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ORIGINAL PAPER

Microbiota and Chemical Compounds in Fermented *Pinelliae Rhizoma* (Banxiaqu) from Different Areas in the Sichuan Province, China

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Abstract

This study focused on the microbiota and chemical compounds of the fermented *Pinelliae Rhizoma* produced in Longchang (LC), Zizhong (ZZ) and Xindu (XD), in Sichuan Province (China). High-throughput sequencing was used to analyze the microbiota. GC-MS and LC-MS were used to detect the compounds produced during the three different *Pinelliae Rhizoma* fermentation processes. The bacteria and fungi of the three fermented *Pinelliae Rhizoma* differed substantially, with the bacterial content mainly composed of the *Bacillus* genus, while the common fungi were only included in four OTUs, which belong to three species of *Eurotiomycetes* and *Aspergillus cibarius*. 51 volatile compounds were detected; they varied between LC, XD, and ZZ fermented *Pinelliae Rhizoma*. C10 and C15 terpenes were most frequently detected, and only curcumene and β-bisabolene were detected in the three fermented *Pinelliae Rhizoma*. 65 non-volatile compounds were detected by LC-MS, most were of C16, C18, C20, C21 and C22 structures. Cluster analysis showed more similarity between LC and XD fermented *Pinelliae Rhizoma* with regards to volatile compound content, but more similarity between the XD and ZZ fermented *Pinelliae Rhizoma* for non-volatiles. Moreover, no correlation between geographical distance and microflora or compounds of fermented *Pinelliae Rhizoma* was observed. These results showed that hundreds of compounds are produced by the natural mixed fermentation of *Pinelliae Rhizoma*, and may mostly relate to the microorganisms of five species.

Key words: Fermented *Pinelliae Rhizoma*, components, microbiota, regions

Introduction

Mixed solid fermentation is advantageous in the formation of many active compounds (Boratyński et al. 2018; Try et al. 2018) and is a widely used process in food production and traditional Chinese medicine for thousands of years (Chen 2013; Zhao et al. 2016; Wu et al. 2018). Fermented *Pinelliae Rhizoma* (Banxiaqu) is a traditional Chinese Medicine made from the *Pinellia ternata* (Thunb) Breit by mixed solid fermentation (Hu et al. 2018). It has been shown to remove phlegm, strengthen the stomach and improve digestion (Wagner et al. 2011; Gong, et al. 2015; Qu et al. 2016; Zhao et al. 2018). The fermentation process follows key sequential steps: 16 kg of licorice is boiled and filtered. The filtrate is then mixed with 20 kg of quick lime, and the supernatant is used to soak 100 kg of *Pinelliae Rhizoma* for 4–5 days. The soaked *Pinelliae Rhizoma* is then removed, washed and ground into fine powder, which is then mixed with 10–20 kg of flour and enough water to form the dough. The dough is subsequently fermented at 30°C–35°C with a humidity of 70–80% for 3–5 days, then dried at 70°C–80°C until the dough becomes loose and porous (Chinese Pharmacopoeia Commission, 2015). As a result of this fermentation process, *Pinelliae Rhizoma* attains better clinical efficacy relative to mouth and throat numbness. A mixture of fungi and bacteria from the surrounding environment carries out complex biochemical reactions during the fermentation. Because the mixed solid fermentation is carried out by different microorganisms in different regions or seasons, the quality of fermented *Pinelliae Rhizoma* may drastically fluctuate, thus explaining the difference in the quality of fermented *Pinelliae Rhizoma* during different seasons, or produced in different districts (Hu et al. 2018). Due to the lack of standardization, the clinical application of fermented *Pinelliae Rhizoma* is severely hindered. As such, only the fermented

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Pinelliae Rhizoma made from certain large traditional factories are still used in hospitals.

Sichuan is an important region of *Pinelliae Rhizoma* production, although this herb grows all over the region. The dried tuber is mainly fermented in the three counties of Longchang (LC), Zizhong (ZZ) and Xindu (XD). The active components of *Pinellia ternate* have not been yet conclusively identified, but clinical applications showed similar efficacies between these three different regionally fermented *Pinelliae Rhizoma,* despite their disparate geographical localizations (Shen et al. 2009). Importantly, as these three preparations of fermented *Pinelliae Rhizoma* are fermented by microorganisms in different locations, and the varied microorganisms produce a range of distinct compounds. It is likely that among these compounds, some contribute more to the clinical efficacy of fermented *Pinelliae Rhizoma* than others, particularly those components that exist across multiple different preparations of fermented *Pinelliae Rhizoma*. It, therefore, stands to reason that studying these common components may reveal the key active compounds in fermented *Pinelliae Rhizoma* and support a model to research the role of microbiota in the fermentation of *Pinelliae Rhizoma*. The purpose of this research is also to help identify the key species of microorganisms involved in *Pinelliae Rhizoma* fermentation, with the objective to thereby improve the controllability of its solid mixed fermentation processes.

Experimental

Materials and Methods

Sample collection. Three fermented *Pinelliae Rhizoma* were produced in three factories located respectively in Longchang (Lianghui company, Shengdeng town, Longchang County, Neijiang city, Sichuan Province), Xindu (Qianfang company, Satellite City Industrial Development Zone, Xindu District, Chengdu, Sichuan Province) and Zizhong (Hongsheng company, Qiuxi town, Zizhong County, Neijiang city, Sichuan Province) using the same raw materials and technology. When the fermentation was complete, three samples were collected and identified respectively as LC, XD, and ZZ.

Physical and chemical testing. The content of all water-soluble saccharides, mannose-oligosaccharides, and polysaccharides in the fermented *Pinelliae Rhizoma* was determined by sulfuric acid-anthrone colorimetry (Cianchetta et al. 2017).

The content of non-protein nitrogen of 0.5 g fermented *Pinelliae Rhizoma* was determined directly by the Kjeldahl method using a Kjeltec-8400 automatic Kjeldahl nitrogen analyzer (FOSS company) (Rédei 2008). Briefly, 0.5 g fermented *Pinelliae Rhizoma* was dissolved for 0.5 h in 20 ml of 98% H_2SO_4, and the content of total nitrogen was determined by the method stated earlier, where the protein nitrogen content corresponded to the difference between total nitrogen and non-protein nitrogen. Protein content was measured as protein nitrogen content multiplied by 6.25, for the average content of nitrogen in proteins is 16%, and therefore use the value to convert nitrogen to protein.

Enzyme activity detection. 5.0 g of fermented *Pinelliae Rhizoma* powder was mixed with 50 ml of ddH_2O, shaken at 40°C for 1 h to fully solubilize the digestive enzymes (amylase, protease, and lipase). The mixture was then centrifuged at 3000 rpm for 5 min, and 5 vml of supernatant was collected for enzymatic activity determination.

The amylase activity was measured as follows: 20 ml of soluble starch blended with 5 ml PBS (pH 6.0) was preheated to 60°C for 8 min. Then 1 ml of the *Pinelliae Rhizoma* supernatant from earlier was added and incubated for 5 min. 1 ml of this solution was then collected and mixed with 0.5 ml of 0.1 M HCl and 5 ml of iodine solution (0.1 g/l iodine and 40 g/l potassium iodide), and its absorbance was measured at 660 nm. One amylase activity unit was defined as: 1 g soluble starch degraded in 1 h at 60°C, pH 6.0 (China National Standard, GB/T 24401-2009).

The protease activity was measured as follows: 1 ml supernatant was preheated to 40°C and added to 10 ml of 1% casein, preheated to 40°C. The mixture was then incubated at 40°C for 10 min, the reaction was quenched by adding 200 ml of 0.4 M $C_2HCl_3O_2$ at the same temperature for 10 minutes. This mixture was left at 40°C for an additional 20 minutes and then centrifuged at 10 000 rpm for 10 minutes. 100 ml of the supernatant was mixed with 0.5 ml of 0.4 M Na_2CO_3 and blended with 1 ml of Folin reagent. After blending, the mixture was kept at 40°C for 20 minutes and the absorbance was measured at 660 nm. One unit of protease activity was defined as 1 μg tyrosine produced per minute at 40°C using casein as a substrate (China Commercial Standard, SB/T 10317-1999).

Lipase activity was determined as follows: 150 ml of polyvinyl alcohol was added to 50 ml of virgin olive oil and the mixture was homogenized for 5 min, and after 2 min again for 3 min. 4 ml of this solution was then added to 5 ml of PBS (pH 7.5) and preheated at 40°C for 5 min, mixed with 1 ml of the *Pinelliae Rhizoma* supernatant from the earlier preparation and left to react for 15 min. 15 ml of 95% CH_3CH_2OH was added to quench the reaction, and the solution was titrated to neutral pH using 0.05 M NaOH. One unit of lipase activity was defined as 1 μmol of titratable fatty acids produced per minute at 40°C using olive oil as a substrate (China National Standard, GB/T 23535-2009).

Microbial community analysis. The fermented *Pinelliae Rhizoma* were crushed under sterile conditions. The total DNA of the samples was extracted using the Omega Kit according to the operating instructions. Bacterial amplification primers were 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) ("H" means A, T or C; "V" means G, A or C; "W" means A or T) (Fan et al. 2014). Fungi primers were ITS1F (CTTGGTCATTTAGAGGAGTAA) and 2043R (GCTGCGTTCTTCATCGATGC) (Pranab et al. 2014). The PCR reaction was performed in a total volume of 25 μl, where the reaction conditions were 95°C 3 min; 95°C 30 sec, 55°C 30 sec, 72°C 45 sec, 27 cycles; 72°C 10 min. The amplification fragments were detected by loading 3 μl of the total product on a 2% agarose gel for electrophoresis and sequencing on the Illumina platform by Pisino Company.

For the sequencing data, the Silva database was used to identify bacterial species (Quast et al. 2013), and the Unite database was used to identify fungal species (Kõljalg et al. 2013). The Mothur software was used to analyze the Alpha diversity which is expressed as the index of Shannon, Chao 1, Simpson, and ACE.

By comparing the existing community composition data with the known reference genome database and correcting the microbial abundance data against the original data, the metabolic function of the community samples was predicted using the software PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al. 2013).

Detection of volatiles. 5.0 g of fermented *Pinelliae Rhizoma* powder was added to a 20 ml headspace vial, with 65 μm PDMS/DVB solid-phase microextraction (Supelco) used to absorb the powder for 40 min at 65°C. This microextraction was then dissolved at 250°C for 5 min for injection on gas chromatography-mass (7890A-5975C, Agilent). Volatile compounds were detected as follows: Helium was used as the carrier gas, which flowed at a rate of 1.0 ml/min. The HP-5MS column (300 mm × 0.25 mm × 0.25 μm) was kept at 50°C for 3 min, then, the temperature was raised to 150°C at 5°C/min and kept for 3 min, then raised to 220°C at 10°C/min and kept for 2 min. The ionization energy was 70 eV; the ionization temperature was 230°C, and the scanning range was 45–550 amu.

Detection of non-volatiles. 20.0 g of fermented *Pinelliae Rhizoma* powder was extracted using 100 ml of 95% CH_3CH_2OH for 15 min, then centrifuged at 3000 rpm for 5 min. The supernatant was collected as an ethanol extraction, and the precipitate was mixed with 50 ml of ddH_2O and extracted by adding 50 ml $CH_3(CH_2)_3OH$ and 50 ml $CH_3COOCH_2CH_3$ sequentially. The extraction was collected sequentially as a butanol extraction, and then an ethyl acetate extraction. All extractions were filtered through 0.22 μm filters and chemical content was detected by liquid chromatography-mass spectrometry (6530 Accurate-Mass Q-TOF, Agilent) using the following conditions: 5 μl samples were injected into the Hermo-C18 column (2.1 mm × 50 mm × 1.8 μm). The mobile phase was a mixture of 0.1% formic acid solution (A) and methanol (B) which flowed at 0.2 ml/min. The entire column elution was introduced into the Q-Tof mass spectrometer. Ion detection was achieved in ESI cation mode using a source capillary voltage of 3.5 kV, and a scanning range of 50 to 1000 m/z. The source temperature was 110°C, drying temperature was 350°C, and the drying gas flowed at 8.0 l/min, with the spray pressure set to 40 psi.

Results

Comparison of physiochemical indexes and enzyme activities. From Table I it is noted that nitrogen in the fermented *Pinelliae Rhizoma* mainly exists in the form of non-protein nitrogen. This indicated successful degradation of the proteins in *Pinellia ternate*. The protease activity and soluble nitrogen content of ZZ fermented *Pinelliae Rhizoma* were significantly higher than those in the other two samples, indicating that the fermentation of the *Pinelliae Rhizoma* carried out in the regions of LC, ZZ and XD may differ in nitrogen metabolism. The starch content in *Pinellia ternate* was about 75% of the total mass of the material (Gong et al. 2015), while the total sugar was roughly 0.1% in fermented *Pinelliae Rhizoma*, which indicates poor degradation of the starch because of low amylase activity. The amylase activity of LC was significantly lower than the other two samples, and in all three fermented *Pinelliae Rhizoma,* the lipase activity was only 0.3 U/g. Protease activity was very elevated, suggesting that increased protein degradation may be more important in the fermentation than the degradation of other macromolecular components. Furthermore, since the digestive enzymes generated during the fermentation of *Pinelliae Rhizoma* are mainly produced by microorganisms, the difference of microbial flora in the three counties may influence the activity of enzymes, and thus influence the efficacy in promoting digestion of fermented *Pinelliae Rhizoma*.

Bacterial community in the three fermented *Pinelliae Rhizoma*. The total number of OTUs of bacteria in the three fermentation samples of *Pinelliae Rhizoma* was 3978, while only 228 common OTUs existed in all three samples (Fig. 1). The differences in unique bacterial identifiers between regions was clear. 629 unique OTUs were found in XD, 300 unique OTUs were found in LC, and 248 unique OTUs were found in ZZ. The 285 OTUs found in common between LC and ZZ, were more numerous than the common OTUs between LC and XD (187) or ZZ and XD (131). Thirteen genera

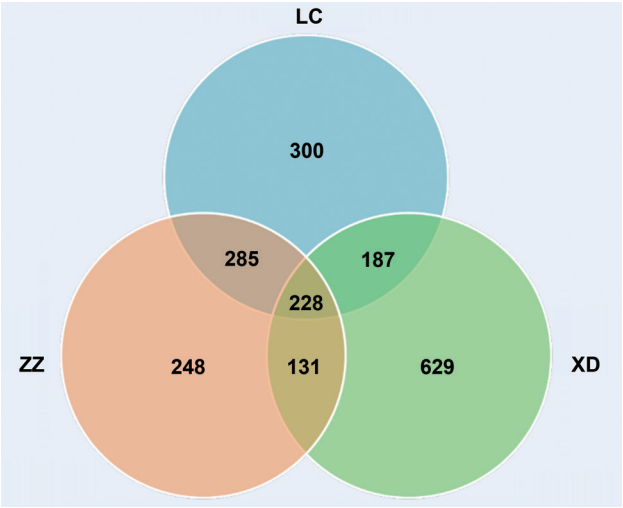

Fig. 1. The similarity of the bacterial communities in three fermented *Pinelliae Rhizoma.*

(*Bacillus*, *Sediminibacterium*, *Ralstonia*, *Lactococcus, Ochrobactrum*, *Carnobacterium*, *Planomicrobium, Sphingomonas*, *Deinococcus*, *Oscillospira*, *Adlercreutzia*, *Bradyrhizobium*, and *Virgibacillus*) were found both in LC and ZZ. Thus, among the three fermented *Pinelliae Rhizoma,* the bacterial similarity between LC and ZZ was the highest, correlating with the distance between Longchang and Zizhong being the shortest at only 76 kilometers. The distance between Longchang and Xindu is 235 kilometers and the distance between Zizhong and Xindu is 144 kilometers). Furthermore, the environment of Longchang and Zizhong are more similar.

The OTUs and alpha diversity of bacteria in XD is significantly higher than that in LC and ZZ (Table II), with 16 genera (*Klebsiella*, *Enterococcus*, *Facklamia, Gemmata*, *Gluconobacter*, *Proteus*, *Aerococcus*, *Aristolochia*, *Zea*, *Rhodospirillum*, *Staphylococcus*, *Anaerobacillus*, *Bacteroides*, *Stenotrophomonas*, *Comamonas*, and *Planococcus*) only detected in XD-fermented *Pinelliae Rhizoma*. This revealed a more complex and unique bacterial community in fermented *Pinelliae Rhizoma* produced in Xindu county.

The abundance of bacterial species in three fermented *Pinelliae Rhizoma* differed from each other (Fig. 2). As the dominant genus in all three fermented *Pinelliae Rhizoma*, the relative abundance of *Bacillus* in LC and ZZ fermented *Pinelliae Rhizoma* was much higher than that in XD.

Differences in biodegradation, nucleic acid metabolism, vitamin metabolism, energy metabolism, lipid metabolism, and amino acid metabolism in the three fermented *Pinelliae Rhizoma* were predicted by KEGG analysis (Fig. 3). The level of carbohydrate metabolism

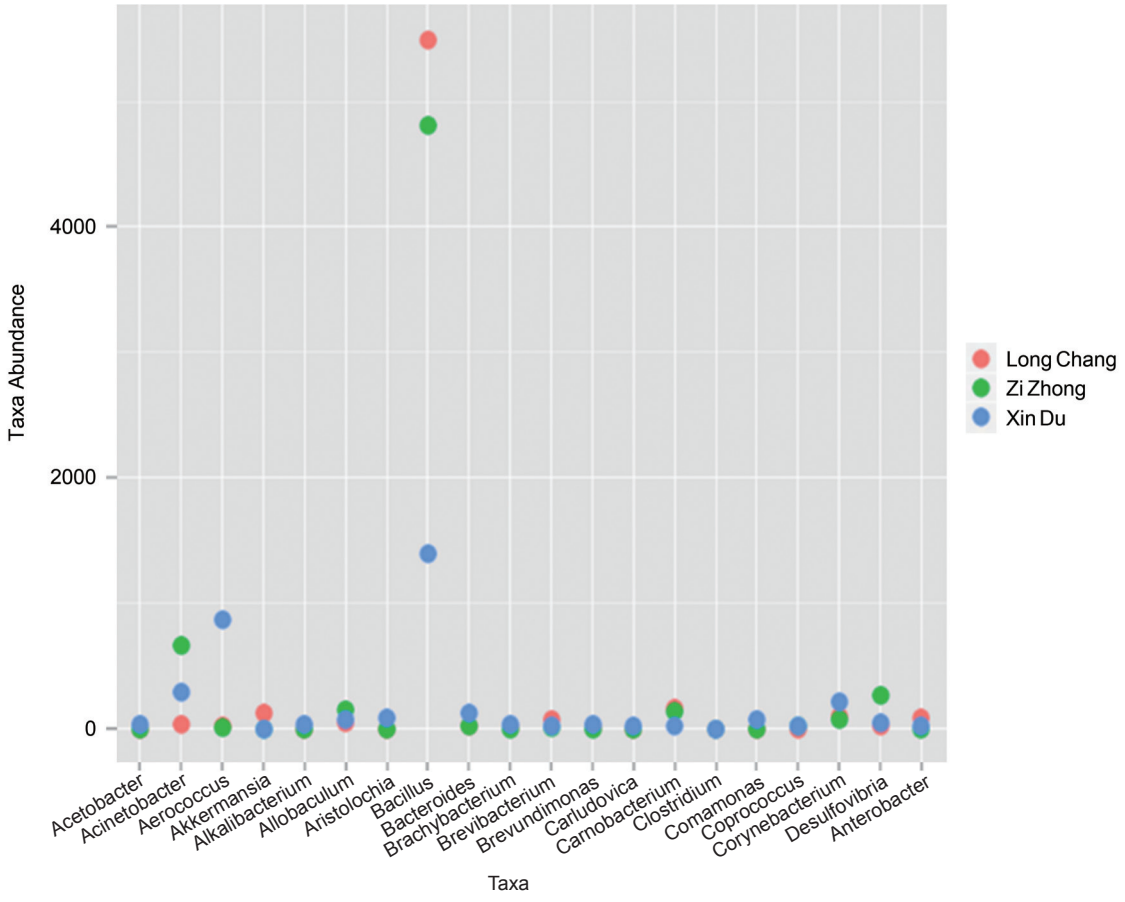

Fig. 2. Taxa abundances of bacterial species in three fermented *Pinelliae Rhizoma.*

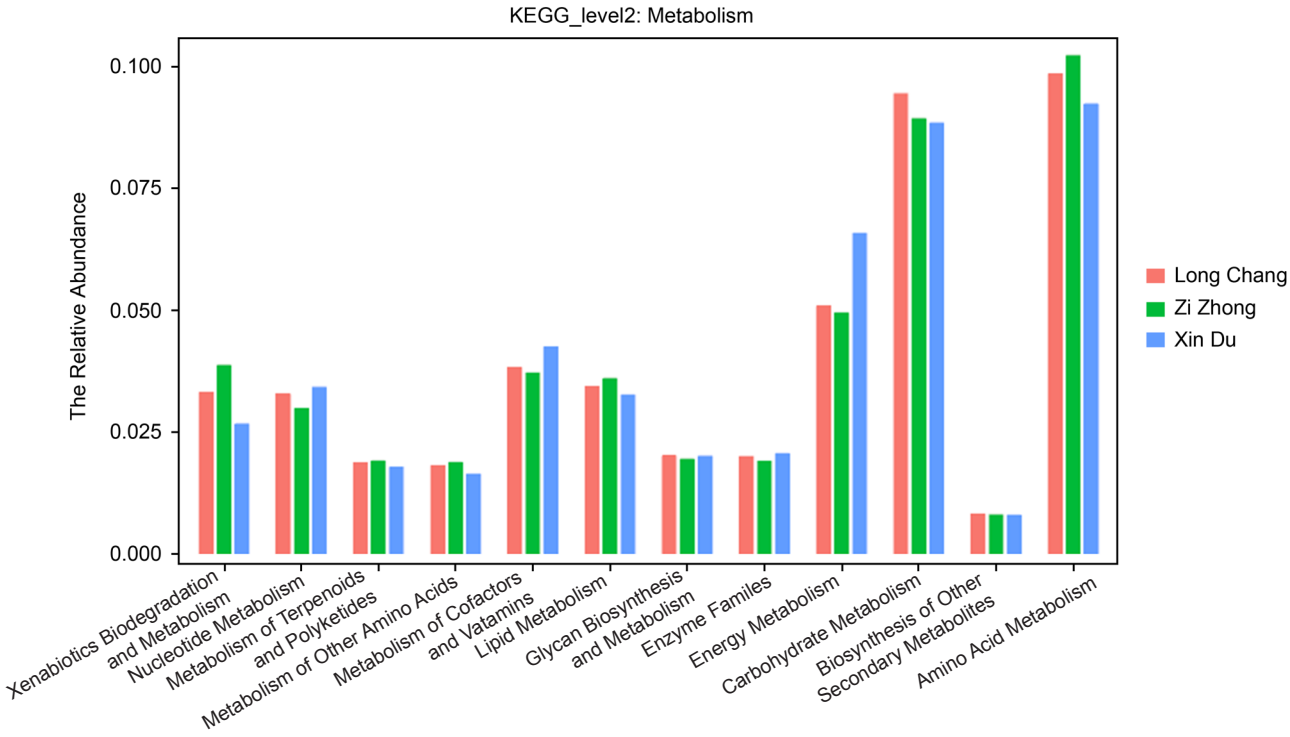

Fig. 3. Comparison of bacterial metabolic differences in the three fermented *Pinelliae Rhizoma*.

by bacteria in LC was significantly higher than ZZ and XD, while the amylase activity of LC was lowest. These results suggest that bacteria were not the main functional microorganisms in the process of carbohydrate metabolism; the level of amino acid metabolism in LC and ZZ fermented *Pinelliae Rhizoma* is higher than that in XD, and the protease activity was also the lowest, which indicated more decomposition of protein by bacteria in the LC and ZZ fermented *Pinelliae Rhizoma*; the level of xenobiotic biodegradation metabolism in XD was weaker than that in LC and ZZ fermented *Pinelliae Rhizoma,* suggesting that the bacterial-degraded xenobiotics occurred less frequently in the Xindu fermentation process of *Pinelliae Rhizoma*. Energy metabolism, cofactors, vitamin metabolism and nucleic acid metabolism related to bacteria in XD fermented *Pinelliae Rhizoma* were higher than LC and ZZ, which may be related to a stronger metabolism by more aerobic bacteria such as *Aerobacoccus* sp. (Fig. 2) in XD fermented *Pinelliae Rhizoma*; since, aerobic bacteria tend to grow faster and produce more energy.

Fungi communities in three fermented *Pinelliae Rhizoma* samples. The alpha diversities of fungi were lower than that of bacteria in all three fermented *Pinelliae Rhizoma*, the alpha diversities of fungi in XD is much lower than that in LC or ZZ (Table III). Over 30 000 total fungal sequences were identified in the three fermented *Pinelliae Rhizoma*, while the number of OTUs was only 100–208, and only 37 OTUs are detected in all three samples (Fig. 4). The number of common OTUs was far less than the number of unique OTUs belonging to each sample.

More than 200 OTUs of fungi detected in three fermented *Pinelliae Rhizoma* were identified and found to belong to four large groups (Fig. 5). The fungi community in XD fermented *Pinelliae Rhizoma* clustered in a single branch, while the fungi community in LC and ZZ clustered in another branch. The genus of *Auricularia delicata* was found in both LC and ZZ at a relative abundance of 47% in LC and 14% in ZZ but was not detected in XD. Four OTUs existed in all three

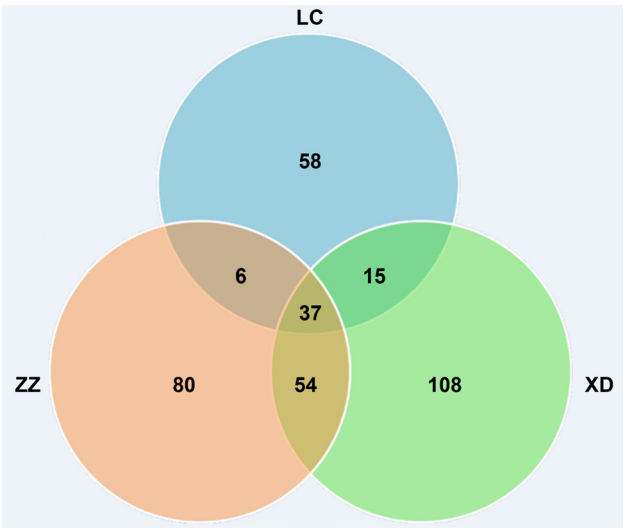

Fig. 4. The similarity of the fungi communities in three fermented *Pinelliae Rhizoma*.

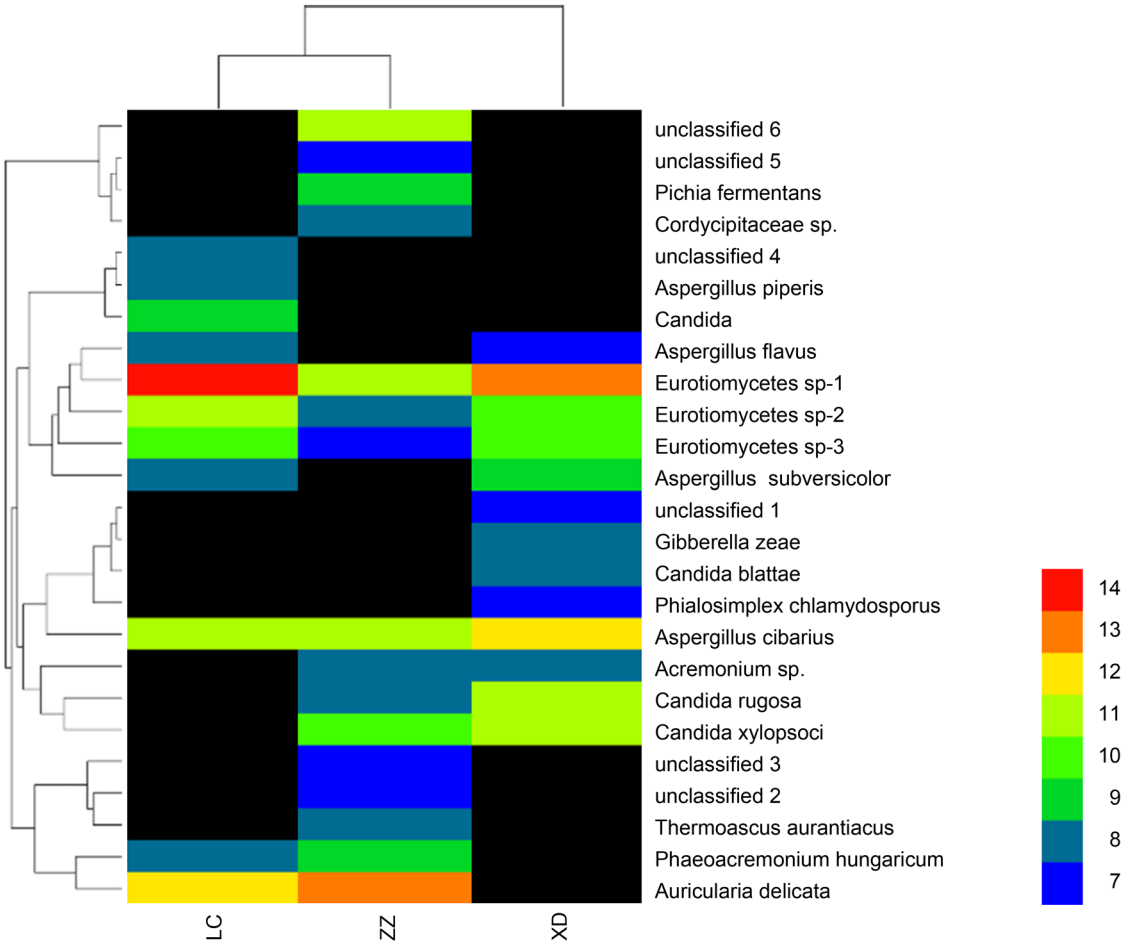

Fig. 5. Heat maps of fungi community in three fermented *Pinelliae Rhizoma.*

fermented *Pinelliae Rhizoma*, which were identified as three species of *Eurotiomycetes* and *Aspergillus cibarius*, and the relative abundance of three *Eurotiomycetes* sp. in XD, ZZ, LC were 46%, 11%, and 67% respectively, with the relative abundance of *Aspergillus cibarius* in XD, ZZ, LC measured at 22%, 9%, and 10%, respectively.

Volatile components in three fermented *Pinelliae Rhizoma.* A total of 51 volatile compounds were detected in the three samples of fermented *Pinelliae Rhizoma*, but each contained roughly 20 different compounds in different abundances (Table IV). Only curcumene and β-bisabolene were found in all three fermented *Pinelliae Rhizoma*. The relative contents of volatile components of C15 structures of bisabolene were 18.8%, 64.09%, and 27.26% in LC, XD, and ZZ, respectively. The relative contents of volatile components of C10 structure were 30.45%, 22.41%, and 7.04% respectively. It seems that steroids or terpenes of C10 and C15 account for most of the total volatile substances and may contribute to the activity of fermented *Pinelliae Rhizoma*.

In LC fermented *Pinelliae Rhizoma*, geraniol, curcumene (ginger), and α-terpineol were three volatile components with the highest relative content of 7.96%, 7.51%, 7.05% respectively. In XD fermented *Pinelliae Rhizoma*, [S-(R*,S*)]-2-methyl-5-(1,5-dimethyl-4-hexenyl)-1,3-cyclohexadiene, curcumene, and [S-(R*,S*)]-3-(1,5-dimethyl-4-hexenyl)-6-methylene-cyclohexene were three volatile components with the highest relative content of 23.07%, 14.21%, 10.62%, respectively. In ZZ fermented *Pinelliae Rhizoma*, curcumene, patchouli alcohol, and (E)-2-nonenal were three volatile components with the highest relative content of 10.98%, 8.55%, and 4.12%, respectively. These results show clear differences between the three fermented *Pinelliae Rhizoma.*

Non-volatile components in three fermented *Pinelliae Rhizoma* samples. More than 400 non-volatile components were detected in the three fermented *Pinelliae Rhizomas*, with 65 of the main non-volatile components (with a peak area over 100 000) shown in Table V. Because these non-volatile components were detected in the ethanol, butanol or ethyl acetate extractions, the relative content is calculated based on the total peak area of all components detected in three extractions.

In the LC, XD and ZZ fermentations, 26, 26 and 29 components were found respectively. In LC fermented *Pinelliae Rhizoma,* glyceride-1,3-dipalmito-

2-sorbate and 7-hydroxycadalenal were the main components detected in the butanol extraction, while 1-methyl-2-[(4Z,7Z)-4,7-tridecadienyl]-4-(1H)-quinolone, pyroglutamic acid, versicolactone D,L-(–)-alpha-monopalmitin, dendroside D, 5-O-methylembelin, ethylpentad ecanoate, ditertbutyl phthalate in the ethanol extraction were detected. In XD fermented *Pinelliae Rhizoma* benzyl formate, albopetasin, glyceride-1,3-dipalmito-2-sorbate, phthalic anhydride, gentiatibetine, capsorubin, muscone, docosandioic acid, ginsenoyne K, and alloisoleucine were detected in the butanol extraction, while styrene, 10-gingediol, villosolside, 10-methoxyheptadeca-1-ene-4,6-diyne-3,9-diol, L-arginine, ethylpentadecanoate were detected in the ethanol extraction. Amarasterone A and linolenic acid were detected in the ethyl acetate extraction. In ZZ fermented *Pinelliae Rhizoma,* non-volatile components mainly extracted in butanol included glyceride-1,3-dipalmito-2-sorbate, pyrrole-2-aldehyde, 8-gingediol, muscone, phthalic anhydride, ambonic acid, 4-methyl heptadecanoic acid, 11-eicosenoic acid, 7-hydroxycadalenal and alpha-monoolein. Although only glyceride-1,3-dipalmito-2-sorbate, linolenic acid and (Z,Z)-9,12-octadecadienoic acid were detected in all three fermented *Pinelliae Rhizoma*, a series of components with C16, C18, C20, C21 and C22 framework were also found in the three fermented *Pinelliae Rhizoma*. Some new active non-volatile components that did not exist in *Pinelliae Rhizoma* were also detected in the three fermented *Pinelliae Rhizoma,* such as 16-carboxytotarol, 21-O-methyl-5,14-pregndiene-3beta,14beta,17beta,21-tetrol-20-one, canavanine, anacardic acid C, kurchiphyllamine, fumotoshidin A, muscone, coronaric acid, 13beta,17beta-epoxyalisol A, lemmasterone, ardisinol II, houpu lignan A, albopetasin, capsorubin, ginsenoyne K, phthalic anhydride, ambonic acid, and 7-hydroxycadalenal. These new components were produced through the fermentation of *Pinelliae Rhizoma*.

Discussion

In our study, *Bacillus* sp. was found to be the dominant bacteria and *Eurotiomycetes* sp. (including 3 OTUs), *Aspergillus cibarius* were found to be the dominant fungi in the three fermented *Pinelliae Rhizoma* produced in Longchang, Xindu, and Zizhong. While Guo et al. (2016) found that the dominant bacteria in the fermentation process of fermented *Pinelliae Rhizoma* were *Streptomyces* sp., *Bacillus pumilus*, *B. subtilis*, *B. aryabhattai*, *Bacillus* sp., and the dominant fungi were *Meyerozyma guilliermondii*, *Paecilomyces variotii*, *Byssochlamy spectabilis* and *Aspergillus niger* by culture methods. This observation difference may be due to the following three reasons: firstly, our samples were dry fermented *Pinelliae Rhizoma*, and some bacteria that grow at higher humidity or occurred occasionally in the fermentation process of *Pinellia ternate* could not be reliably detected, especially those which do not produce spores. Secondly, high-throughput sequencing can supply more comprehensive information about the microbiota compared to the culture method. Finally, the results of our study were summarized from three fermented *Pinellia ternate*, that ensured most common species could be found and less accidental microorganisms caused by different environmental factors.

Five genera may play important roles in the fermentation process of *Pinelliae Rhizoma* according to our study. From the aspect of bacteria, the abundance of *Bacillus* was higher in LC and ZZ, and the fermented *Pinelliae Rhizoma* produced in Longchang and Zizhong have been found to be generally more clinically effective than the one from Xindu, so *Bacillus* may closely correlate with the quality of fermented *Pinelliae Rhizoma*. For the fungi, three fermented *Pinelliae Rhizoma* have very different fungi community, four fungal species may play more important roles in the fermentation process of *Pinelliae Rhizoma* and could be isolated on selected medium for further study. Some detected OTUs could not be successfully identified using the UNITE or Genbank databases, strongly suggesting that some new fungal species may exist in these fermentations. As such, the traditionally fermented Chinese medicines could supply fungal resources as novel and important materials.

These dominant microorganisms species are mainly derived from raw materials as the endophyte of *Pinelliae Rhizoma*, while some other genera of microorganisms from local environmental sources may also influence the fermentation. For the bacteria, the OTUs and alpha diversity of bacteria in XD is significantly higher than that in LC and ZZ, and the microbiota was more similar between LC and ZZ; it may be attributed to the fact that Xindu county is only 25 kilometers from Chendu, the capital of Sichuan. The average PM 2.5 value in the air of Xindu in May 2016 is 53 $\mu g/m^3$, while it is 40 $\mu g/m^3$ in Zizhong and 35 $\mu g/m^3$ in Longchang. These data suggest that the fermentation of *Pinelliae Rhizoma* could have been influenced by more complex environmental factors stemming from a modern metropolis. So do the fungi, the genus of *Auricularia delicata* was found in both LC and ZZ, but was not detected in XD, so it may be associated with the local environment of Longchang and Zizhong.

Although above five genera are thought to be effective in the fermentation of *Pinelliae Rhizoma*, the composition of volatile or non-volatile components obviously differed among three fermented *Pinelliae Rhizoma* produced from the same material in Longchang,

Zizhong and Xindu counties. For example, non-volatile components in LC and ZZ fermented *Pinelliae Rhizoma* were similar in total peak area, while these same components yielded only half the peak area in XD, which indicated that the composition of the three fermented *Pinelliae Rhizoma* was greatly different. Therefore, it is not that the closer the two areas are, the more similar the components are, and the differences among samples seem to change geographically in an unregular way. The unique identities of fermented *Pinelliae Rhizoma* were also related to the region where the fermentation was carried out, in addition to the variety and cultivation management of *Pinellia ternate* (Lu 2012), for the results of fermentation also reflects the complexity of local microbiota.

For the most common volatile components, the activity of [S-(R*,S*)]-2-methyl-5-(1,5-dimethyl-4-hexenyl)-1,3-cyclohexadiene and [S-(R*,S*)]-3-(1,5-dimethyl-4-hexenyl)-6-methylene-cyclohexene have not been discovered. However, curcumene (Grzanna et al. 2005; Shirvani et al. 2015), geraniol (Carnesecchi et al. 2004), α-terpineol (de Sousa et al. 2007), geraniol (Chen et al. 1980) in fermented *Pinelliae Rhizoma* have been reported to have at least one kind of function. Other components containing β-bisabolene, endo-borneol, citronellol, patchouli alcohol, camphor, alloaromadendrene, citral have been shown to have antioxidant and anti-inflammatory activities (Miguel 2010), but these components were only detected in one or two fermented *Pinelliae Rhizoma*. Furthermore, fungi is more closely related to the formation of terpenoids or terpenoid volatiles (Lemfack et al. 2018), [S-(R,S)]-2-methyl-5-(1,5-dimethyl-4-hexenyl)-1,3-cyclohexadiene and [S-(R,S)]-3-(1,5-dimethyl-4-hexenyl)-6-methylene-cyclohexene were detected as main volatile components in the XD fermented *Pinelliae Rhizoma*, while not found in LC or ZZ fermented *Pinelliae Rhizoma*. This indicated that these compounds may be derived from its different fungal flora from the other two fermentations.

Non-volatile components have been shown to have many activities, for example, alkaloids contained in *Pinelliae Rhizoma* or fermented *Pinelliae Rhizoma* have the effects of sedation, hypotension and saliva secretion (Zhou et al. 2006; Xiao et al. 2016). Gentiatibetine has certain anticonvulsant and brain protective effects (Peng 2016), gingediol (curcumin) could be used to treat cold and vomiting (Hatcher et al. 2008), amarasterone A can reduce blood sugar (Catalan et al. 1982), linolenic acid has been suggested to have anti-tumor properties (Huan et al. 2012). Houpu lignan A has therapeutic effects on dyspepsia, abdominal distention, constipation and also has certain anti-cancer properties (Si et al. 2002), albopetasin can relieve swelling and pain (Zhang 2009), ginsenoyne K could invigorate the spleen and benefit the lung (He et al. 1993), ambonic acid has antibacterial effect (Omar et al. 2017), 7-hydroxycadalenal can relieve pain (State Administration of Traditional Chinese Medicine 2005), etc. These activities may attribute to the function of fermented *Pinelliae Rhizoma*.

Fermented *Pinelliae Rhizoma* produced in different counties showed equivalent or similar clinical efficacy, while in this study, on the whole, the composition of volatile and non-volatile components in LC, ZZ and XD fermented *Pinelliae Rhizoma* seem to differ substantially and be affected by their location, only a few of the same volatile or non-volatile components were found in all three fermented *Pinelliae Rhizoma*. Of note, the clinical holistic effect of fermented *Pinelliae Rhizoma* cannot be achieved by these several components alone, instead, a variety of bioactive volatile components with C10 and C15 structures as well as non-volatile components with C16, C18, C20, C21, and C22 structures were also detected in the three fermented *Pinelliae Rhizoma*. This indicates that the efficacy of fermented *Pinelliae Rhizoma* may originate from a series of structurally similar components. In the fermentation of *Pinelliae Rhizoma* by a mix of environmental microorganism, the same component in the raw material is transformed into a variety of substances with similar structures and possibilities of mutual transformation through secondary metabolism. The metabolic pathways of secondary metabolism are very complex, so despite different mixtures of microorganisms, these groups may act on similar secondary metabolite pathways creating a plethora of different compounds. The components of fermented *Pinellia ternata* neither reflect the composition of its microbiota microscopically nor geographical features macroscopically. The results also help to explain why no unique active components have been identified in those traditional Chinese medicines which demonstrate wide and definite curative effects.

With such a wide range of microbes and active compounds present in the fermentation process, we were unable to achieve a one-to-one correspondence between certain species and active compounds. Therefore, we do not yet know which species produce certain active compounds in the context of controlled mixed fermentation. These results do however provide clear evidence that many complex active compounds are produced through the solid-state mixed fermentation of *Pinelliae Rhizoma*, and as such, similar solid-state mixed fermentation processes may be also used in the preparation of other traditional Chinese medicines or natural products. Further study may be focused on the identification of the main microorganisms and their metabolic products, that may initiate controlled mixed fermentation by functional pure microbial strains with the variety of traditional Chinese herbs.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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