one-way ANOVA was used to analyze the effects of different year and licorice species on the contents of effective components. Spearman correlations (r) were run among the effective components, the soil physicochemical properties and nutritional components of leaves.

Abbreviations

HPLC
high-performance liquid chromatography
PDA
potato dextrose agar
GIA
glycyrrhizic acid
LI
liquiritin
GTF
total flavonoid
SOM
organic matter
STN
total nitrogen
STP
total phosphorus
STK
total potassium
TS
total salt
SNN
nitrate nitrogen
SAN
ammonium nitrogen
POC
organic carbon
PWC
water content
PTN
total nitrogen
PTP
total phosphorus
PTK
total potassium
CF
crude fiber
OTUs
operational taxonomic units
db-RDA
distance-based redundancy analysis
LDA
linear discriminant analysis
LEfSe

linear discriminant analysis effect size
ANOVA
analysis of variance

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was financially supported by the Chinese National Basic Research Program (2014CB954203) and the National Natural Science Foundation of China (31360139, 41561010 and 31560177). All funding agencies provided financial support only and did not participate in the design of experiments, sampling and manuscript writing tasks.

Authors' contributions

L.Z., H.D. and T.Z., designed the experiment. H.D and T.Z. performed methodology, data analysis, investigated and were a major contributor in writing the manuscript. G. Li., Y. Mu, X. Lv. and Z. W. collected the samples and modified the manuscript. H.D. and T.Z. contributed equally to this work and should be considered co-first authors. All authors read and approved the final manuscript.

Acknowledgments

In this study, we would like to thank professor L.Z for hers guidance, all the authors for their joint efforts. We would like to thank many graduate students who are not listed as co-authors and to the staff involved in the maintenance of farmland and the collection of soil samples.

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Supplementary Files Legends

Supplementary table S1 Sequencing results of each sample.

Description: Raw reads refers to the sequence filtering out low-quality bases; clean reads refers to the sequence finally used for subsequent analysis after filtering chimeras; base refers to the number of bases of final clean reads; Avglen refers to the average length of clean reads. Q20 refers to the percentage of bases whose quality value is greater than 20 (sequencing error rate is less than 1%); GC (%) refers to the content of GC bases in clean reads; effective (%) refers to the percentage of the number of clean reads and the number of raw reads. Sample name: E, R and S: years 1, 2, and 3, respectively; W, G and D: *Glycyrrhiza uralensis Fisch*., *Glycyrrhiza glabra L*.and *Glycyrrhiza inflata Batal*., respectively; the third number representing the replicate number.

Supplementary table S2 Composition information of dominant bacteria at each classification level

Description: Others: The sum of the undefined and unannotated parts.

Supplementary table S3 The alpha diversity indices in each group.

Description: Community richness was identified using the Chao1 and ACE estimator. Community diversity was identified using the Shannon and Simpson indexes. Sequencing depth was characterized by Good's coverage, good's coverage estimator values ranged from 99.6–99.8%, indicating that the sequence numbers per group were high enough. Group: E, R and S: years 1, 2, and 3, respectively; W, G and D: *Glycyrrhiza uralensis Fisch*., *Glycyrrhiza glabra L*.and *Glycyrrhiza inflata Batal*., respectively.

Supplementary table S4 Spearman correlation analyses testing the relationship between the Biomarker and the relationship between the physicochemical properties of soil, leaf nutrients and medicinal components of licorice root.

Description: The values are the correlation coefficients. ** means $P < 0.01$; * means $P < 0.05$.

Supplementary table S5 Correlations between environmental factors and endophytic bacterial communities in each group.

Description: Variable is the information of environmental factors, *r* is the correlation coefficient, and *P* value is the *p*-value of significance test. The larger *r* value is, the greater the correlation between environmental factors and species abundance information is. $P < 0.05$ indicates statistical significance.

Figures

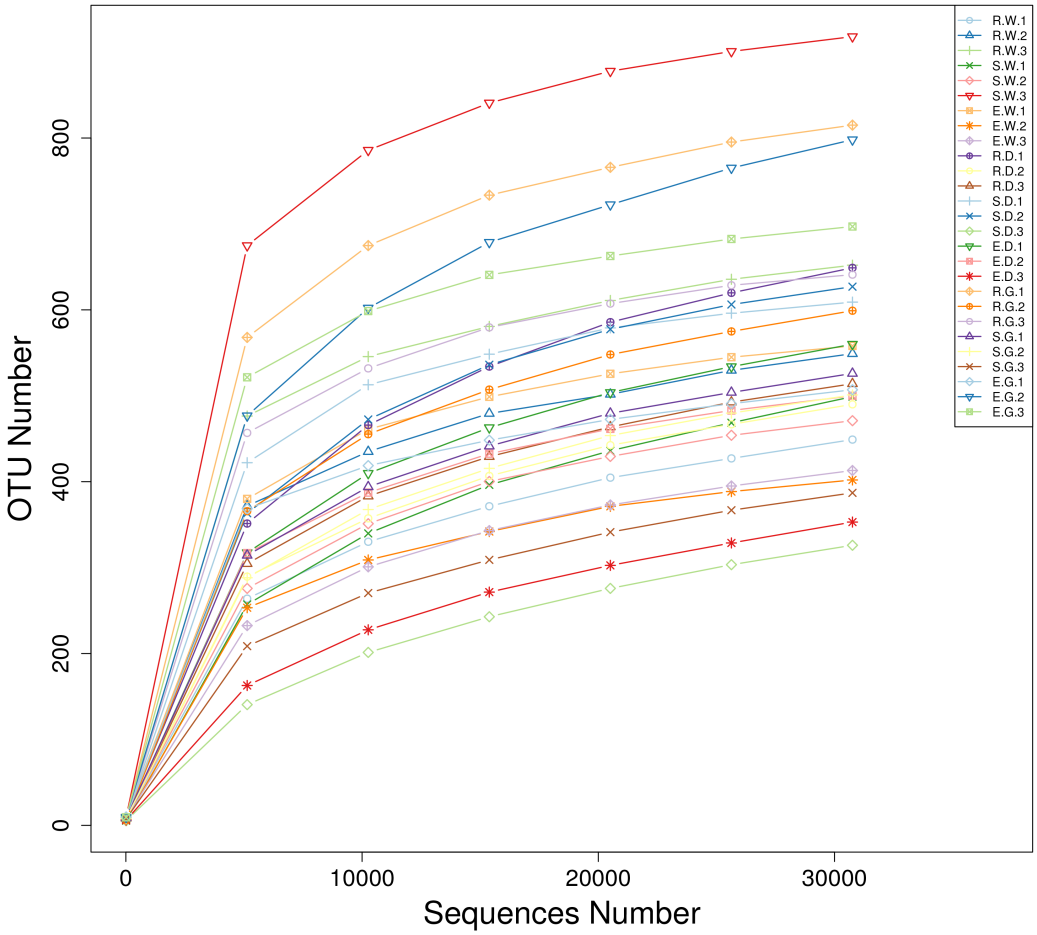


Figure 1

Rarefaction curves of bacterial community composition in 27 samples. Description: The rarefaction curves different colors represent different samples (E, R and S: years 1, 2, and 3, respectively; W, G and D: Glycyrrhiza uralensis Fisch., Glycyrrhiza glabra L.and Glycyrrhiza inflata Batal., respectively; the third number representing the replicate number).

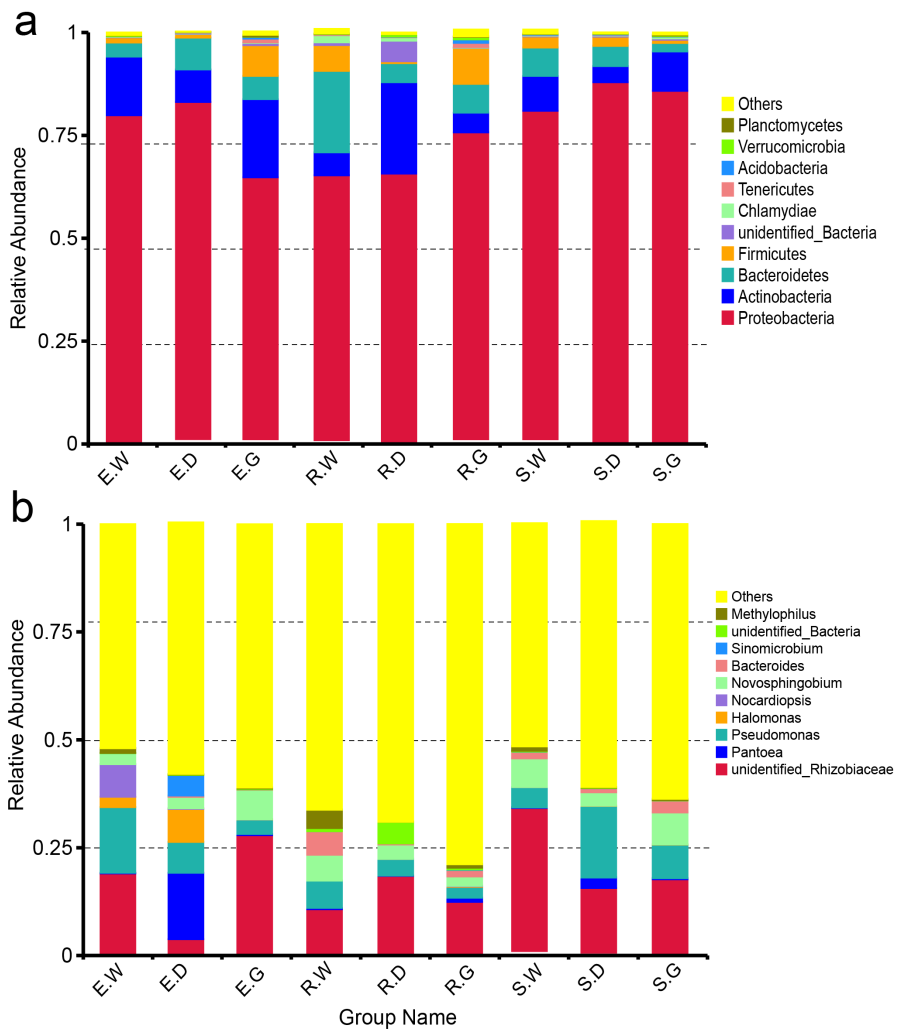


Figure 2

Histograms of relative abundance of the top 10 bacteria at the phyla (a) and genera (b) level of taxonomy. Description: Ordinate is the relative abundance; others refers to are sequences with less or not be annotated; abscissa is the group name (E, R and S: years 1, 2, and 3, respectively; W, G and D: Glycyrrhiza uralensis Fisch., Glycyrrhiza glabra L.and Glycyrrhiza inflata Batal., respectively).

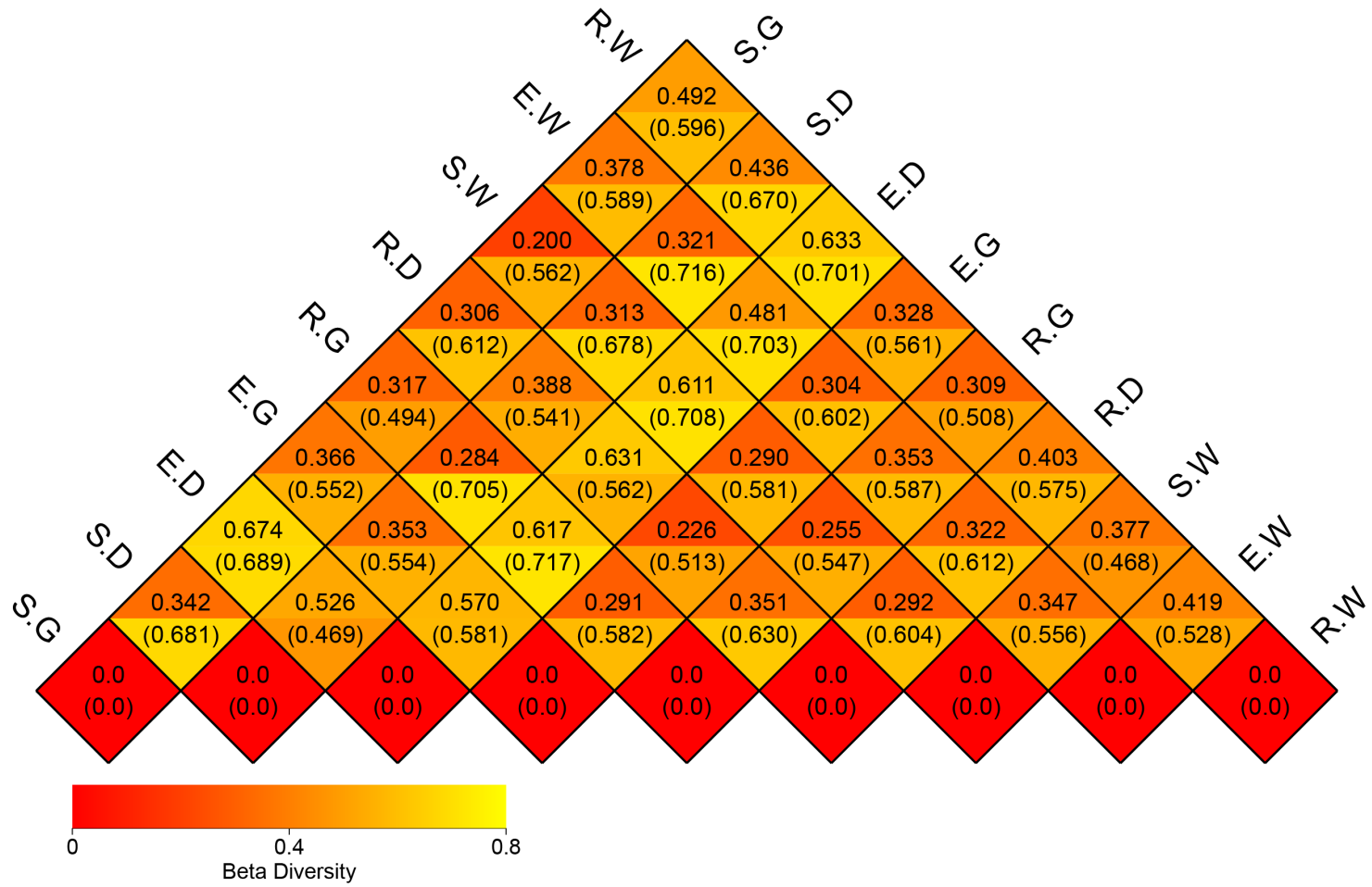


Figure 3

Beta diversity heat map based on the weighted-UniFrac and unweighted-UniFrac distance. Description: The numerical of upper triangle is the weighted distance. The numerical from the lower triangles is the unweighted distance. The smaller the numerical, the smaller the difference between the two groups in terms of species diversity. Abbreviations is the group name: E, R and S: years 1, 2, and 3, respectively; W, G and D: Glycyrrhiza uralensis Fisch., Glycyrrhiza glabra L.and Glycyrrhiza inflata Batal., respectively.

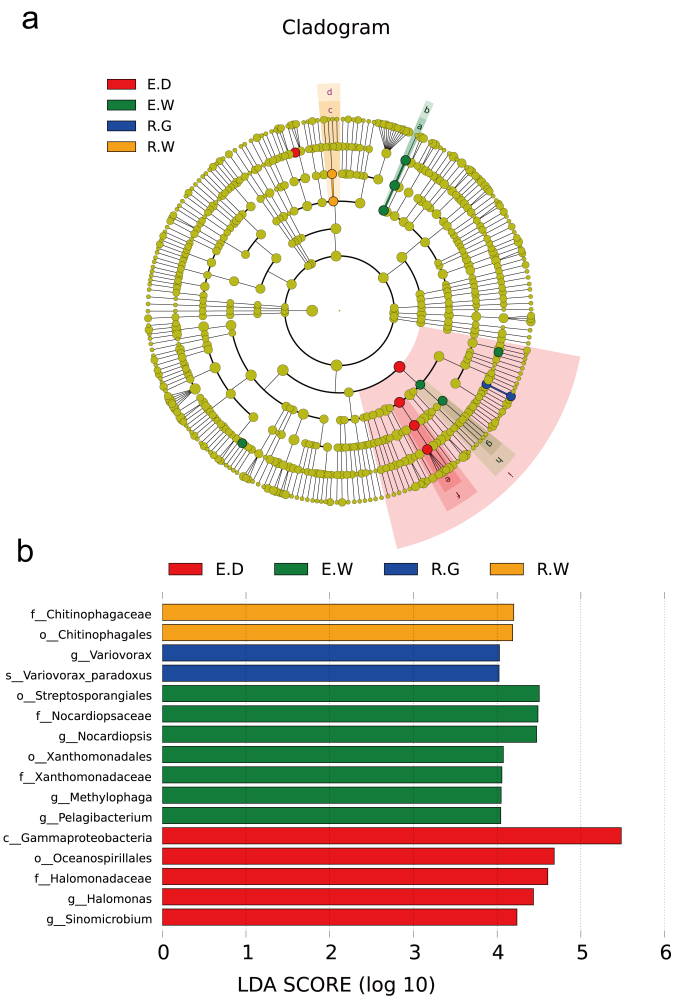


Figure 4

Cladograms (a) and LDA value distribution histogram (b) in 27 samples. Description: In cladograms (a), the circle radiating from inside to outside represents the taxonomic level from the Phylum to the species. Each small circle at a different taxonomic level represents a taxonomic at that level, and the diameter of the small circle is proportionate to the relative abundance of species. The figure shows the species with LDA Score greater than the set value (default setting is 4) (b), that is, species with significant differences in different groups. The length of the histogram represents the size of the influence of species with significant differences. The English letters in the figure is the group name (E, R and S: years 1, 2, and 3, respectively; W, G and D: Glycyrrhiza uralensis Fisch., Glycyrrhiza glabra L.and Glycyrrhiza inflata Batal., respectively).

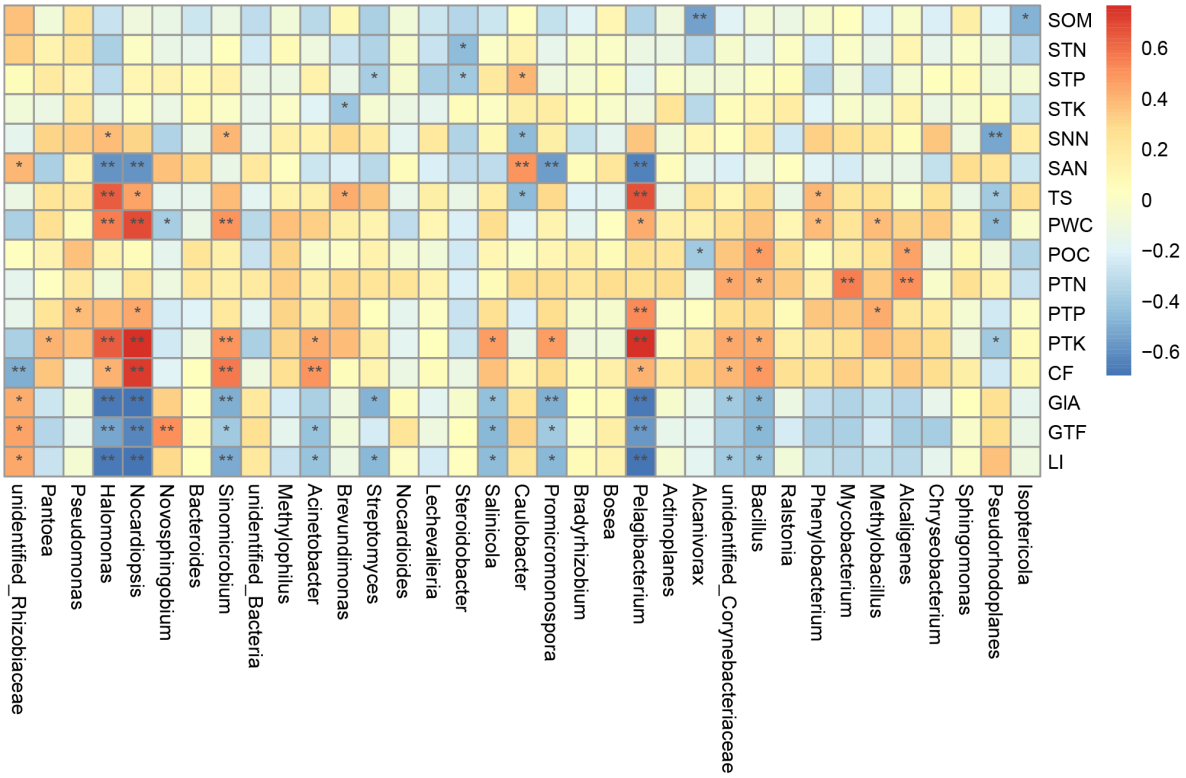


Figure 5

Heat maps of Spearman correlation analysis. Description: Ordinate is the information of environmental factors, and abscissa is the information of species at the genera level of taxonomy. The correlation coefficient r of Spearman is between -1 and 1, $r < 0$ is negative correlation, $r > 0$ is positive correlation, and the mark * is significance test $p < 0.05$.

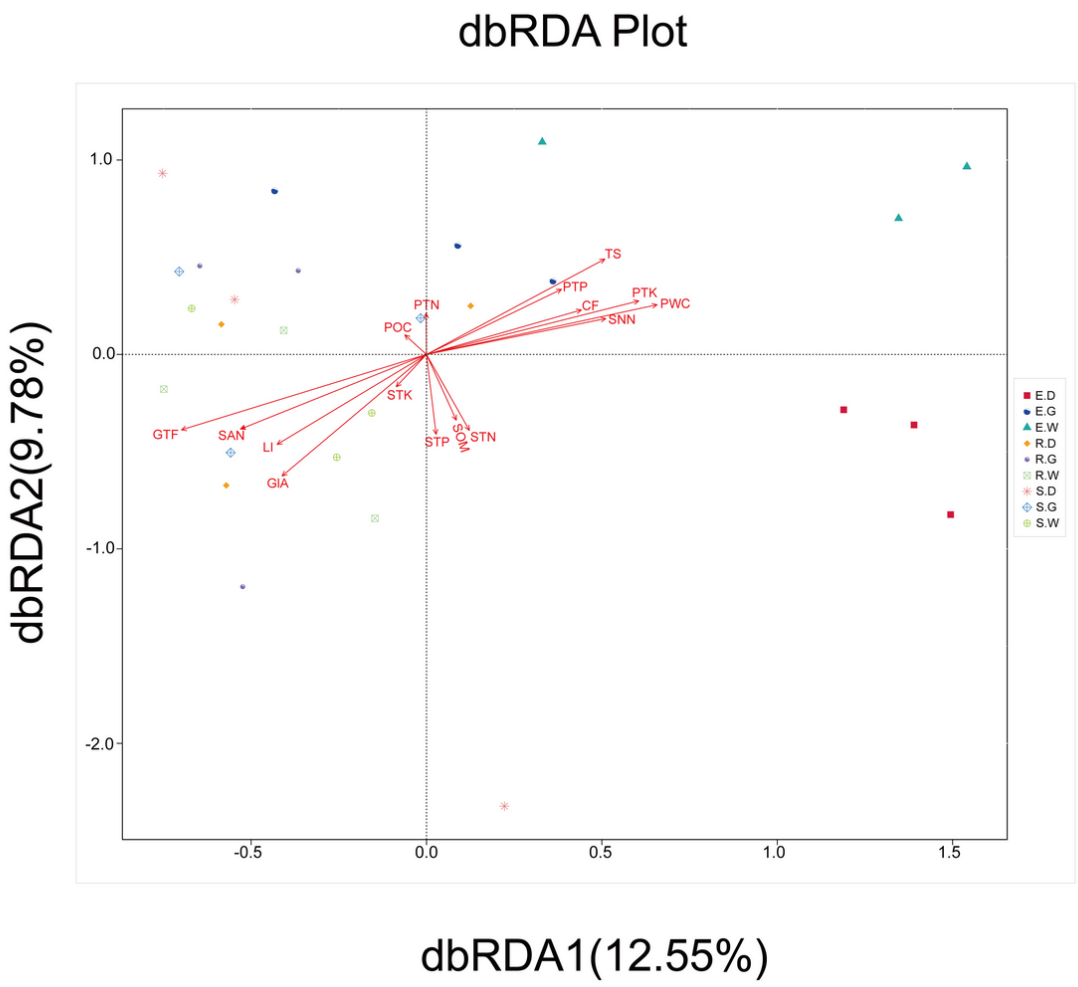


Figure 6

Heat maps of Spearman correlation analysis. Description: Ordinate is the information of environmental factors, and abscissa is the information of species at the genera level of taxonomy. The correlation coefficient r of Spearman is between -1 and 1, r < 0 is negative correlation, r > 0 is positive correlation, and the mark * is significance test p < 0.05.

Supplementary Files

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