



# Identification of core microbiota in the fermented grains of a Chinese strong-flavor liquor from Sichuan



Wenjing Jiao <sup>a</sup>, Fei Xie <sup>b</sup>, Lei Gao <sup>a</sup>, Liqian Du <sup>b</sup>, Yanxia Wei <sup>a</sup>, Jian Zhou <sup>a, \*\*</sup>, Guiqiang He <sup>a,\*</sup>

<sup>a</sup> School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, Sichuan, 621010, China

<sup>b</sup> Forgood Distillery Co., Ltd, Mianyang, Sichuan, 621000, China

## ARTICLE INFO

### Keywords:

Chinese liquor  
Microbial community  
Core microbiota  
Synthetic fermentation  
Co-occurrence analysis

## ABSTRACT

In traditional fermentation process of Chinese *strong-flavor* liquor, composition, dynamic succession, and interaction of microbial community in the fermented grains (FG) are fluctuant, resulting in instability and inconsistency of liquor flavor and quality. It is therefore important to reveal the core microbiota of FG from different pit ages and layers of fermentation cellars in this study. Firstly, Venn analysis showed that 14 and 13 ubiquitous bacterial and fungal genera were respectively observed. Meanwhile, *Lactobacillus*, *Aquabacterium*, *Thermoactinomyces*, *Aspergillus*, *Thermoascus*, *Kazachstania*, *Pichia*, and *Rhizomucor* with a relative abundance over 1% in all samples were identified as dominant microbiota. Additionally, 8 bacterial and 3 fungal genera were significantly correlated with flavor compounds including esters, alcohols, acids, and aldehydes by RDA, indicating these genera were flavor-associated microbiota. Furthermore, co-occurrence network analysis demonstrated that 7 bacterial and 5 fungal genera were highly connected with other genera, which were defined as co-occurring microbiota. Taken together, *Lactobacillus*, *Thermoactinomyces*, *Aquabacterium*, *Aspergillus*, and *Kazachstania* were identified as the core microbiota in Chinese *strong-flavor* liquor brewing process. Core microbiota of liquor brewing microecosystem were defined in this work, it is of paramount important to construct synthetic microbiota for stable and homogenous production of high-quality liquor.

## 1. Introduction

Chinese liquor, an ancient alcoholic beverage in the world, occupies an important position in the brewing history with its skillful manufacturing technique and valuable cultural heritage (Jin, Zhu, & Xu, 2017). In particular, *strong-flavor* liquor, one of three top flavor types in China, is the most popular among consumers, and accounts for more than 50% of the Chinese liquor market share because of its unique flavor and aroma (Liu & Sun, 2018). Traditional production of Chinese *strong-flavor* liquor is a typical solid-state fermentation process in an open environment involving synergistic interactions by multi-microorganisms (Zou, Zhao, & Luo, 2018). Dynamic succession and evolution of these microorganisms are drove by the environmental factors such as moisture, temperature, oxygen, and acidity in the fermentation process (Wang, Du, Zhang, & Xu, 2017). Also because of this, it is difficult to control microbial metabolism during the brewing process that affects the quality of liquor products.

Based on this, many researchers have focused on how to transform to

the synthetic fermentation for production of high-quality food from spontaneous fermentation. For example, acetoin-producing microbial community composed by *Acetobacter pasteurianus*, *Lactobacillus brevis*, and *Lactobacillus fermentum* was synthesized and applied in traditional vinegar fermentation, the content of acetoin in vinegar significantly increased compared with the control group (Lu et al., 2016). It seems impossible to construct the synthetic microbiota for regulation and control of the flavor compound formation in such an open, complicated, and dynamic multi-microorganisms fermentation process like Chinese liquor brewing. However, statistical and *meta*-omics methods make it possible by identification and isolation of the core microbiota with the development of systems biology. For instance, synthetic microbiota was constructed in Chinese *light-flavor* liquor by core functional microbiota including *Lactobacillus*, *Saccharomyces*, *Pichia*, *Geotrichum*, and *Candida*, 77.27% of the flavor compounds produced by the synthetic microbiota exhibited a similar dynamic profile with that in the natural fermentation, and the flavor profile presented a similar composition (Wang, Wu, Nie, Wu, & Xu, 2019).

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [zhoujian@swust.edu.cn](mailto:zhoujian@swust.edu.cn) (J. Zhou), [guiqianghe@swust.edu.cn](mailto:guiqianghe@swust.edu.cn) (G. He).

Fermentation with optimized synthetic microbiota is an efficient approach to regulate and control the flavor metabolism in the traditional fermented food. In addition, identification of core microbiota in ecosystem and construction of synthetic microbiota has become the most frequently used method for revealing the performance and stability of microbial community (De Roy, Marzorati, Van den Abbeele, Van de Wiele, & Boon, 2014). But one of the critical problems for construction of designed synthetic microbiota is to identify the core microbiota in the complex fermentation microecosystem (Großkopf & Soyer, 2014; Wolfe & Dutton, 2015). Currently, the methods for defining core microbiota are mainly by identification of ubiquitous microbiota, dominant microbiota, flavor-associated microbiota, and co-occurring microbiota (Chaillou et al., 2015; Hu, Du, Ren, & Xu, 2016; Parente et al., 2016; Wang, Lu, Shi, & Xu, 2016). For example, *Gluconacetobacter*, *Lactobacillus*, *Lactococcus*, *Pichia*, *Wickerhamomyces*, and *Saccharomyces* were determined as the core functional microbiota in rice wine, because these genera were the largest contributors to the production of the flavor compounds (Huang et al., 2018). It was reported that 24 widely distributed and culturable genera as dominant community in cheese rinds, and these dominant genera were isolated and reconstructed the synthetic microbiota, then the manipulation and repeatability of microbial succession and dynamic were observed during the synthetic fermentation process of cheese rind (Wolfe, Button, Santarelli, & Dutton, 2014).

Unfortunately, there may still exist omissions of some important species by a single method to identifying core microbiota in the research process. Therefore, we suggest that the ubiquitous microbiota, dominant microbiota, flavor-associated microbiota, and co-occurring microbiota should be considered together and comprehensively for determining the core microbiota in the fermentation process. In the present study, these methods were carried out concurrently to identify core microbiota in the fermentation process of Chinese *strong-flavor* liquor by different pit ages and layers of fermentation cellars. Identification of core microbiota is of great significance to understand and regulate the spontaneous fermentations of Chinese liquor.

## 2. Materials and methods

### 2.1. Sampling

The FG samples were collected from fermentation cellars with different pit muds (PM) in a *strong-flavor* liquor industry, located in Mianyang city, the northwest part of Sichuan province in China. The samples were collected according to the method as shown in Supplementary Fig. 1. Normally, the flavor and quality of liquor distilled from upper layer of FG was lower than that from the next layers during the production process, so the microbial community in the middle and bottom layers of FG was more comprehensive and representative. For each cellar, five plots (200 g/each sample plot) were taken from FG in the middle and bottom layer of cellar, respectively. These five positions were mixed evenly, taken 500 g as one sample, transferred to sterile polyethylene bags, then immediately stored at -80 °C. The FG samples fermented with new PM and 40-year PM in the middle layer were numbered as N-M-FG and 40-M-FG, respectively. Likewise, the bottom layer of samples were marked N-B-FG and 40-B-FG, respectively.

### 2.2. DNA extraction, PCR amplification, and sequencing analysis

Total microbial genomic DNA of samples was extracted using the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The quantity and quality of extracted DNAs were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. The specific primers 338F/806R and ITS5/ITS1 with the Illumina barcodes were used to amplify the V3–V4 regions of bacterial 16S rRNA gene and ITS1 regions of fungal rRNA

gene, respectively (He, Huang, Zhou, Wu, & Jin, 2019a). PCR amplification procedures were performed as reported previously (He et al., 2019b). After purification and quantification of the PCR amplicon, an equal amount of amplicon was collected and the pair-end of 2 × 300 basis point sequencing was analyzed (Shanghai Personal Biotechnology Co., LTD, China).

The sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME, Version 1.8.0) pipeline (Caporaso et al., 2010). Chimeric sequences and low-quality sequences including read length less than 200 bp, mean base quality score less than 30, and mononucleotide repeats over 8 bp were filtered out (Chen & Jiang, 2014). Subsequently, paired-end reads were aligned and assembled using FLASH (Version 1.2.7) (Mago & Salzberg, 2011). The high-quality sequences were clustered into operational taxonomic units (OTUs) at the 97% sequence identity by UCLUST (Edgar, 2010). OTU taxonomic classification was conducted by BLAST searching the representative sequences set against the Greengenes Database (Release 13.8) (Desantis et al., 2006) and UNITE Database (Release 5.0) (Käppljalg et al., 2013).

### 2.3. Determination of flavor compounds

The volatile compounds in FG samples were extracted and detected by headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS). A 50/30 μm DVB/CAR/PDMS fibre (Supelco, Bellefonte, PA, USA) was used for extracting volatile compounds. For each extraction, 1.0 g of FG sample and 20 μL of internal standard (2-octanol, 0.0063 g/100 mL) were placed into a 15-mL headspace vial. The vial was transferred to a thermostatic block stirrer for thermal equilibrium with 10 min at 55 °C, and subsequently extracted for another 50 min.

After extraction, SPME fibre was withdrawn into the needle and immediately inserted into the injection port of GC-MS system, where the extracted volatiles were desorbed thermally for 5 min at 250 °C. The volatiles of FGs were transferred into a DB-WAX capillary column (30.0 m × 0.25 mm × 0.25 μm, Agilent Technology, Santa Clara, CA, USA) and detected by Trace GC Ultra gas chromatograph-DSQ II mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA). The analytical method of GC-MS was carried out as previously reported by the literature (Ding, Huang, Wu, & Zhou, 2017). The results were compared with those of NIST12 library to obtain the identification of volatile compounds.

### 2.4. Statistical and network analysis

One-way analysis of variance (ANOVA) was performed to assess the significant differences ( $P < 0.05$ ) in volatile compounds by using SPSS 19.0 software (SPSS Inc. Chicago, IL, USA). QIIME (Version 1.8.0) and R package (Version 3.2.0) were used for sequencing analysis and figure mapping. Alpha diversity indices including Chao1, Shannon, and Simpson were calculated using the QIIME at OTU level. Rarefaction curves were plotted by R packages. Redundancy analysis (RDA) between the microbial community and volatile compounds was performed with Canoco 5.0 software.

In order to analyze the correlations among microbiota in the liquor fermentation process, Spearman's rank correlations between the genera were calculated. When the absolute value of Spearman's rank correlation coefficient was over 0.8 and statistically significant ( $P < 0.01$ ), a valid correlation event to be a robust correlation was defined (Shannon et al., 2003). Cytoscape was used to create the co-occurrence network analysis and visualize the interaction among genera.

## 3. Results and discussion

### 3.1. Sequencing results and alpha diversity analysis

After removing the low-quality sequences, chimeric sequences, and

PCR primers, effective sequences with different phylogenetic OTUs were obtained by 97% sequence identity cutoff (Supplementary Table 1). The numbers of high-quality sequences ranged from 77925 to 87917 for bacteria and 90667 to 103848 for fungi, respectively. In addition, the proportions of high-quality sequences were more than 90% in all FG samples. Moreover, the rarefaction curves based on the observed species of bacteria tended to be flattened and that of fungi were saturated with increasing the sequencing depth (Supplementary Fig. 2), indicating that the obtained sequences were sufficient to represent the microbial structure of the FG samples.

The richness (Chao1 and Observed species) and diversity (Shannon and Simpson) indexes were calculated to characterize the  $\alpha$ -diversity of the microbial community in different FG samples (Table 1). For new fermentation cellar, the combined data showed that higher microbial richness (Chao1 and Observed species) in the bottom layer of FG (N-B-FG) was observed than that in the middle layer (N-M-FG). Three possible reasons were as follows. Firstly, microbiota in FG of the middle layer infiltrated into the bottom layer along with the Huangshui, consequently resulting in higher abundance in the bottom layer. In addition, microbiota seemed to prefer to grow and reproduce in the bottom layer because of the anaerobic environments during the liquor fermentation process. Lastly, the bottom PM was considered to be a source of inoculum of microbes into the bottom layer of FG (Wang, Du, & Xu, 2017). For 40-year fermentation cellar, otherwise, the diversity indexes Shannon and Simpson in the bottom layer of FG (40-B-FG) were higher than those in the middle layer (40-M-FG). For the same layer of FGs, it was worth noting that the species richness and diversity in the 40-year fermentation cellar were higher than those in the new cellar. This could be attributed to the fact that Chinese *strong-flavor* liquor brewing is a cyclic and polyphasic fermentation process, in which *Daqu* starter, PM, and Huangshui can provide microbial source for FG during the long-term production (Ding, Wu, Huang, & Zhou, 2015; Zheng & Han, 2016). In general, the quantities and proportions of microbial community in fermentation cellar were continuously evolved and until mature and stable with the increase of cellar age. For example, the prokaryotic diversity increased with cellar age in succession period (from 1 to 25 years), but there was no significant differences between the 25-year and 50-year samples in the diversity and richness indices (Tao et al., 2014).

### 3.2. Identification of ubiquitous microbiota

The similarity and difference of microbial community between the FG samples were revealed by a Venn diagram (Fig. 1). The ubiquitous microbiota were distinguished by the shared genera in all the samples. Microbial community of the FG was composed by a total of 99 bacterial and 81 fungal genera. For bacteria, 14 ubiquitous genera including *Lactobacillus*, *Aquabacterium*, *Weissella*, *Thermoactinomyces*, *Azospirillum*, *Novosphingobium*, *Caulobacter*, *Flavobacterium*, *Pseudomonas*, *Pelomonas*, *Bacillus*, *Pantoea*, *Acinetobacter*, and *Sedimentibacter* were observed (Fig. 1A). Most of them have been usually detected in the FG of Chinese *strong-flavor* liquor (Sun et al., 2016; Wang, Du, & Xu, 2017). As for fungi, 13 genera containing *Aspergillus*, *Thermoascus*, *Rasamonia*, *Malassezia*, *Penicillium*, *Russula*, *Pichia*, *Thermomyces*, *Mycosphaerella*, *Apotrichum*, *Fusarium*, *Kazachstanica*, and *Hizomuco* were defined as ubiquitous microbiota (Fig. 1B). These genera were mainly composed by thermophilic fungi, yeast, and mould, also uncovered in previous studies

(He, Huang, Wu, Jin, & Zhou, 2019c; He et al., 2019a).

### 3.3. Identification of dominant microbiota

Relative abundance of microbial taxa was analyzed at the phylum level, as shown in Fig. 2. For bacteria, two dominant phyla *Firmicutes* and *Proteobacteria* were observed (Fig. 2A). The total relative abundance of both phyla was ranged from 89.97% to 98.79% in these FG samples. *Firmicutes* was the predominant in FG samples with its proportion over 65%. For fungi, the dominant phylum was *Ascomycota*, and its abundance accounted for 20.67%–87.12% in the samples (Fig. 2B). It was interesting that most sequences could not be classified to fungal taxa by UNITE Database (Käpljalg et al., 2013), suggesting that some novel microorganisms have not been fully uncovered in the liquor brewing process.

The genera with a relative abundance over 1.0% in all samples were considered as dominant microbiota. For bacteria, dominant microbiota was composed by three genera including *Lactobacillus*, *Aquabacterium*, and *Chloroplast* (Fig. 3A). *Lactobacillus* exhibited the highest abundance in all samples, which was ranged from 62.49% to 98.69%. Abundant *Lactobacillus* could produce lactic acid in the brewing process, which was of great significance to inhibit the harmful microorganisms and maintain food quality and safety (Cizeikiene, Juodeikiene, Paskevicius, & Bartkiene, 2013). In addition, lactic acid is the precursor of ethyl lactate, which is helpful to the formation of the mellow flavor in Chinese liquor (Gao, Fan, & Xu, 2014). In particular, the abundance of *Lactobacillus* in the middle layer of FG was higher than that in the bottom layer for the same fermentation cellar. *Lactobacillus*, facultative anaerobe, maybe like to inhabit the middle layer, in which oxygen content was higher than that in the bottom layer. The dominant genus of *Aquabacterium*, a denitrifying bacterium (Zhang, Li, Szewzyk, & Ma, 2016), was the first reported for Chinese liquor production. In our study, the abundance of *Aquabacterium* in 40-year fermentation cellar was higher than that of new cellar, and the bottom layer of FG exhibited higher proportion than that in the middle layer. Although the functions of *Aquabacterium* in liquor brewing process remain unclear so far, it was reported that *Aquabacterium* could produce glucan branching enzymes and affect the starch hydrolysis (Xia et al., 2021). Taken together, *Aquabacterium* may contribute to carbon cycling in the liquor fermentation system by involving the biological anaerobic digestion process. *Chloroplast* was considered as one of dominant genera, and it was uncovered in vinegar fermentation (Peng, Yang, Guo, & Han, 2015). Additionally, *Chloroplast* also was observed in the *strong-flavor* liquor brewing process (Sun et al., 2016), and it was produced by PCR amplification of 16S rRNA gene in *Cyanobacteria*.

For fungi, dominant genera including *Aspergillus*, *Thermoascus*, *Kazachstanica*, *Pichia*, and *Rhizomucor* were observed (Fig. 3B). Among them, *Aspergillus* was obtained with the abundance over 7% in all samples, and the N-B-FG presented the highest proportion of 68.63%. *Aspergillus* was defined as an important genus in the fermentation starter (*Daqu*) for production of Chinese liquor (Zheng, Tabrizi, Nout, & Han, 2012). The main function of *Aspergillus* is to secrete a variety of enzymes, such as saccharification, liquefaction enzymes, and proteases (Masayuki, Osamu, & Katsuya, 2008), which plays a crucial role in promoting the decomposition of starch, protein, and other macromolecules of the brewing raw materials, laying a foundation for the

**Table 1**  
Alpha diversity indexes of the microbial community in FG samples.

Samples	Chao1		Observed species		Shannon		Simpson	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
N-M-FG	69	183	66	182	1.36	3.65	0.50	0.86
N-B-FG	193	284	180	284	2.26	2.69	0.65	0.53
40-M-FG	422	525	397	525	2.73	5.15	0.64	0.87
40-B-FG	302	916	289	915	3.43	6.65	0.79	0.96

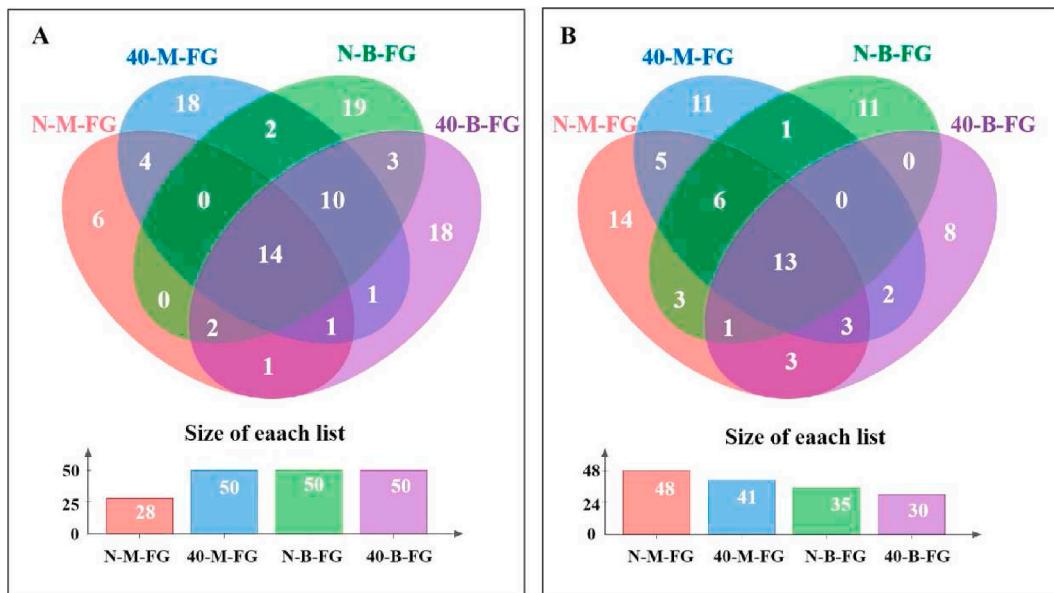


Fig. 1. Comparison of bacterial (A) and fungal (B) genera between the FG samples by Venn diagram.

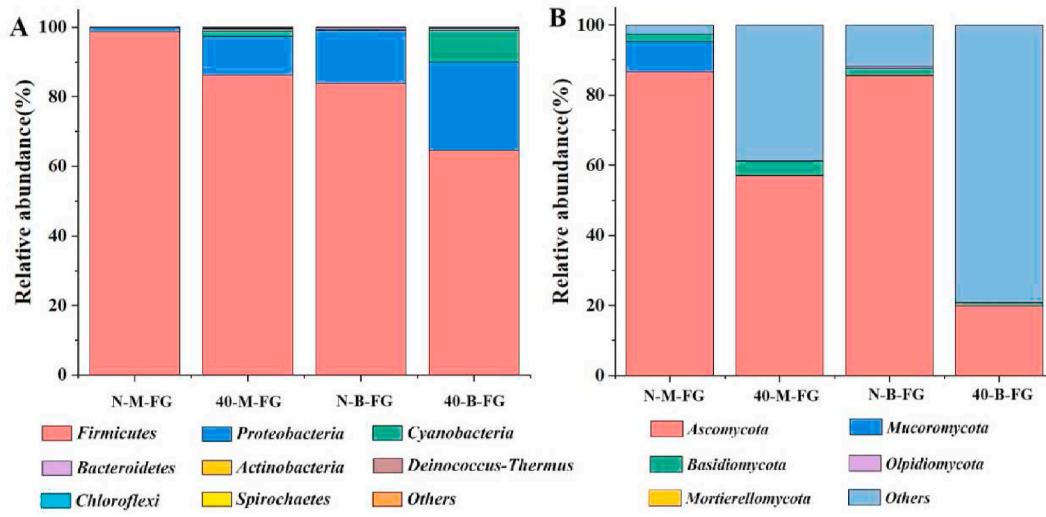


Fig. 2. Microbial compositions of bacteria (A) and fungi (B) in FG at phylum level.

subsequent formation of liquor flavor. The abundance of *Thermoascus* in the middle layer of FG was higher than that of bottom layer. This thermophilic genus has been reported to be high production of thermophilic enzymes for carbohydrate degradation (Mcclendon et al., 2012), which might have beneficial effects on liquor production. *Kazachstania* and *Pichia*, both of dominant yeast in the strong-flavor liquor fermentation process, played key roles in the production of ethanol and flavor alcohols (Ling et al., 2021). In addition, *Kazachstania* could produce blue-phenylethyl alcohol and ethyl fatty acid, which contributed to the flower and fruit flavor characteristics of liquor (Dashko et al., 2015).

Hierarchical clustering analysis was used to determine whether the microbial structure differentiated between different FG samples. For bacteria, the comparisons by clustering indicated that the identified genera partitioned the FG samples into three groups. FGs fermented in new fermentation cellar (N-M-FG and N-B-FG) were clustered into a group, 40-M-FG and 40-B-FG were clustered another group, respectively (Fig. 3A). These results showed that the bacterial community was sensitively affected by different cellar ages. For fungi, the middle layer of

FGs (N-M-FG and 40-M-FG) were gathered into a group, N-B-FG and 40-B-FG were clustered another group, respectively (Fig. 3B). The results demonstrated that different layers of fermentation cellar easily resulted in the distinction of the fungal community of the FG.

### 3.4. Identification of flavor-associated microbiota

Firstly, the flavor compounds of FG samples were determined in our study. A total of 63 volatile compounds were identified and classified in these samples (Supplementary Table 2). These compounds could be divided into esters (40), acids (8), alcohols (7), aldehydes and ketones (4), and phenols (4), according to their chemical structures and characteristics. As shown in Fig. 4, the concentrations of volatile compounds exhibited significant differences between the FG samples. The heterogeneity of flavor compounds in different cellar ages and layers of FG was in accordance with different  $\alpha$ -diversity indexes and community compositions in FG samples (Table 1 and Fig. 3). Among these compounds, the highest concentration of volatile compound was esters, followed by acids, alcohols, and a small amount of phenols, aldehydes and ketones.

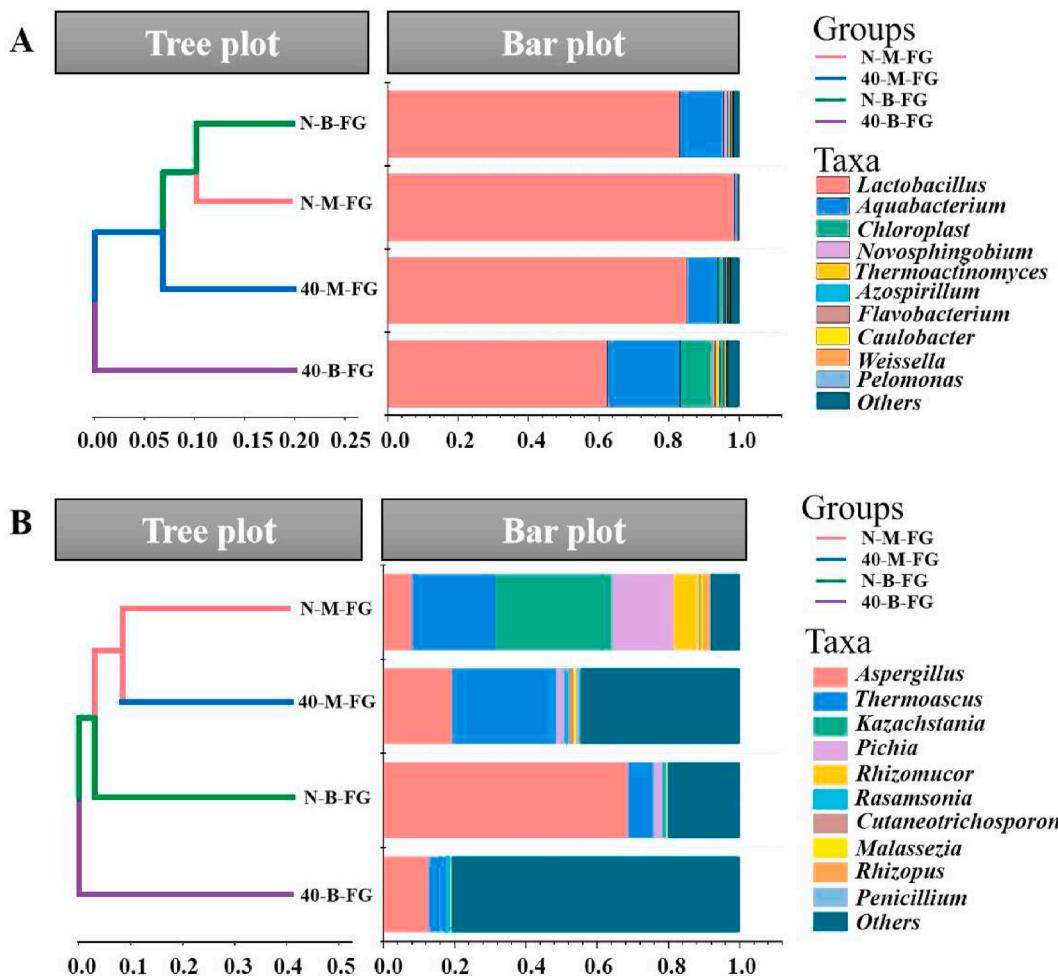


Fig. 3. Hierarchical clustering analysis based on genus level of bacterial (A) and fungal (B) community.

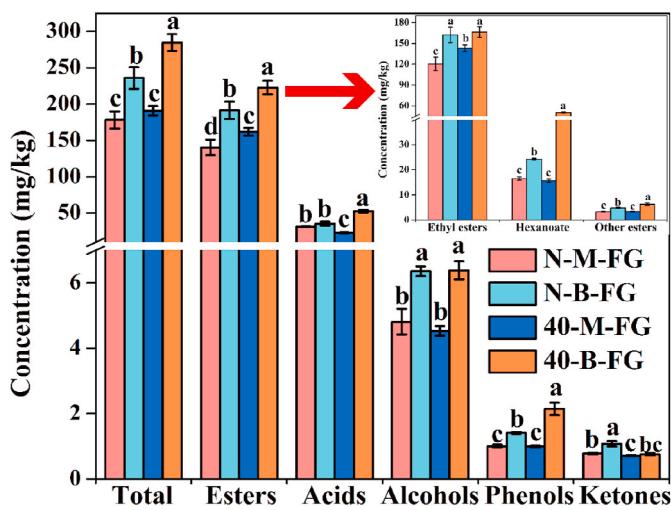
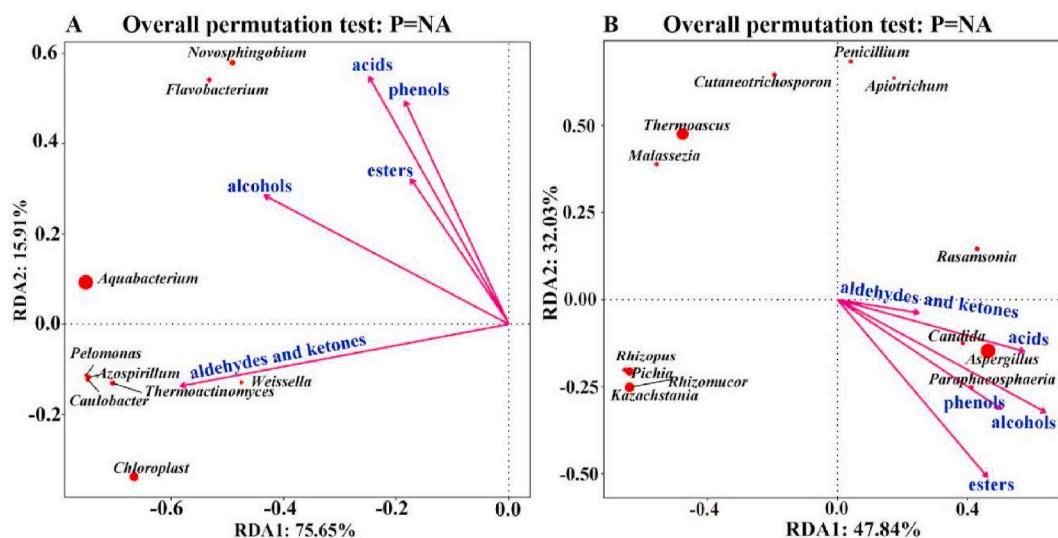


Fig. 4. Concentration of the volatile compounds in FG samples. Different letters obtained by ANOVA indicated significant differences at  $P < 0.05$  ( $n = 3$ ).

Compared with the middle layer of FG, a remarkable increase in the total concentration of volatile compounds was observed in the bottom layer of FG, in which the 40-B-FG exhibited higher contents than that of N-B-FG. In particular, the esters composed by ethyl esters, hexanoate esters, and other esters, in which ethyl esters were considered as skeleton flavor

compounds in Chinese *strong-flavor* liquor (Fan & Qian, 2006), and accounted for 74.6%–88.3% in these samples.

Furthermore, the correlations between microbial genera and volatile compounds were revealed by redundancy analysis (RDA), as shown in Fig. 5. As a whole, the two axes explained 91.56% and 79.87% of the total variation in bacterial and fungal community differentiation, respectively, suggesting the strong correlations between microbial community and flavor compounds. For bacteria, *Flavobacterium* and *Novosphingobium* were positively correlated with esters, acids, alcohols, and phenols (Fig. 5A). In addition, positive correlations between *Pelomonas*, *Azospirillum*, *Caulobacter*, *Weissella*, and *Thermoactinomyces* with aldehydes and ketones were observed. It was worth noting that *Thermoactinomyces* as a thermotolerant genus in medium-temperature *Daqu* could secrete the esterase amylase, which was critical to the flavor production of Chinese liquor (Xiao et al., 2017). In particular, abundant *Aquabacterium* was positively related to all the compounds, which indicated again that the *Aquabacterium* was an important genus for Chinese *strong-flavor* liquor brewing. Among fungi, the flavor compounds were mainly correlated with *Candida*, *Aspergillus*, and *Paraphaeosphaeria* (Fig. 5B). Therefore, the flavor-associated microbiota in FG included 8 bacterial genera (*Novosphingobium*, *Flavobacterium*, *Aquabacterium*, *Pelomonas*, *Azospirillum*, *Caulobacter*, *Weissella*, and *Thermoactinomyces*) and 3 fungal genera (*Candida*, *Aspergillus*, and *Paraphaeosphaeria*). These results demonstrated that these genera were the main contributors to flavor formation in the liquor brewing process.



**Fig. 5.** Correlations of bacterial (A) and fungal (B) genera with volatile compounds were constructed by redundancy analysis (RDA).

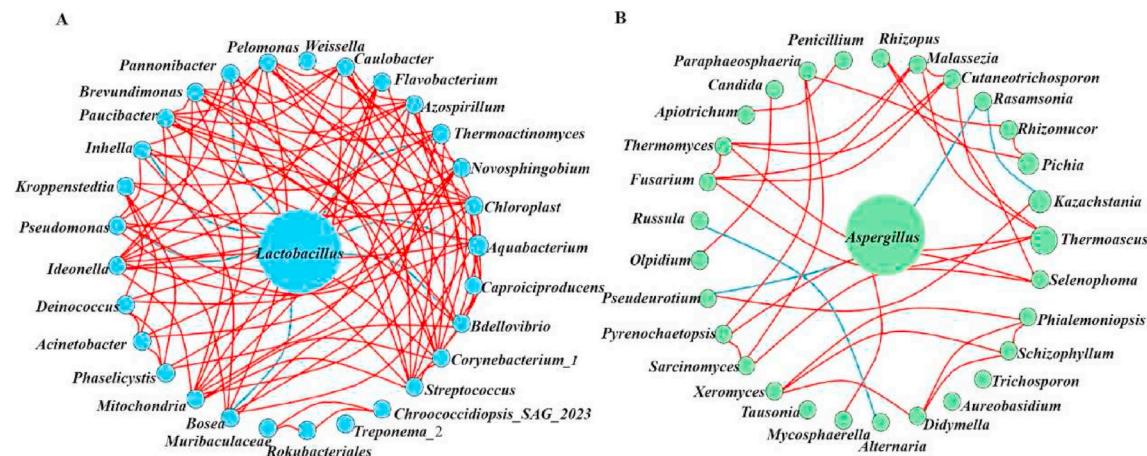
### 3.5. Identification of co-occurring microbiota

To reveal the interactions between bacterial and fungal communities, we calculated Spearman correlation coefficients and explored the co-occurrence and co-exclusion patterns of the dominant genera (Fig. 6). Bacterial results showed that the networks consisted of total 92 pairs (edges) of significant and robust correlations from 30 genera (nodes) (Fig. 6A). Among them, 85 and 7 pairs of positive and negative correlations respectively were identified from these genera. 7 critical nodes (strongly connected genera,  $\geq 8$  edges per node) were mainly distributed by *Lactobacillus*, *Aquabacterium*, *Thermoactinomyces*, *Caproiciproducens*, *Caulobacter*, *Streptococcus*, and *Azospirillum*. In particular, *Caproiciproducens* was an important genus for production of hexanoic acid and it was positively correlated with ethyl hexanoate and hexyl hexanoate in the liquor brewing microecosystem (Liu et al., 2017; He et al., 2019c), so highly complex interactions by *Caproiciproducens* might contribute to the formation of typical characteristics for Chinese strong-flavor liquor. It was worth noting that 7 pairs of negative correlations presented in the bacterial co-occurrence network were all related to *Lactobacillus*. And its co-exclusion correlations including *Aquabacterium*, *Thermoactinomyces*, *Bosea*, *Pannonibacter*, *Flavobacterium*, *Bdellovibrio*, and *Inhella* were obtained. *Lactobacillus* was the dominant

genus in Chinese liquor fermentation process, providing important flavor substances such as ethanol, acetic acid, and lactic acid. These substances can rapidly change the brewing ecological environment during fermentation and inhibit the growth of other intolerant microorganisms (De Angelis, Calasso, Cavallo, Di Cagno, & Gobbetti, 2016), which was an important reason why the *Lactobacillus* was negatively correlated with many other bacteria. For the fungal network, 5 highly connected genera including *Aspergillus*, *Malassezia*, *Thermoascus*, *Fusarium*, and *Thermomyces* were observed (Fig. 6B). These genera were mainly composed by mould and thermophilic fungi, and they can produce a series of enzymes including amylase, lipase, and protease (Masayuki et al., 2008; Mcclendon et al., 2012), which could be beneficial for hydrolysis of the macromolecular substances from fermented cereals. So more complex interactions by these genera might accelerate the brewing process and subsequently the flavor compounds conversion.

### 4. Conclusion

Identification of the core microbiota in the liquor brewing is a critical factor for construction of the synthetic microbiota. In the present study, core microbiota in the fermentation process of Chinese strong-flavor liquor was revealed by different pit ages and layers of fermentation



**Fig. 6.** Co-occurrence networks of bacterial (A) and fungal (B) communities based on correlation analysis. A connection stands for a statistically significant ( $P < 0.01$ ) and strong positive (red, Spearman's rho  $> 0.8$ ) or negative (blue, Spearman's rho  $< -0.8$ ) correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cellars. Five genera were identified to be the core microbiota including *Lactobacillus*, *Thermoactinomyces*, *Aquabacterium*, *Aspergillus*, and *Kazachstania* by finding ubiquitous microbiota, dominant microbiota, flavor-associated microbiota, and co-occurring microbiota together. These results would lay the foundation of construction the synthetic microbiota for controlling the liquor brewing process and achieving constancy of product quality.

### CRediT authorship contribution statement

**Wenjing Jiao:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Fei Xie:** Methodology, Investigation, Visualization. **Lei Gao:** Data curation, Methodology, Investigation. **Liquan Du:** Methodology, Investigation, Formal analysis. **Yanxia Wei:** Data curation, Methodology. **Jian Zhou:** Methodology, Writing – review & editing. **Guqiang He:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

### Declaration of competing interest

The authors declare no conflict of interest.

### Acknowledgement

The authors acknowledge the financial support from the Sichuan Province Key Research and Development Project (2021YFS0340) and Doctoral Scientific Fund Project of Southwest University of Science and Technology (20zx7130).

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2022.113140>.

### References

- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7, 335–336.
- Chailhou, S., Chauvel-Talmon, A., Caekebeke, H., Cardinal, M., Christeans, S., Denis, C., et al. (2015). Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood spoilage. *The ISME Journal*, 9, 1105–1118.
- Chen, H., & Jiang, W. (2014). Application of high-throughput sequencing in understanding human oral microbiome related with health and disease. *Frontiers in Microbiology*, 5, 508.
- Cizekiene, D., Juodeikiene, G., Paskevicius, A., & Bartkiene, E. (2013). Antimicrobial activity of lactic acid bacteria against pathogenic and spoilage microorganism isolated from food and their control in wheat bread. *Food Control*, 31, 539–545.
- Dashko, S., Zhou, N., Tinta, T., Sivilotti, P., Lemut, M. S., Trost, K., et al. (2015). Use of non-conventional yeast improves the wine aroma profile of Ribolla Gialla. *Journal of Industrial Microbiology and Biotechnology*, 42, 997–1010.
- De Angelis, M., Calasso, M., Cavallo, N., Di Cagno, R., & Gobbetti, M. (2016). Functional proteomics within the genus *Lactobacillus*. *Proteomics*, 16, 946–962.
- De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., & Boon, N. (2014). Synthetic microbial ecosystems: An exciting tool to understand and apply microbial communities. *Environmental Microbiology*, 16, 1472–1481.
- Desantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimeric-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, 72, 5069–5072.
- Ding, X. F., Huang, J., Wu, C. D., & Zhou, R. Q. (2017). Effects of different distillation patterns on main compounds of Chinese *Luzhou-flavour* raw liquors. *Journal of the Institute of Brewing*, 123, 442–451.
- Ding, X. F., Wu, C. D., Huang, J., & Zhou, R. Q. (2015). Interphase microbial community characteristics in the fermentation cellar of Chinese *Luzhou-flavor* liquor determined by PLFA and DGGE profiles. *Food Research International*, 72, 16–24.
- Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, 26, 2460–2461.
- Fan, W. L., & Qian, M. C. (2006). Characterization of aroma compounds of Chinese “Wuliangye” and “Jiannanchun” liquors by aroma extract dilution analysis. *Journal of Agricultural and Food Chemistry*, 54, 2695–2704.
- Gao, W. J., Fan, W. L., & Xu, Y. (2014). Characterization of the key odorants in light aroma type Chinese liquor by gas chromatography-olfactometry, quantitative measurements, aroma recombination, and omission studies. *Journal of Agricultural and Food Chemistry*, 62, 5796–5804.
- Großkopf, T., & Soyer, O. S. (2014). Synthetic microbial communities. *Current Opinion in Microbiology*, 18, 72–77.
- He, G. Q., Dong, Y., Huang, J., Wang, X. J., Zhang, S. Y., Wu, C. D., et al. (2019b). Alteration of microbial community for improving flavor character of *Dagu* by inoculation with *Bacillus velezensis* and *Bacillus subtilis*. *LWT-Food Science and Technology*, 111, 1–8.
- He, G. Q., Huang, J., Wu, C. D., Jin, Y., & Zhou, R. Q. (2019c). Bioturbation effect of fortified *Dagu* on microbial community and flavor metabolite in Chinese strong-flavor liquor brewing microecosystem. *Food Research International*, 129, Article 108851.
- He, G. Q., Huang, J., Zhou, R. Q., Wu, C. D., & Jin, Y. (2019a). Effect of fortified *Dagu* on the microbial community and flavor in Chinese strong-flavor liquor brewing process. *Frontiers in Microbiology*, 10, 56.
- Huang, Z. R., Hong, J. L., Xu, J. X., Li, L., Guo, W. L., Pan, Y. Y., et al. (2018). Exploring core functional microbiota responsible for the production of volatile flavour during the traditional brewing of Wuyi Hong Qu glutinous rice wine. *Food Microbiology*, 76, 487–496.
- Hu, X. L., Du, H., Ren, C., & Xu, Y. (2016). Illuminating anaerobic microbial community and cooccurrence patterns across a quality gradient in Chinese liquor fermentation pit muds. *Applied and Environmental Microbiology*, 82, 2506–2515.
- Jin, G. Y., Zhu, Y., & Xu, Y. (2017). Mystery behind Chinese liquor fermentation. *Trends in Food Science & Technology*, 63, 18–28.
- Käpäljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., et al. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, 22, 5271–5277.
- Ling, Y., Zhao, D., Zhou, R. Q., Tan, Y., Wang, T., & Zheng, J. (2021). Distribution and function of dominant yeast species in the fermentation of strong-flavor *baijiu*. *World Journal of Microbiology and Biotechnology*, 37, 108–116.
- Liu, H., & Sun, B. G. (2018). Effect of fermentation processing on the flavor of *baijiu*. *Journal of Agricultural and Food Chemistry*, 66, 5425–5432.
- Liu, M. K., Tang, Y. M., Guo, X. L., Zhao, K., Tian, X. H., Liu, Y., et al. (2017). Deep sequencing reveals high bacterial diversity and phylogenetic novelty in pit mud from *Luzhou Laojiao* cellars for Chinese strong-flavor *Baijiu*. *Food Research International*, 102, 68–76.
- Lu, Z. M., Liu, N., Wang, L. J., Wu, L. H., Gong, J. S., Yu, Y. J., et al. (2016). Elucidating and regulating the acetoxy production role of microbial functional groups in multispecies acetic acid fermentation. *Applied and Environmental Microbiology*, 82, 5860–5868.
- Mago, T., & Salzberg, S. L. (2011). Flash: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27, 2957–2963.
- Masayuki, M., Osamu, Y., & Katsuya, G. (2008). Genomics of *Aspergillus oryzae*: Learning from the history of *koji* mold and exploration of its future. *DNA Research*, 15, 173–183.
- McClendon, S. D., Batth, T., Petzold, C. J., Adams, P. D., Simmons, B. A., & Singer, S. W. (2012). *Thermoascus aurantiacus* is a promising source of enzymes for biomass deconstruction under thermophilic conditions. *BioTechnology for Biofuels*, 5, 54.
- Parente, E., Cocolin, L., De, F. F., Zotta, T., Ferrocino, I., Osullivan, O., et al. (2016). Foodmicrobionet: A database for the visualisation and exploration of food bacterial communities based on network analysis. *International Journal of Food Microbiology*, 219, 28–37.
- Peng, Q., Yang, Y., Guo, Y., & Han, Y. (2015). Analysis of bacterial diversity during acetic acid fermentation of Tianjin Dului aged vinegar by 454 pyrosequencing. *Current Microbiology*, 71, 195–203.
- Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Research*, 13, 2498–2504.
- Sun, W., Xiao, H., Peng, Q., Zhang, Q., Li, X., & Han, Y. (2016). Analysis of bacterial diversity of Chinese *Luzhou-flavor* liquor brewed in different seasons by Illumina MiSeq sequencing. *Annals of Microbiology*, 66, 1293–1301.
- Tao, Y., Li, J. B., Rui, J. P., Xu, Z. C., Zhou, Y., Hu, X. H., et al. (2014). Prokaryotic communities in pit mud from different-aged cellars used for the production of Chinese strong-flavored liquor. *Applied and Environmental Microbiology*, 80, 2254–2260.
- Wang, X. S., Du, H., & Xu, Y. (2017). Source tracking of prokaryotic communities in fermented grain of Chinese strong-flavor liquor. *International Journal of Food Microbiology*, 244, 27–35.
- Wang, X. S., Du, H., Zhang, Y., & Xu, Y. (2017). Environmental microbiota drives microbial succession and metabolic profiles during Chinese liquor fermentation. *Applied and Environmental Microbiology*, 84, e02369–17.
- Wang, Z. M., Lu, Z. M., Shi, J. S., & Xu, Z. H. (2016). Exploring flavour-producing core microbiota in multispecies solid-state fermentation of traditional Chinese vinegar. *Scientific Reports*, 6, 26818.
- Wang, S., Wu, Q., Nie, Y., Wu, J., & Xu, Y. (2019). Construction of synthetic microbiota for reproducible flavor metabolism in Chinese light aroma type liquor produced by solid-state fermentation. *Applied and Environmental Microbiology*, 85, 0 e03090–18.
- Wolfe, B. E., Button, J., Santarelli, M., & Dutton, R. (2014). Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell*, 158, 422–433.
- Wolfe, B. E., & Dutton, R. J. (2015). Fermented foods as experimentally tractable microbial ecosystems. *Cell*, 161, 49–55.
- Xiao, C., Lu, Z. M., Zhang, X. J., Wang, S. T., Ao, L., Shen, C. H., et al. (2017). Bio-Heat is a key environmental driver shaping the microbial community of medium-temperature *Dagu*. *Applied and Environmental Microbiology*, 83, e01550–17.
- Xia, C. Y., Qiao, Y., Dong, C. N., Chen, X. P., Wang, Y. X., & Li, D. (2021). Enzymatic properties of an efficient glucan branching enzyme and its potential application in starch modification. *Protein Expression and Purification*, 178, Article 105779.

- Zhang, X. X., Li, A., Szewzyk, U., & Ma, F. (2016). Improvement of biological nitrogen removal with nitrate-dependent Fe (II) oxidation bacterium *Aquabacterium parvum* B6 in an up-flow bioreactor for wastewater treatment. *Bioresource Technology*, 219, 624–631.
- Zheng, X. W., & Han, B. Z. (2016). *Baijiu* (白酒), Chinese liquor: History, classification and manufacture. *Journal of Ethnic Foods*, 3, 19–25.
- Zheng, X. W., Tabrizi, M. R., Nout, M. J. R., & Han, B. Z. (2012). *Daqu*-a traditional Chinese liquor fermentation starter. *Journal of the Institute of Brewing*, 117, 82–90.
- Zou, W., Zhao, C. Q., & Luo, H. B. (2018). Diversity and function of microbial community in Chinese strong-flavor *baijiu* ecosystem: A review. *Frontiers in Microbiology*, 9, 671.