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Original Article

Assembly and network of *Rhei Radix* et *Rhizoma* surface microbiome shaped by processing methods and sampling locations

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ABSTRACT

Objective: *Rhei Radix* et *Rhizoma* has five types of products, namely, raw rhubarb (RR), wine rhubarb (WR), vinegar rhubarb (VR), cooked rhubarb (CR), and rhubarb charcoal (RC). However, *Rhei Radix* et *Rhizoma* is easily contaminated with fungi and mycotoxins if not harvested or processed properly. Here, we intend to analyze how microbiome assemblies and co-occurrence patterns are influenced by sampling locations and processing methods.

Methods: High-throughput sequencing and internal transcribed spacer 2 (ITS2) were carried out to study the diversities (α- and β-diversity), composition (dominant taxa and potential biomarkers), and network complexitity of surface fungi on RR, WR, VR, CR, and RC collected from Gansu and Sichuan provinces, China.

Results: The phyla Ascomycota and Basidiomycota; the genera *Kazachstania*, *Malassezia*, and *Asterotremella*; and the species *Kazachstania exigua*, *Asterotremella pseudolonga*, and *Malassezia restricta* were the dominant fungi and exhibited differences in the two provinces and the five processed products. The α-diversity and network complexity were strongly dependent on processing methods. Chao 1, the Shannon index, and network complexity and connectivity were highest in the CR group. The α-diversity and network complexity were influenced by sampling locations. Chao 1 and network complexity and connectivity were highest in the Gansu Province.

Conclusion: The assembly and network of the surface microbiome on *Rhei Radix* et *Rhizoma* were shaped by processing methods and sampling locations. This paper offers a comprehensive understanding of microorganisms, which can provide early warning for potential mycotoxins and ensure the safety of drugs and consumers.

1. Introduction

Rhei Radix et *Rhizoma* (Dahuang in Chinese) is the dried rhizomes and roots of perennial plant *Rheum palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinate* Baill. within the family Polygonaceae (Chinese Pharmacopoeia Committee, 2020). *Rhei Radix* et *Rhizoma* is an important traditional Chinese medicine (TCM) and food with great economic and medicinal values whose main bioactive ingredients are anthraquinone, anthrone, stilbene, tannin, acylglucoside, butyrophenone, and naphthealene glycoside derivatives (Fan, Niu, Xu, Yang, & Yang, 2019). *Rhei Radix* et *Rhizoma* has a wide range of pharmacological activities, such as purgative, anti-inflammatory, anti-fungal, anti-tumor, and liver protecting effects (Dai et al., 2021; Gecibesler et al., 2021; Yang, Zhou, Liao, & Lv, 2023).

TCM processing is a distinguished and unique pharmaceutical technique in China that has been applied for thousands of years (Sun et al., 2020). TCM processing may affect chemical components, thereby increasing medical potencies, retaining active ingredients, altering pharmacological properties, decreasing side effects, and even altering efficacy (Lei et al., 2021; Guo, Brand, & Zhao, 2015). *Rhei Radix et Rhizoma* is a typical example that is used either

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raw or processed in clinical applications. *Rhei Radix* et *Rhizoma* includes the following products: raw rhubarb (RR), wine rhubarb (WR), cooked rhubarb (CR), vinegar rhubarb (VR) and rhubarb charcoal (RC) (Chinese Pharmacopoeia Committee, 2020; Li et al., 2009). Different processing methods will lead to changes in the active ingredients of *Rhei Radix* et *Rhizoma* and even change its pharmacological action and clinical therapeutic effect (Zhu, Liu, Wang, Zhu, & Cai, 2016). If mastered and applied improperly, then it will even produce toxic side effects.

Thus far, researchers have focused on the differences in chemical components and pharmacological effects of *Rhei Radix* et *Rhizoma* before and after processing (Gao, Guo, Xu, & Yang, 2017; Bai et al., 2024). *Rhei Radix* et *Rhizoma*, particularly after being processed, is easily contaminated with fungi and mycotoxin if harvested or processed improperly. In particular, the mycotoxins contamination levels of *Rhei Radix* et *Rhizoma* samples were notable (13.91–52.69 μg/kg aflatoxins, 6.81–8.35 μg/kg ochratoxins, and 18.73–6 052.52 μg/kg fumonisims) (Wei et al., 2023a). Mycotoxins, especially aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, and aflatoxin M1, have a variety of toxic side effects on humans (Han et al., 2012).

Mycotoxins are produced by a variety of fungi. Some strains of *Aspergillus*, *Fusarium*, *Alternaria*, and *Penicillium* can produce mycotoxins such as aflatoxins, fumonisins and ochratoxins (Zheng, Wang, Xu, Zhan, & Chen, 2014). However, traditional isolation and identification methods are time consuming and have difficulty fully monitoring the diversity and composition of fungal communities. High-throughput sequencing can provide a large amount of data on low-abundance microbial community and has been widely applied in fungal research (Tedersoo et al., 2014; Lee, An, Xu, & Yamamoto, 2016), thereby providing a promising new prospect to study the fungal diversity and composition of surface microbiome in herbs (Wei et al., 2023a). The diversity and composition of microorganisms in *Arecae semen*, *Semen Persicae*, and *Polygoni Multiffori Radix* were analyzed by high-throughput sequencing (Wei et al., 2023b; Wei et al., 2023c; Wei et al., 2024). However, the microbiome assemblies of surface fungal communities of *Rhei Radix* et *Rhizoma* and whether they are shaped by the sampling location and processing methods remain unknown.

The current study analyzed fungal diversity and composition on the surface of five types of *Rhei Radix* et *Rhizoma* products collected from two Gansu and Sichuan provinces, China by targeting the internal transcribed spacer 2 (ITS2) through high-throughput sequencing. The study intends to analyze how microbiome assemblies and co-occurrence patterns are influenced by sampling locations and processing methods. This study reports the surface microbiome of *Rhei Radix* et *Rhizoma* samples and will provide a reference for the safe application and quality improvement of *Rhei Radix* et *Rhizoma*.

2. Materials and methods

2.1. Sample collection

A total of thirty *Rhei Radix* et *Rhizoma* samples were collected from Sichuan Province (SC, n = 15) and Gansu Province (GS, n = 15), China (Table S1). The samples we collected were identified by Professor Shilin Chen, Chengdu University of Traditional Chinese Medicine. The samples were divided into two groups by production areas, namely, SC and GS. The samples were divided into five processed groups: raw rhubarb (RR, n = 6), wine rhubarb (WR, n = 6), vinegar rhubarb (VR, n = 6), cooked rhubarb (CR, n = 6), and rhubarb charcoal (RC, n = 6). These products were processed according to the methods recorded in previous studies (Wang et al., 2022; Xie et al., 2022). Each processing method in each sampling area had at least three samples. In order to ensure the reliability of the samples, the samples were stored under the same methods and same environments. Each sample was collected approximately 500 g and stored at −20 °C until the DNA extraction.

2.2. DNA extraction, PCR amplification, and high-throughput sequencing

A total of 10 g of each *Rhei Radix* et *Rhizoma* was transferred to a 100 mL sterilized conical flask. Then, sterilized water (25 mL) was added, and the mixture was shaken for 20 min. The mixture was centrifuged for 20 min at 7 830 r/min to obtain surface microorganisms of *Rhei Radix* et *Rhizoma* samples (Zhao et al., 2007). Next, total DNA was extracted by using the PowerSoil® DNA Isolation Kit (MoBio, New York, USA) according to the instructions. The quality of DNA was measured by NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, New York, USA) and 1% agarose gel electrophoresis.

The fungal primer pairs fITS7/ITS4 (5′-GTGARTCATC GAATCTTTG-3′) / (5′-TCCTCCGCTTATTG ATATGC-3′) were applied to amplify the ITS region (Adams, Miletto, Taylor, & Bruns, 2013). The PCR reaction mixture consisted of Phusion® Hot Start Flex 2X Master Mix (12.5 μL), each primer (2.5 μL, 200 mmol/L), template DNA (50 ng), and PCR-grade water with a final volume of 25 μL (Lin, Wang, Li, Xu, & Li, 2022). The PCR amplification program was performed under the following conditions: pre-denaturation at 98 °C for 30 s; 32 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s; elongation at 72 °C for 45 s, and final extension at 72 °C for 10 min. The PCR products were detected with 2% agarose gel, purified using the DNA gel extraction kit (Axygen, Guigu, USA), and sequenced on the NovaSeq PE250 platform (Illumina, USA) for paired-end reads (2 × 250 bp). The fast files were uploaded to the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) with the accession number PRJNA1102931.

2.3. Sequence analysis

The raw fastq files were demultiplexed and quality-filtered using UPARSE (v8) (Edgar, 2013) and QIIME (v1.8) (Caporaso et al., 2010). The sequences matching the mitochondria and chloroplast were removed, and then the paired reads were truncated, and an average Phred quality score of ≥ Q20 equivalent to a 0.01% error rate was acquired (Marasco, Rolli, Fusi, Michoud, & Daffonchio, 2018). The obtained amplified sequence variants (ASVs) were used to explore α- and β-diversity and taxonomic assignment (Izawa et al., 2020). Taxonomy (at the phylum, class, order, family, genus, and species levels) was assigned to the representative sequences of the ASVs on the basis of the UNITE and INSDC databases (Nilsson et al., 2019b; Kõljalg et al., 2013).

The α-diversity indices (i.e., Chao 1 and Shannon) were calculated by mothur (v.1.30.1, https://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity) (Schloss, Flanagan, Culler, & Wright, 2009). Chao 1 and Shannon indices are two important diversity estimators, high Shannon index represents higher fungal community diversity and high Chao index reflects higher fungal community richness (Roswell, Dushoff, & Winfree, 2021). Non-metric multidimensional scaling (NMDS) was performed on the basis of the unweighted UniFrac distance matrices to analyze the difference of β-diversity among different groups with R software (v.3.3.1, https://www.r-project.org/). Anova analysis was performed to measure significant differences among different groups (Oksanen, Kindt, Legendre, O'Hara, & Wagner, 2007). A Venn diagram was drawn to identify the unique and shared ASVs among different groups using R software (v.3.3.1, https://www.r-project.org/). Linear discriminant analysis effect size (LEfSe) with linear discriminant analysis (LDA) score higher than 4.0 and P-values less than

0.05 was used to determine the discriminant taxa among different groups using LEfSe software (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload) (Segata et al., 2011). Circos diagrams showing the distribution proportion of core microbiota were developed using the Circos-0.67–7 software (Krzywinski et al., 2009). The co-occurrence network analysis was used to explore the significant relations among the taxa by using Cytoscape software (v3.7.2) (Faust et al., 2012). Furthermore, only highly and statistically significant correlations (Spearman's $|r|>0.8$ and $P < 0.05$) were kept, and the network was visualized with Gephi (Bastian, Heymann, & Jacomy, 2009).

3. Results

3.1. Microbiome diversity on surface of Rhei Radix et Rhizoma affected by processing methods and sampling locations

Approximately 2 220 921 fungi reads were achieved on the surface of *Rhei Radix* et *Rhizoma* samples (Table S2). According to Venn profiles, a total of 15 ASVs were shared in five processing methods; 71, 87, 50, 166, and 38 ASVs were unique in RR, WR, VR, CR, and RC, respectively (Fig. 1A). The α-diversity values of fungi were strongly dependent on processing methods (ANOVA, $P < 0.05$; Fig. 1C and Table S3). The Chao 1 index was significantly higher in the CR (53.15) and WR (45.06) groups than in the RR (32.00), VR (32.00), and RC (22.08) groups. The Shannon index was highest in the CR group (3.80) and lowest in the RR group (2.27). The β-diversity variation of fungal community was visualized by NMDS, and the results showed that microbiome was clustered in different sampling locations (stress = 0.20; Fig. 1E).

The difference of fungal community was acquired between two sampling locations but was not significant. Venn profiles showed that 96 ASVs were shared in two sampling locations; 194 and 236 ASVs were unique in SC and GS, respectively (Fig. 1B). The α-diversity was slightly influenced by sampling locations, but the difference was not significant (ANOVA, $P > 0.05$; Fig. 1D and Table S4). The Chao 1 index was slightly higher in the GS group (38.67) than in the SC group (33.77). The Shannon index was higher in the SC group (3.17) than in the GS group (2.90). The β-diversity similarities of fungal communities between two sampling locations were clustered on the basis of processing methods (Fig. 1F).

3.2. Microbiome composition on surface of Rhei Radix et Rhizoma affected by processing methods and sampling locations

At the phylum level, Ascomycota, Basidiomycota, Fungi unclassified, and Zygomycota were the top four phyla and demonstrated differences in five processing methods (Figs. 2A and 3A, and Table S5). The abundance of Ascomycota was highest in the RR group (66.64%) and lowest in the RC group (26.25%) ($P < 0.05$). Basidiomycota had the highest proportion in the RC group (69.94%) and the lowest in the RR group (29.58%) ($P < 0.05$). At the genus level, *Kazachstania*, *Malassezia*, *Asterotremella*, *Wallemia*, *Cochliobolus*, and *Cryptococcus* were the dominant genera and presented differences in five processing methods ($P < 0.05$; Figs. 2B and 3B, and Table S5). *Kazachstania* was the most abundant in the RR group (47.10%), followed by the WR group (16.54%), and was lowest in the RC group (1.43%). *Malassezia* had the highest abundance in the CR group (25.32%), followed by the VR group (18.29%) and the RC group (18.01%). *Asterotremella* had the highest abundance in the RC group (27.58%) and the lowest abundance in the VR group (0.78%). *Wallemia* had the highest abundance in the RC group (21.42%), followed by the WR group (9.45%). Moreover, *Cochliobolus* was highest in the VR group (23.66%), while *Cryptococcus* was highest in the RR group (9.98%). At the species level, *Kazachstania exigua*, *Asterotremella pseudolonga*, *Malassezia restricta*, *Wallemia sebi*, *Ascomycota unclassified*, and *Cochliobolus verruculosus* were the predominant species and revealed differences in five processing methods (Figs. 2C and 3C, and Table S5). *Kazachstania exigua* was highest in the RR group (47.10%), followed by the WR group (16.54%) and the VR group (11.91%) ($P < 0.05$). The abundance of *Asterotremella pseudolonga* was highest in the RC group (27.58%) and lowest in the VR group (0.78%) ($P < 0.05$). The abundance of *Malassezia restricta* (14.88%) and *Wallemia sebi* (20.82%) was highest in the RC group, while the abundance of *Cochliobolus verruculosus* was highest in the VR group (19.54%).

At the phylum level, significant differences existed in the abundances of Ascomycota and Basidiomycota between the two provinces ($P < 0.05$; Figs. 2D and 3D, and Table S6). Ascomycota was more abundant in SC Province (57.70%) than in GS Province (40.68%), while Basidiomycota had a higher proportion in GS Province (52.64%) than in SC Province (35.40%). The dominant genus (*Kazachstania*, *Malassezia*, *Asterotremella*, and *Wallemia*) showed significant differences in the two provinces ($P < 0.05$; Figs. 2E and 3E, and Table S6). *Kazachstania* had a higher abundance in SC Province (25.46%) than in GS Province (7.4%). *Malassezia*, *Asterotremella*, and *Wallemia* had higher abundances in GS Province (20.31%, 15.91%, and 10.53%, respectively) than in SC Province (8.13%, 7.70%, and 3.78%, respectively). Moreover, the dominant species (*Kazachstania exigua*, *Asterotremella pseudolonga*, *Malassezia restricta*, *Wallemia sebi*, *Cochliobolus verruculosus* and *Cryptococcus albidus sp 1*) exhibited significant differences in GS and SC provinces ($P < 0.05$; Figs. 2F and 3F, and Table S6). *Kazachstania exigua*, *Cochliobolus verruculosus* and *Cryptococcus albidus sp 1* had higher abundances in SC Province (25.46%, 7.82%, and 5.28%, respectively) than in GS Province (7.37%, 0.01%, and 0.99%, respectively). *Asterotremella pseudolonga*, *Malassezia restricta*, and *Wallemia sebi* had higher abundances in GS Province (17.04%, 12.61%, and 10.24%, respectively) than in SC Province (7.80%, 4.87%, and 3.40%, respectively).

3.3. Microbial biomarkers of Rhei Radix et Rhizoma surface microbiome influenced by processing methods and sampling locations

LEfSe analysis displayed that the potential biomarkers were different among the five processing methods (Fig. 4). Of the 30 bacterial biomarkers (LDA > 3 and $P < 0.05$), seven, seven, ten, and six were enriched in RR, WR, CR, and RC, respectively (Fig. 4A and B). The class Saccharomycetes, order Saccharomycetales, the family Debaryomycetaceae, the genera *Kazachstania* and *Zygowilliopsis*, and the species *Kazachstania exigua* and *Zygowilliopsis unclassified* were enriched in the RR group. Meanwhile, the orders Cystofilobasidiales and Agaricales, the families Cystofilobasidiaceae and Nectriaceae, the genus *Cystofilobasidium*, and the species *Cystofilobasidium infirmominiatum* and *Aspergillus penicillioides* were enriched in the WR group. The orders Malasseziales and Hypocreales; the classes Ustilaginomycotina Incertae sedis and Sordariomycetes; the families Malasseziales incertae sedis and Corynesporascaceae; the genera *Malassezia*, *Corynespora*, and *Neosartorya*; and the species *Corynespora cassiicola* were enriched in the CR group. The phylum Basidiomycota, the order Trichosporonales, the family Trichosporonaceae, the genus *Asterotremella*, and the species *Asterotremella pseudolonga* and *Malassezia restricta* were enriched in the RC group.

LEfSe was used to probe the potential biomarkers between SC and GS provinces (Fig. 4C and D). Among the ten biomarkers (LDA > 3.0 and $P < 0.05$), six and four were enriched in the SC and GS groups, respectively. The class Eurotiomycetes, the family Saccharomycetales incertae sedis, the genera *Mucor* and *Candida*, and the species *Rhizopus microsporus var chinensis* and *Mucor hiemalis f coricola* were enriched in SC Province. Meanwhile, the fam-

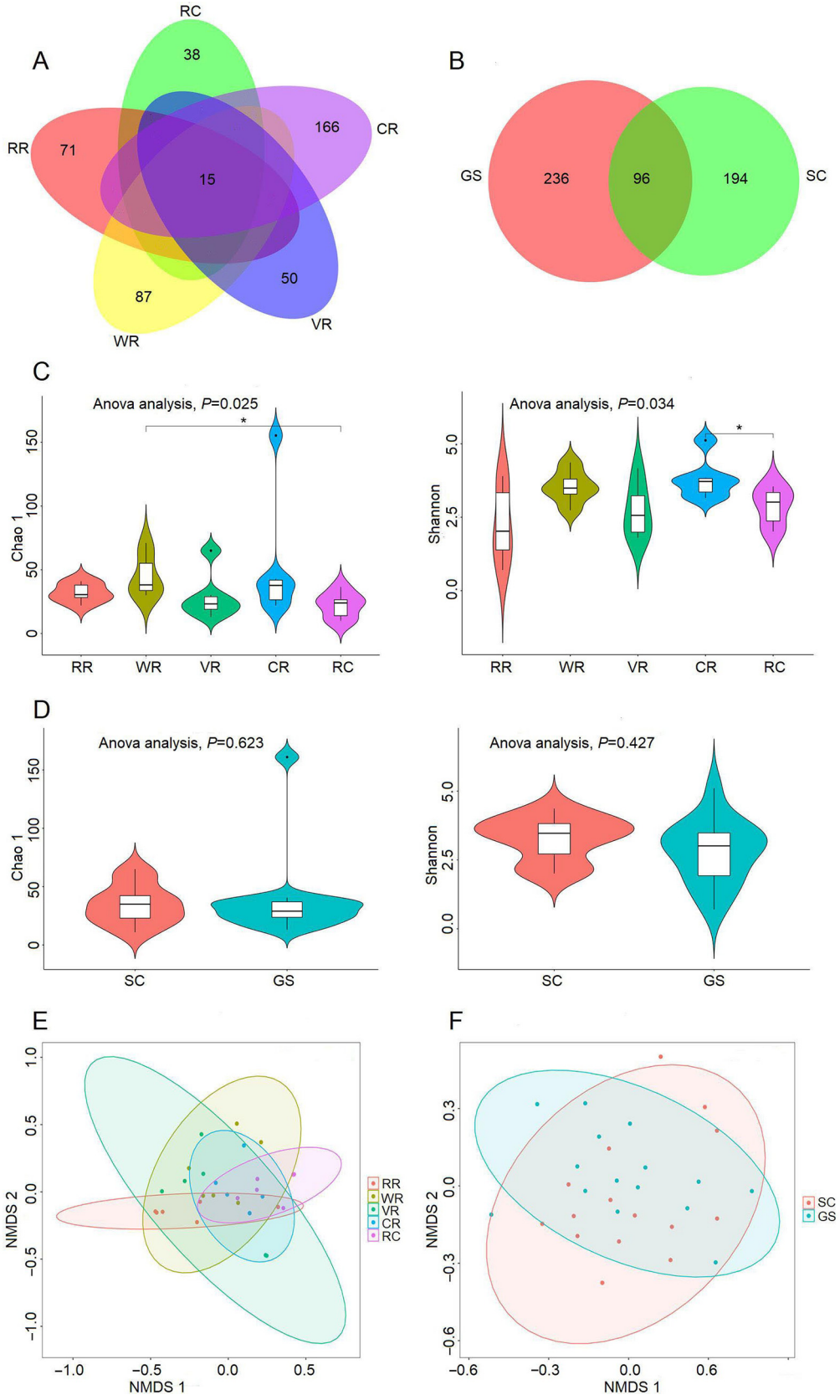

Fig. 1. Diversity of fungal community on surface of *Rhei Radix* et *Rhizoma* samples. (A and B) Venn profiles according to processing methods and sampling locations, respectively. (C and D) α-Diversity indices (i.e., Chao 1 and Shannon) according to processing methods and sampling locations, respectively. Anova analysis were carried out by SPSS 22.0, and * represent significant differences between different groups. (E and F) NMDS analysis of unweighted distance matrices according to processing methods and sampling locations, respectively. RR: raw rhubarb, WR: wine rhubarb, VR: vinegar rhubarb, CR: cooked rhubarb, RC: rhubarb charcoal; SC: Sichuan Province, GS: Gansu Province.

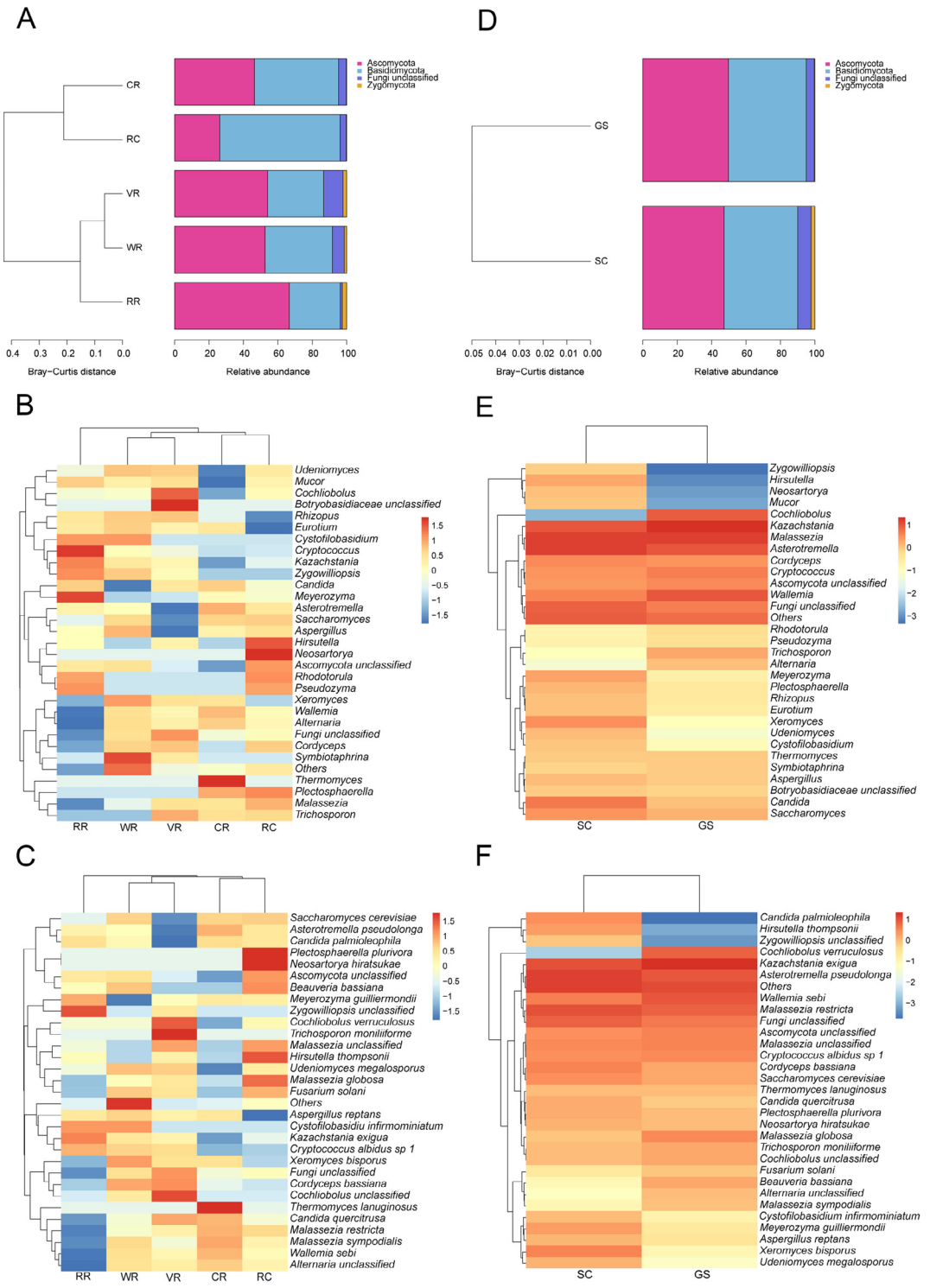

Fig. 2. Composition of fungal community on surface of *Rhei Radix* et *Rhizoma* samples. (A–C) Fungal composition at phylum, genus, and species levels on basis of processing methods. (D–F) Fungal composition at phylum, genus, and species levels on basis of sampling locations.

ily Pleosporaceae, the genus *Talaromyces*, and the species *Malassezia globosa* and *Cryptococcus magnus* were enriched in GS Province.

3.4. Network of Rhei Radix et Rhizoma surface microbiome influenced by processing methods and sampling locations

The co-occurrence patterns of microbial communities from different processing methods exhibited different network complexity (as indicated by average degree) and connectivity (Fig. 5A and Table S7). The average degree was significantly higher in the CR group (28.30) than in the other four groups (RR, 4.05; WR, 6.63; VR, 7.28; and RC, 6.24). The values of topological properties (i.e., number of nodes, number of edges, positive edges, average clustering coefficient, average weighted degree, density, and total triangles) were highest in the CR group (79, 1 118, 1 109, 0.93, 53.62, 0.36, and 14 697, respectively). The values of topological properties

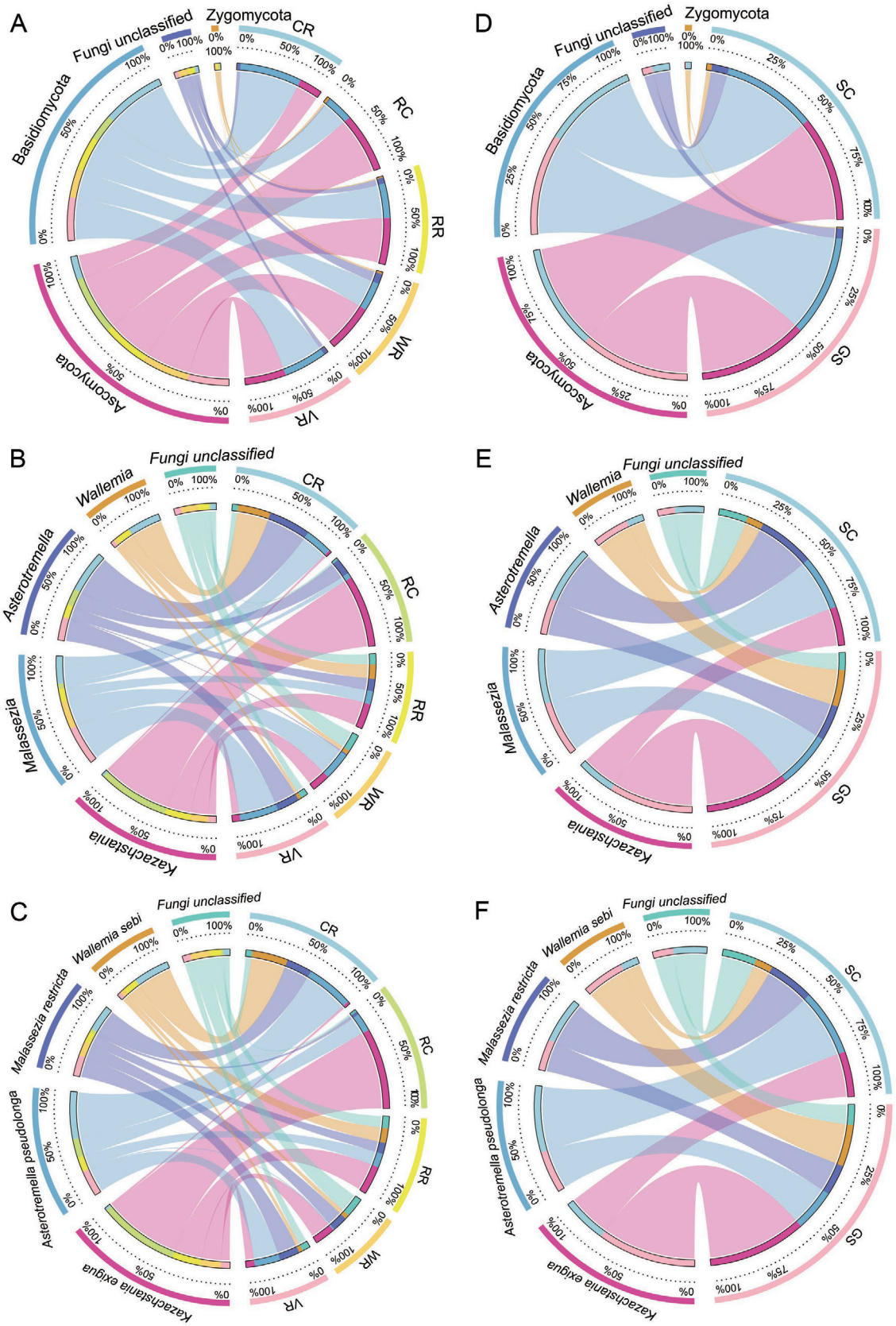

Fig. 3. Circos plots of fungal community on surface of *Rhei Radix* et *Rhizoma* samples. (A–C) Circos plots of predominant taxa at phylum, genus, and species levels based on processing methods. (D–F) Circos plots of predominant taxa at phylum, genus, and species levels based on sampling locations.

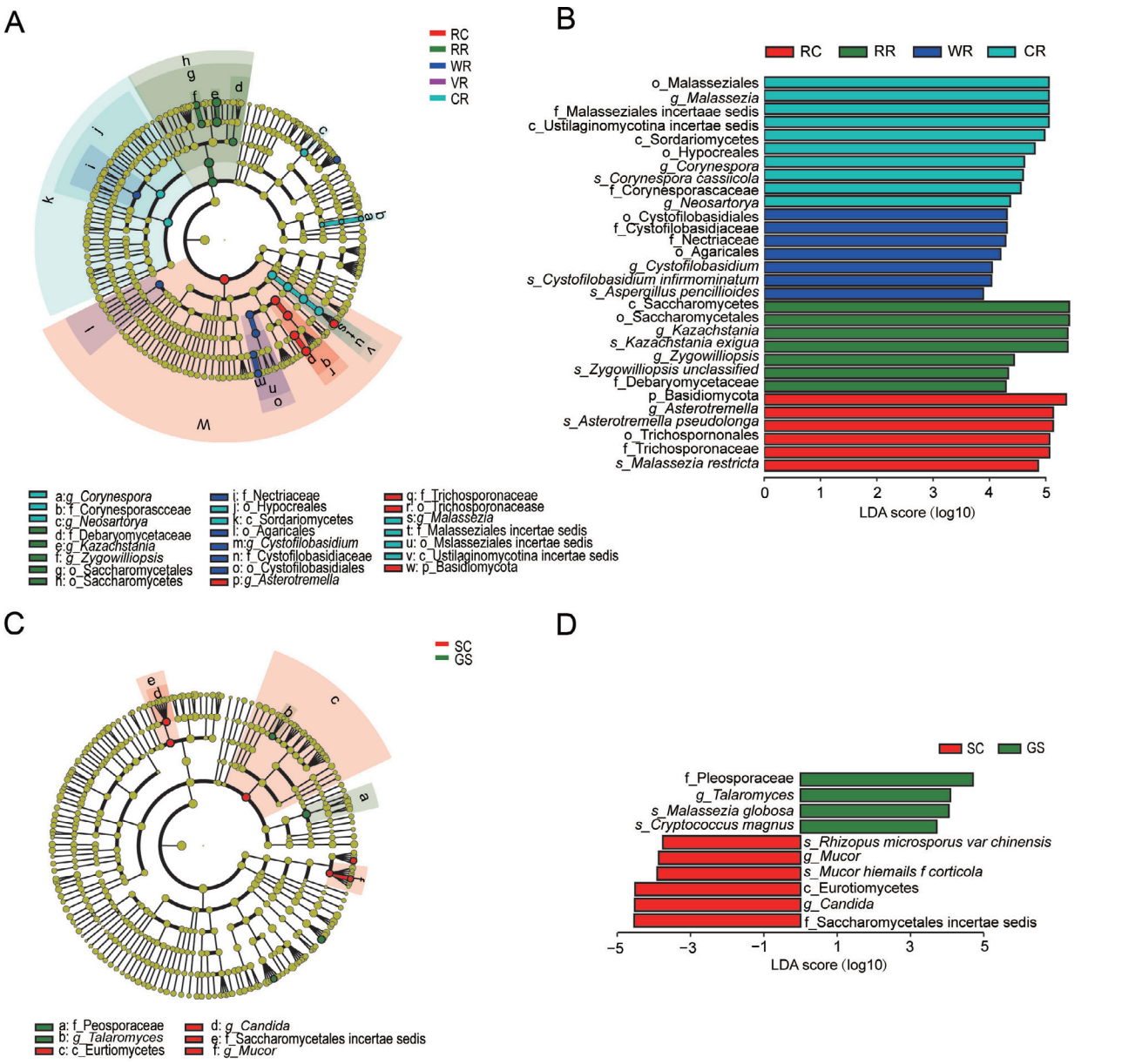

Fig. 4. LEfSe of fungal community with an LDA score higher than 4.0 and *P* values < 0.05 on surface of *Rhei Radix* et *Rhizoma* samples. (A and B) LEfSe and LDA analysis based on processing methods, respectively. (C and D) LEfSe and LDA analysis based on sampling locations, respectively.

(i.e., modularity, average path length, and modularity with resolution) were highest in the RR group (0.87, 2.68, and 0.87, respectively).

The network complexity (as indicated by average degree) and connectivity were influenced by the sampling locations (Fig. 5B and Table S8). The average degree was higher in the GS group (22.02) than in the SC group (3.90). The values of topological properties (i.e., number of nodes, number of edges, positive edges, average clustering coefficient, average weighted degree, density, and total triangles) were higher in the GS group (92, 1 013, 1 013, 0.99, 42.03, 0.24, and 12 737, respectively) than in the SC group (76, 148, 148, 0.94, 5.78, 0.05, and 111, respectively). By contrast, the values of topological properties (i.e., modularity, number of communities, and modularity with resolution) were higher in the SC group (0.82, 46, and 0.82, respectively) than in the GS group (0.04, 38, and 0.04, respectively).

4. Discussions

Fungal contamination in medicinal herbs has been reported worldwide and received considerable public attention (Su et al., 2018; Rocha-Miranda & Venâncio, 2019). In a previous study, 83.3% of 48 medicinal herbs were contaminated with fungi (Chen et al., 2020). Next-generation sequencing, especially the combination of high-throughput sequencing with ITS, can rapidly and effectively analyze the diversity and composition of a microbial community with low abundances (Nilsson et al., 2019a). The ITS region of rDNA, which is considered a better DNA barcode for fungi owing to its more prevalent primer sites and less variation in length despite some bias in amplication, is the most frequently used target in high-throughput sequencing-based metabarcoding (Schloss, Flanagan, Culler, & Wright, 2012; Tedersoo et al., 2015; Dao et al., 2024).

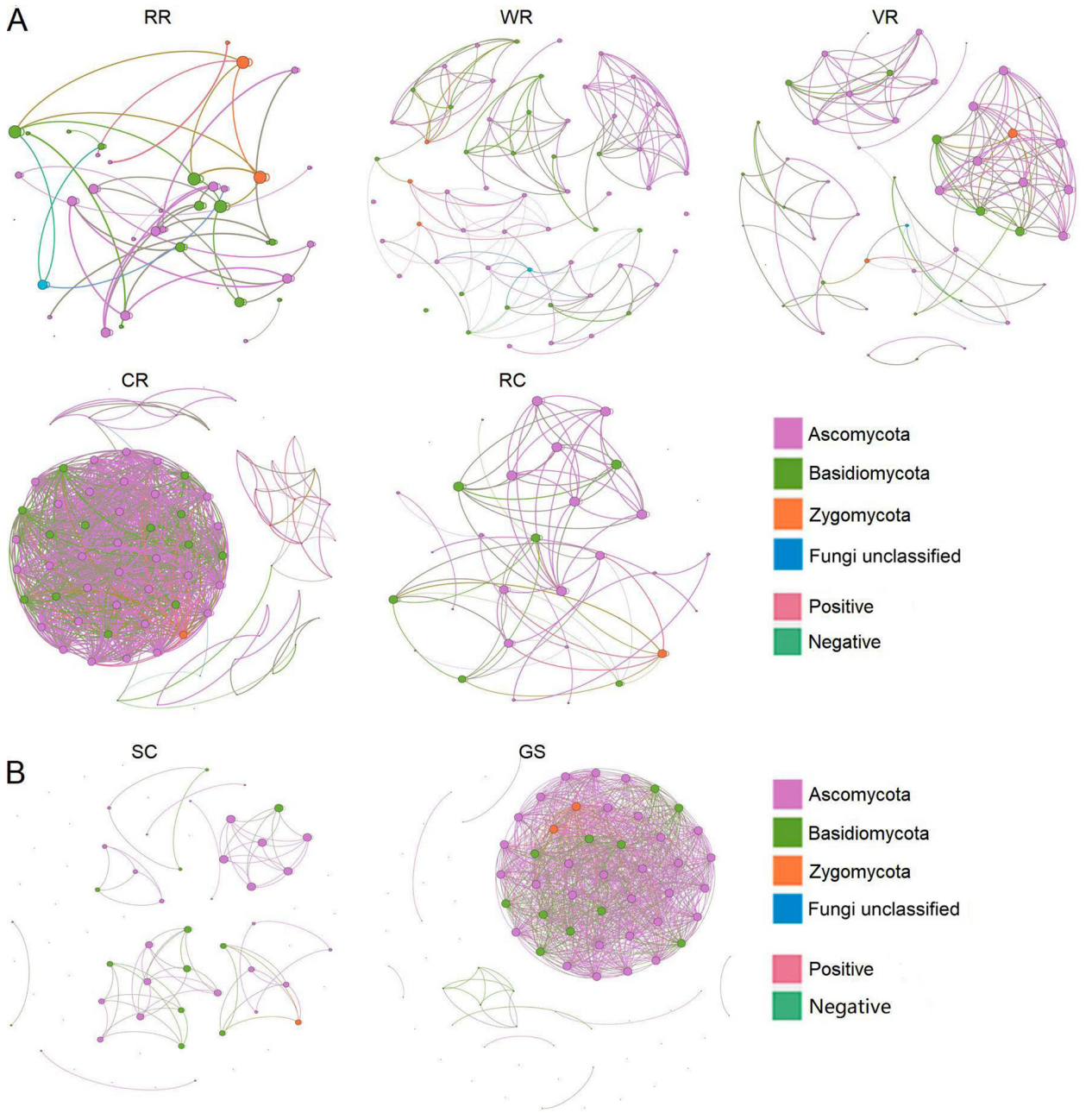

Fig. 5. Co-occurrence network analysis of fungal microbial communities on surface of *Rhei Radix* et *Rhizoma* samples (Spearman's |r| > 0.8 and $P < 0.05$). (A) Network based on processing methods. (B) Network based on sampling locations.

A total of 30 *Rhei Radix* et *Rhizoma* samples from different provinces were applied to analyze the fungal contamination on the surface of *Rhei Radix* et *Rhizoma*. Our relatively small sample size will lead to poor reproducibility and representative results, which is the limitations of the study. In order to ensure the accuracy of the experiment, problems such as sample sizes, batches, and storage conditions should be considered in future experiments. High-throughput sequencing results showed that Ascomycota and Basidiomycota were the dominant phyla; *Kazachstania*, *Malassezia*, *Asterotremella*, and *Wallemia* were the dominant genera; and *Kazachstania exigua*, *Asterotremella pseudolonga*, *Malassezia restricta*, and *Wallemia sebi* were the predominant species in *Rhei Radix* et *Rhizoma* samples. The presence of these predominant taxa in other herbs has been reported previously (Guo, Jiang, Luo, Yang, & Pang, 2018; Yu, Jiang, Guo, Dao, & Pang, 2022; Guo, Jiang, Yang, Dou, & Pang, 2020). In *Ziziphi Spinosae Semen* samples, the phylum Ascomycota and three genera (*Aspergillus*, *Candida*, and *Wallemia*) were the most predominant fungi (Guo, Jiang, Luo, Yang, & Pang, 2018). For *Lycii Fructus* samples, Ascomycota, Dothideomycetes, Pleosporales, and Pleosporaceae were the predominant fungi (Yu, Jiang, Guo, Dao, & Pang, 2022). For *Cassiae Semen* samples, Ascomycota was the prevailing phylum, and *Aspergillus*, *Cladosporium*, and *Penicillium* were the most dominant genera (Guo, Jiang, Yang, Dou, & Pang, 2020). Some taxa are found to be the dominant flora for the first time, and the differences between the results of the present study and previous studies may be related to the different nutritional composition of medicinal herbs (Wei et al., 2024). Mycotoxins are synthesized by mycotoxin-producing fungi under appropriate conditions. The emergence of potentially toxic fungi is a necessary condition for the production of mycotoxins. Some

potential mycotoxin-producing fungi, such as *Aspergillus restrictus*, were detected in our *Rhei Radix* et *Rhizoma* samples. A rapid method for detecting toxigenic fungi based on toxigenic genes was established in our previous paper (Wei et al., 2023a). So, early identifying mycotoxin-producing fungi in *Rhei Radix* et *Rhizoma* using this rapid detection method is of great significance for preventing further mycotoxin contamination. And, the fungi should be isolated from the surface of *Rhei Radix* et *Rhizoma* samples in the future, and the ability for producing mycotoxins should be investigated.

The fungal communities in five *Rhei Radix* et *Rhizoma* groups were compared based on processing methods, and the differences in fungal communities were obtained. The Chao 1 and Shannon indices were highest in the CR group, this finding is consistent with the results of *Cassiae Semen* and *Arecae Semen* (Guo, Jiang, Yang, Dou, & Pang, 2020; Wei et al., 2023b). The present results indicated that processing increased the α-diversity and the abundance of Basidiomycota, *Wallemia*, and *Cryptococcus*, while it decreased the abundance of Ascomycota and *Malassezia*. In *Cassiae Semen*, processing decreased the relative abundance of *Neofusicoccum* species, whereas it increased the proportion of *Penicillium* and *Periconia* species (Guo, Jiang, Yang, Dou, & Pang, 2020). The diversity and abundances of beneficial endophytic fungi decreased after processing in *Polygala tenuifolia* (He et al., 2020). In addition, CR samples had higher network complexity and connectivity, that was a more stable co-occurrence pattern. Processing dramatically changed the chemical composition and contents of herbs (Huang et al., 2018). Therefore, we infer that processing alters the substrate composition and contents of *Rhei Radix* et *Rhizoma* leading to variations in the fungal structure and/or toxigenic fungal contamination (Zheng et al., 2017).

The diversity, structure, and network of the *Rhei Radix* et *Rhizoma* samples exhibited differences between Gansu and Sichuan provinces. In our previous *Polygoni Multiflori Radix* study, fungal α-diversities (i.e., Chao 1 and Shannon) were highest in Yunnan Province and then incrementally decreased from Sichuan Province to Anhui Province and Guangdong Province (Wei et al., 2024). In another study of ours, the α-diversity exhibited significant differences in *Arecae Semen* samples from the four sampling locations ($P < 0.05$), being higher in Yunnan and Hainan provinces (Wei et al., 2023b). In addition, the composition of *Rhei Radix* et *Rhizoma* surface microbiome also showed significant differences. For example, Ascomycota and *Kazachstania* had higher abundance in Sichuan Province, however, Basidiomycota, *Malassezia*, *Asterotremella*, and *Wallemia* had higher proportion in Gansu Province. The phyla Ascomycota and Basidiomycota and the genera *Xeromyces*, *Cystofilobasidium*, *Eurotium*, and *Aspergillus* were the dominant fungi, and significant differences were presented in the *Polygoni Multiflori Radix* collected from four areas (Wei et al., 2024). Differences in the fungal composition of the *Fritillariae Cirrhosae Bulbus* and *Platycladi Cacumen* samples were also obtained in different provinces at various taxonomic levels (Yu, Guo, Jiang, Yang, & Pang, 2020; Yu, Jiang, Guo, Dao, & Pang, 2022). The difference in the distribution of these taxa may be caused by the local storage conditions, which may be more suitable for the growth of some taxa in some locations than in others. Different storage factors, such as temperature, humidity, and pH, in distinct environments might influence the growth of certain fungi (Darko, Kumar Mallikarjunan, Kaya-Celiker, Frimpong, & Dizisi, 2018). For example, *Alternaria* and *Fusarium* usually grow under low temperature and high humidity, respectively (EFSA Panel, 2011; Liao, Sun, Wei, Zhou, & Kong, 2020). In addition, the links among genera in our network were predominantly positive, and network complexity and connectivity were higher in Gansu Province. These results indicate that most taxa have extensive cooperative interactions with their micro-environments (Qian et al., 2019). *Rhei Radix* et *Rhizoma* are easily susceptible to contamination by various fungi producing mycotoxins, and the process is accompanied by complex internal (i.e., substrate composition) and external environmental (i.e., temperature and humidity) factors. So, *Rhei Radix* et *Rhizoma* should be dried as soon as possible after harvesting, and appropriate processing and storage measures should be performed.

5. Conclusions

In this study, the diversity, composition, and network of fungal microbiome on the surface of *Rhei Radix* et *Rhizoma* samples were investigated. The assembly and network of the *Rhei Radix* et *Rhizoma* surface microbiome were shaped by sampling locations and processing methods. The phyla Ascomycota and Basidiomycota; the genera *Kazachstania*, *Malassezia*, and *Asterotremella*; and the species *Kazachstania exigua*, *Asterotremella pseudolonga*, and *Malassezia restricta* were the dominant fungi and exhibited differences in two provinces and five processed products. This paper highlights the importance of the roles of sampling locations and processing methods in the *Rhei Radix* et *Rhizoma* surface microbiome.

CRediT authorship contribution statement

Guangfei Wei: Data curation, Formal analysis, Visualization, Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Xiao Chen:** Data curation, Formal analysis, Visualization, Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Guozhuang Zhang:** Data curation, Formal analysis, Visualization, Writing – original draft. **Conglian Liang:** Data curation, Visualization. **Zhaoyu Zhang:** Data curation, Visualization. **Bo Zhang:** Data curation, Visualization. **Shilin Chen:** Supervision, Project administration, Writing – review & editing. **Linlin Dong:** Supervision, Project administration, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2024.11.006.

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