

Analysis of Microbial Diversity and Variation Law During Xiaoqu Baijiu Fermentation using High-throughput Sequencing Technology

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Abstract

In this study, high-throughput sequencing (HTS) was used to compare and analyze the microbial diversity and variation law during the brewing process of *xiaoqu Baijiu*. The results showed that 34 phyla, 378 genera of bacteria and 4 phyla, 32 genera of fungi were detected. At the phylum level, Firmicutes, Proteobacteria, Bacteroidetes, Ascomycota and Bacteroidetes were the dominant groups. During the brewing process of *xiaoqu Baijiu*, the dominant bacteria were *Weissella* and unidentified *Rickettsiales* 2 days before brewing and *Lactobacillus* 3 days after brewing until the end of brewing. The dominant fungi were *Rhizopus*, *Saccharomyces* and *Issatchenkia*. The relative abundance of *Rhizopus* decreased with the extension of brewing time, while the relative abundance of *Saccharomyces* increased and became the dominant bacteria after the second day of brewing. This study revealed the diversity and variation of microbial community in the brewing process of *xiaoqu Baijiu*, and provide theoretical support and lay the foundation for future study on the contribution of microbial metabolism during brewing of *xiaoqu Baijiu*, thereby promote the development of *xiaoqu baijiu* industry.

Introduction

Chinese liquor (*Baijiu*) is one of the oldest solid-state fermented, distilled spirits [1]. *Baijiu* can be categorized into three major types, sauce-flavour *Baijiu*, strong-flavour *Baijiu*, and light-flavour *Baijiu* according to the aroma components [2]. *Xiaoqu Baijiu* is an important component of light-flavour *Baijiu* in China, which is produced by *xiaoqu*. *Xiaoqu* is one types of *jiuqu* used in *Baijiu* production. *Xiaoqu* is usually made of rice, rice bran and wheat bran as raw materials, which is artificially inoculated with *Rhizopus* and yeast [3, 4]. *Xiaoqu Baijiu* had a short brewing period and a higher yield compared with *Daqu Baijiu* [4].

The research of microbial diversity is mostly carried out by traditional microbial culture technology in early stage. In recent years, with the rapid development of molecular biotechnology, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and high-throughput sequencing (HTS) technology are applied to analyze microbial community diversity [5]. HTS is a simple technique that generated information rich data [6]. HTS technology has been more and more applied in the research of *Daqu Baijiu*, including *Daqu*, pit mud and fermented grains to study diversity and structure of microorganisms [7–10]. Bacterial flora plays an important role for *Baijiu* production and quality according to the research of sauce-flavour *Baijiu* and strong-flavour *Baijiu* [11, 6]. However, few studies investigated the microbial diversity and community structure of light-flavour *Baijiu* [12], especially *xiaoqu Baijiu*. Studies for microbial diversity in *xiaoqu Baijiu* mainly focused on *xiaoqu* from different producing areas in China, which possessed different bacterial compositions leading to the difference of volatile compounds in *xiaoqu Baijiu* [13, 5, 14]. *Lactobacillus*, *bacillus*, *Acinetobacter* and *glucobacillus* are dominant bacteria in *xiaoqu* from Hubei and Sichuan in China [15]. Bacterial community structure is relatively complex than the fungal community in *xiaoqu*. However, there is little research focused on the microbial community structure and diversity in *xiaoqu Baijiu* brewing process. Though bacterial community structure was analyzed in brewing process, the structure and diversity of fungal were not

studied [16]. Changes in the microbial communities of Sichuan Xiaoqu baijiu fermented grains which used Dazhu *xiaoqu* not pure *xiaoqu* were evaluated using HTS [17]. The difference of microbial community diversity of *xiaoqu* may lead to significant differences in the quality of *xiaoqu Baijiu* [13]. Tracking the microbial community structure and dynamic succession law during *xiaoqu Baijiu* brewing will provide a scientific basis for the correlation between microorganisms and the formation of *xiaoqu Baijiu*.

In the present study, we investigated microbial community structure and diversity during *xiaoqu Baijiu* brewing. It is helpful to understand of the contribution of various microorganisms to the quality of *xiaoqu Baijiu*. Pure cultured *xiaoqu* was used to better understand the source and role of functional bacteria in *xiaoqu Baijiu*, deeply excavating the functional bacteria and improving the quality of *xiaoqu Baijiu* in the future.

Materials And Methods

Sample preparation and collection

Samples were collected from the distillery of Wangxian Co.Ltd, Chongqing, China. The pure culture *xiaoqu* was prepared by mixing *Aspergillus niger*, *Rhizopus nigrum*, *Rhizopus oryzae* and *Aspergillus oryzae* with 7:2:3:1 inoculated with wheat bran according to 0.4% of the weight of raw materials. The aroma-producing yeast was added according to 0.5% of the weight.

After soaking, steaming, cooling, mixed with *xiaoqu*, stacking and saccharifying for 24 h, sorghum was put into the pit and mud sealed. The pit was made of cement, and the brewing cycle was 7 days (Fig.1). The microbial species and abundance change greatly in the early stage of brewing, and tend to be stable in the late stage of brewing. Therefore, the samples were taken at the end of the stacking brewing stage (Day0), every day in the first three days (Day1, Day2, Day3), and on the fifth and last day (Day5, Day7) in the *Baijiu* brewing stage. The samples were taken at 30 cm below the surface of fermented grains. The samples were frozen in liquid nitrogen immediately after collection, and then stored in -80 °C ultra-low temperature refrigerator for subsequent DNA extraction.

DNA extraction and HTS

Genomic DNA of the samples was extracted by CTAB method. Then, the purity and concentration of DNA were detected by agarose gel electrophoresis. A suitable sample of DNA was used in the centrifuge tube, and the sample was diluted with sterile water to 1 ng/ L.

The V4 hypervariable regions of 16S rDNA in bacterial were amplified with primer set 515F/806R (515F: gtagcagcmccgcgtaa, 806R: ggactacnvgggtwtctaa). The ITS1 hypervariable regions in fungal were amplified with primer set 1737F/2043R (1737F: ggaagtaaagctgtaacagg, 2043R: gctgcgttctcatcatgatgc).

All PCR products were purified and then constructed library using TruSeq® DNA PCR-Free Sample Preparation Kit. After the library was qualified by qubit and Q-PCR, samples were loaded on the Illumina NovaSeq6000 platform (Novogene Co., Ltd. China) for HTS.

Data analyses

The raw HTS data, spliced the reads using FLASH software [18], was processed with QIIME [19]. Final effective data is obtained by Tags interception and length filtering, and then were clustered into operational taxonomic units (OTUs) with 97% sequence identity by VSEARCH software [20,21]. The OTU sequence was annotated using Mothur method compared with database, and the relative content of OTU in each sample was calculated [22]. Shannon diversity and Chao1 richness were obtained on the basis of the OTUs [23]. Bacterial community composition map was drawn by R package software.

Results

Microbial abundance and OTU diversity

In order to study the species composition of each sample, OTUs was clustered with 97% identity, and then the OTUs sequence was annotated. The results of HTS showed that 1609 OTU of 16S rRNA V4 region and 57 OUT of ITS1 were obtained by clustering. A total of 34 phyla, 378 genera and 215 species level bacteria and 4 phyla, 32 genera and 44 species level fungi were annotated after comparing each OTU with the taxonomy database.

The OTUs of bacteria in all sample was counted and compared in *xiaoqu Baijiu* brewing process. Venn diagram was created to investigate whether exclusively shared OTUs existed. As shown in Fig.2, at a 97% similarity level, 60 OTUs and 14 OTUs coexisted in the six samples for bacterial and fungal. The results suggested the fungal community is relatively less complex than bacterial community during *xiaoqu Baijiu* brewing. The number of unique OTUs decreased during brewing. The OUT number was different which may be a result of the production process.

The community abundance and diversity indexes, including Shannon, Simpson, ACE and Chao values, were calculated to assess the abundance and diversity of microbial (Table 1). The Chao and Shannon of each sample were used to evaluate the sequencing abundance and diversity of species, respectively. The Chao was positively correlated with the microbial community abundance. The average coverage of all samples exceeds 0.99, indicating that the identified sequences represented the sample. ACE and Chao decreased on the first day of brewing, and then gradually increased, and reached the highest value on the fifth day of brewing. Shannon and Simpson were used to estimate the microbial diversity in samples. Shannon and Simpson had the highest at the second day of brewing. Generally, the abundance and diversity of bacterial of *xiaoqu Baijiu* samples decreased as brewing days increased.

For fungal, ACE index in samples increased at the first day, decreased on the second day. Chao index is the community richness index, and the highest index is 38.6 at the first day of brewing. Shannon index is the minimum at the beginning of brewing and the maximum at the third day of brewing. Simpson index can also be used to estimate the microbial diversity index in the sample, which is also the lowest at the beginning of brewing.

Table 1

Richness and diversity indexes of microbial community in the samples

Samples	Bacterial				Fungi			
	Shannon	Simpson	Chao1	Ace	Shannon	Simpson	Chao1	Ace
Day0	2.95	0.81	179	183	0.75	0.21	28	30.22
Day1	2.33	0.64	134	135	1.57	0.57	39	42.79
Day2	3.52	0.81	155	159	1.71	0.65	28	31.70
Day3	2.27	0.59	146	150	1.77	0.66	26	27.00
Day5	2.79	0.77	171	176	1.62	0.65	28	32.34
Day7	3.06	0.77	145	147	1.43	0.56	28	33.20

Diversity analysis of bacterial community

At the phylum level, 34 bacterial species were identified in samples. The top 10 species in abundance level and other species merged into others were shown in Fig. 3. The dominant bacteria phylum are Proteobacteria and Firmicutes as shown in Fig. 3a. In the early stage of brewing, the dominant bacteria were Proteobacteria and Firmicutes. The proportion of Proteobacteria decreased during the brewing of *xiaoqu Baijiu*, and Firmicutes became dominant bacteria, which may be because some aerobic bacteria of Proteobacteria may be difficult to survive in the environment of low oxygen, high acid and alcohol. There is still a certain relative abundance of Actinobacteria before 2 days brewing, and reduced to a very low relative abundance.

A total of 378 bacterial species were detected in *xiaoqu Baijiu* samples at the genus level. The top 20 species in abundance level and other species merged into others were shown in Fig. 3b. Although the dominant bacterial genera (top 20) were almost identical, there were significant differences in bacterial abundance among the samples. The top 5 species are *Lactobacillus*, *Weissella*, unidentified *Rickettsiales*, *Bacillus*, and *Pantoea*. The relative abundance of *Weissella* and unidentified *Rickettsiales* accounted for a large proportion before brewing and decreased during brewing. The relative abundance of *Bacillus* increased before the second day of brewing. The relative abundance of *Pantoea* did not change much during brewing period. The relative abundance of *Lactobacillus* did not change much in the first two days

of brewing, but increased rapidly with the brewing, and became the dominant bacteria in *xiaoqu Baijiu* samples.

Diversity Analysis of fungi community

At the phylum level, 4 species were identified in samples. As shown in Fig. 4, the dominant fungi are Ascomycota and Mucoromycota at phylum level. With the development of brewing, the starch content in the substrate becomes lower and lower, and the relative abundance of Mucoromycota decreased. The community of Ascomycota increased and occupied an absolute dominant position with the brewing process, which indicated that Ascomycota played an important role in the brewing of *xiaoqu Baijiu*.

At the genus level, a total of 32 fungal species were detected in *xiaoqu Baijiu* samples. The top 10 species and other species merged into others were shown in Fig. 4b. *Rhizopus* and *Aspergillus* were the dominant genera before entering the pit, the relative abundance of which decreased during brewing. The relative abundance of *Saccharomyces* increased during whole brewing process and became the dominant genus at the end of brewing.. The relative abundance of *Issatchenkia* increased after first of brewing, and did not change much during brewing.

Sample clustering in bacterial and fungal communities

Principal coordinate analysis (PCA) was used to elucidate the abundance and microbial community. The maximum variations of bacterial communities were 30.55% (PC1) and 25.44% (PC2). The PCA showed that there were apparent differences in the bacterial community structure among the *xiaoqu* samples (Fig. 5a). PCA revealed that no obvious difference was found among the samples after third day for bacterial community structure. In fungal communities, the maximum variations were 35.73% (PC1) and 23.13% (PC2) as shown in Fig. 5b.

Discussion

Microorganisms produce the metabolites that form flavour substances during the brewing of *Baijiu* [24]. The difference of microbial species and community structures affect the formation of different flavour [2]. Therefore, there is an increasing interest in revealing the characteristics of microbial community and the important influence of changes of microbial community on *Baijiu* brewing [25].

Bacillus in starter can secret various hydrolases including amylases, proteases, and lipases for macromolecular hydrolysis and produce specific flavour compounds improving the sensory experience and confer health benefits, such as acetate, higher alcohols, diacetyl, pyrazines and aromatic compounds (Zhang et al., 2017). Moreover, cooperation *Bacillus* and *Aspergillus* produce more aromatic compounds [10]. *Pantoea* bacteria is an important heterotrophic bacterium with metabolizing and brewing. Although the abundances of *Acetobacter* and *Gluconobacter* are not high during brewing, they can produce

precursor acetic acid of ethyl acetate contributing positively to the flavour, which is the main flavour substance of *xiaoqu Baijiu* (Wang et al., 2018b).

Lactobacillus, *Weissella*, *Pediococcus*, *Leuconostoc* and *Lactococcus* belong to Lactic acid bacteria (LABs). The increase trend of Firmicutes in the process of brewing *Baijiu* was mainly related to the increase in abundance of *Lactobacillus* [26, 27]. LABs, facultative anaerobic or anaerobic bacteria [28], have important role in regulating biological structure in *Baijiu* brewing. LABs also predominate in sauce-flavour, strong-flavour, and light-flavour [26, 11, 6] during the brewing stage. Lactic acid and acetic acid, produced by lactic acid bacteria, can react with ethanol to produce ethyl lactate and ethyl acetate, which are important flavour substances in *xiaoqu Baijiu*. [23]. But too much ethyl lactate will bring a undesirable flavour in light-flavour *Baijiu*. Lactic acid, produced with the increase of LABs, reduced the pH in fermented grains influencing community structure [23] and inhibited the propagation of other spoilage bacteria [29]. A negative correlation between LABs and other prokaryotic genera were observed during the brewing process [23]. LABs, also possessing different enzymes such as glucosidase, esterase and proteases, played a critical role in the formation of volatile compounds in fermented foods [30, 31]. The type, quantity and dynamic change of LABs are very important for improving *Baijiu* taste. LABs may be introduced from the environment of *xiaoqu Baijiu* brewing workshop during sorghum cooling, because no LABs added in *xiaoqu*. The abundance of LABs was very high on the surface of ground and tools in the brewing workshop [32].

Molds and yeasts are mainly responsible for saccharification and alcoholic brewing to produce *Baijiu* [33]. *Rhizopus* and *Aspergillus* are considered as important functional microorganisms in many fermented foods [13]. The relative abundance of *Aspergillus* and *Rhizopus* decreased due to the continuous increased concentration of acids and alcohols. *Rhizopus oryzae* has high amylase production capacity, which can degrade starch in sorghum, and is widely used in *koji* and *Baijiu* brewing [34]. It affects *Baijiu* flavour by produce glycerol, lactic acid, enzymes and volatile compounds, such as ethanol, 2-methyl-1-butanol, and 3-methyl-1-butanol [35]. *Aspergillus* produce a wide spectrum of proteolytic and other lytic enzymes, playing an important role in saccharification of starch and protein hydrolysis in sorghum [36]. *Aspergillus* can also promote the synthesis of some esters, improving the *Baijiu* quality.

Saccharomyces, having strong ethanol brewing capacity under anaerobic conditions, is responsible not only for the alcohol yield but also for the flavour and taste feature of *xiaoqu Baijiu* [13, 37].

Saccharomyces also interact with other microorganisms such as mold and bacteria, improving the quality of *xiaoqu Baijiu*. *Saccharomyces* could tolerate anaerobic and high ethanol concentration. Therefore, relative abundance of *Saccharomyces* increased in the *xiaoqu Baijiu* brewing process.

Issatchenkia, abundant in soil and fermented grains, can produce ethanol and ethyl acetate with acid resistance, high temperature resistance and ethanol resistance. It is a functional fungal in the brewing process of *Baijiu*, and has been reported in many *Baijiu* Daqu and fermented grains with different flavour types [32, 38, 39].

Baijiu could maintain its character due to the similar microbial communities of different batches in the same period. The difference of microbial communities in different workshops or periods may cause the different flavour profile even same processing procedures were used [25]. The quantity and species of microorganisms such as bacteria, yeast and molds in fermented starters, more conducive to the yielding of the flavour compound, are the key factors to produce high-quality *Baijiu* [40]. They were altered due to the change the micro-environment, such as reduction of substrate, decrease of pH, increase of alcohol concentration, anaerobic situation, biotic and abiotic interactions [41, 42].

Xiaoqu and *xiaoqu Baijiu* are produced in an open environment [1]. Microorganisms is derived not only from the starters, but also the environment encountered in the process of material pretreatment [3, 43], such as water, machine, tools, ground and air in material pretreatment room. Therefore, the quality of *Baijiu* depends on well-balanced microbial community from the environment and starter. Microorganisms such as *Lactobacillus*, *Bacillus*, and *Saccharomyces* were supposed to be introduced into sorghum from brewing workshop environment during cooling and entered into the alcoholic brewing stage [32]. *Lactobacillus* and *Saccharomyces* were the predominant bacterial and fungal genera at the later stage of brewing, respectively. They are closely related to the formation of various flavours during *xiaoqu Baijiu* brewing.

Conclusions

In summary, HTS technology was used to characterize the microbial community diversity in the brewing process of *xiaoqu Baijiu* for 7 days. A more comprehensive insight to the microbial diversity and dynamic succession during brewing process of *xiaoqu Baijiu* was obtained. Some low abundance microbial populations during brewing were also revealed. *Lactobacillus*, *Weissella*, *Rickettsiales* and *Bacillus* were the main bacterial groups and *Rhizopus*, *Saccharomyces* and *Issatchenkia* were the main fungi groups during *xiaoqu Baijiu* brewing based on HTS technology. The microbial community structure and diversity showed a dynamic succession in the *xiaoqu Baijiu* brewing process. The abundance of *Weissella*, *Rickettsiales* and *Rhizopus* was higher in the early period, and the abundance of *Lactobacillus*, *Issatchenkia* and *Saccharomyces* increased significantly during the brewing period. This study contributes to expanding our understanding of microbial community diversity of *xiaoqu Baijiu* during the brewing process and provides useful information for future in depth studies of the microbial diversity and process improvement of *xiaoqu Baijiu*.

Declarations

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Authors' contributions

Guoqiang Han and Qing Wang participated in the design of this study, and they both performed the statistical analysis. Xiaoqing Xiang and Pengfei Wu carried out the study and collected important background information. Guoqiang Han drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declared that they have no conflict of interest.

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Figures

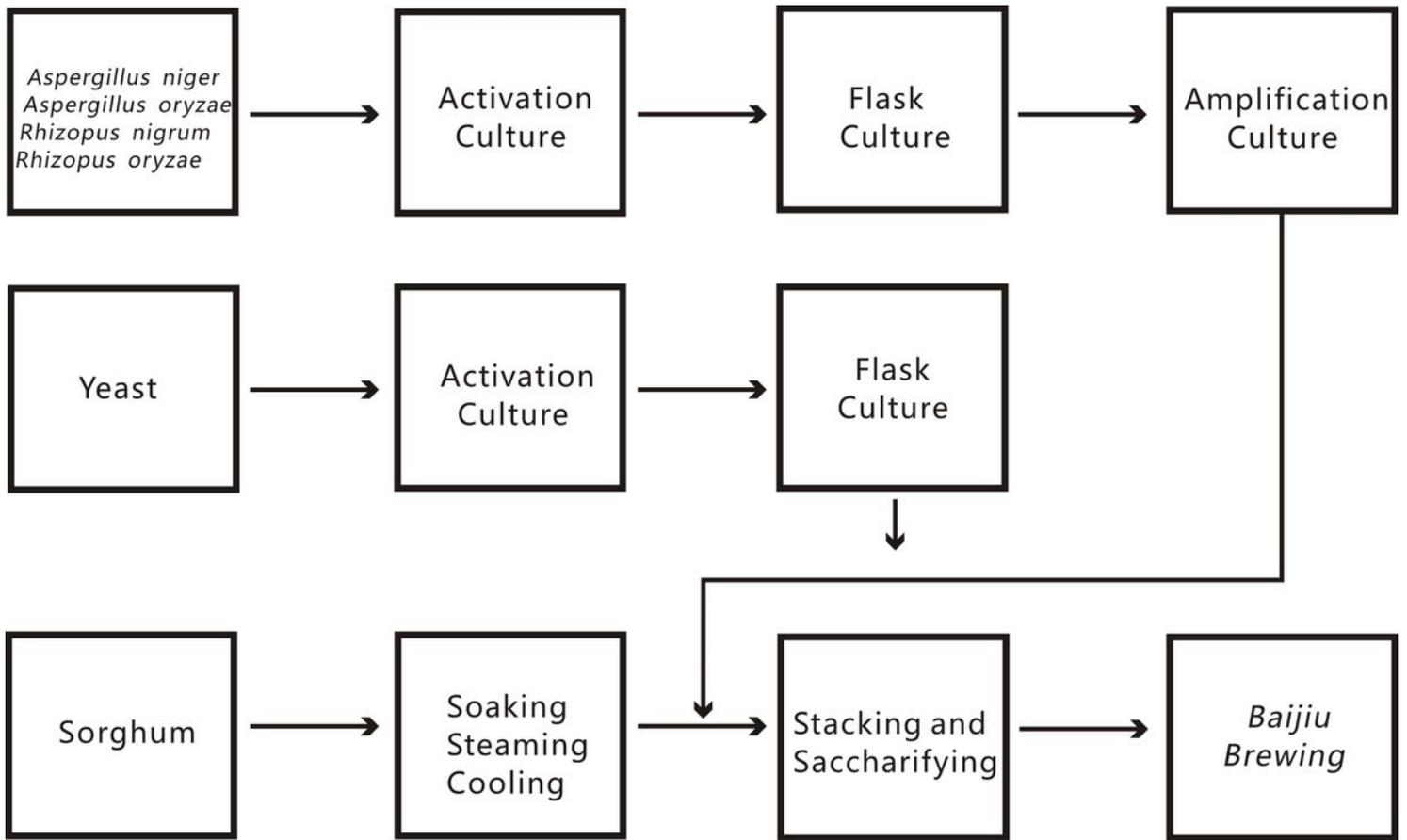


Figure 1

After soaking, steaming, cooling, mixed with xiaoqu, stacking and saccharifying for 24 h, sorghum was put into the pit and mud sealed. The pit was made of cement, and the brewing cycle was 7 days (Fig.1).

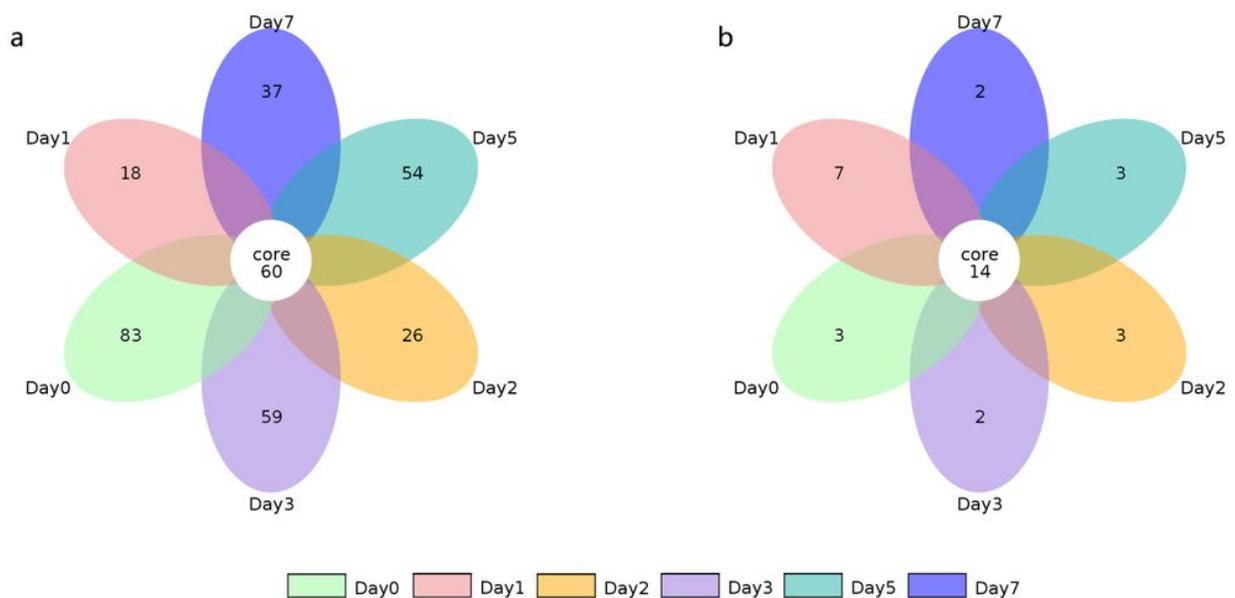


Figure 2

The OTUs of bacteria in all sample was counted and compared in xiaoqu Baijiu brewing process. Venn diagram was created to investigate whether exclusively shared OTUs existed. As shown in Fig.2, at a 97% similarity level, 60 OTUs and 14 OTUs coexisted in the six samples for bacterial and fungal. The results suggested the fungal community is relatively less complex than bacterial community during xiaoqu Baijiu brewing. The number of unique OTUs decreased during brewing. The OUT number was different which may be a result of the production process.

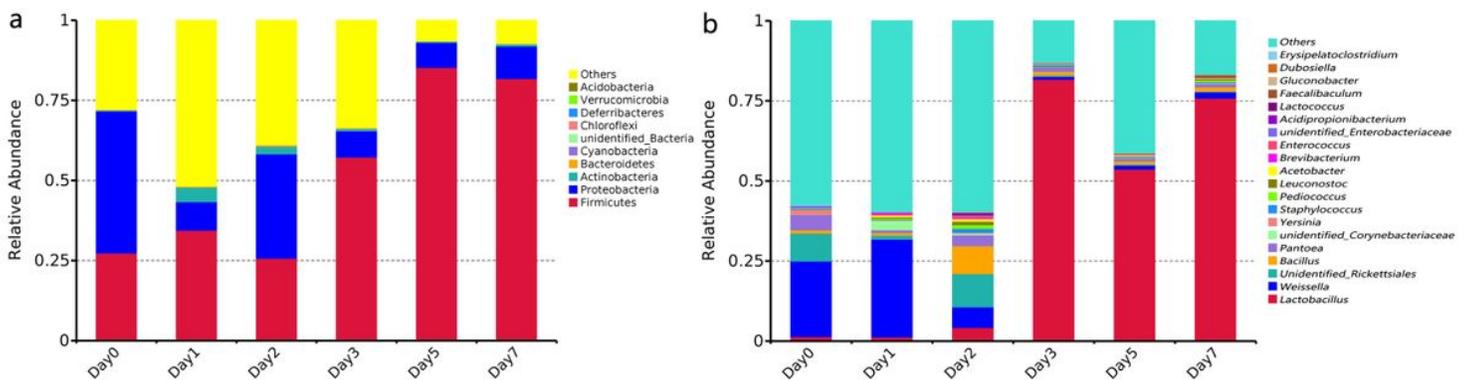


Figure 3

At the phylum level, 34 bacterial species were identified in samples. The top 10 species in abundance level and other species merged into others were shown in Fig. 3.

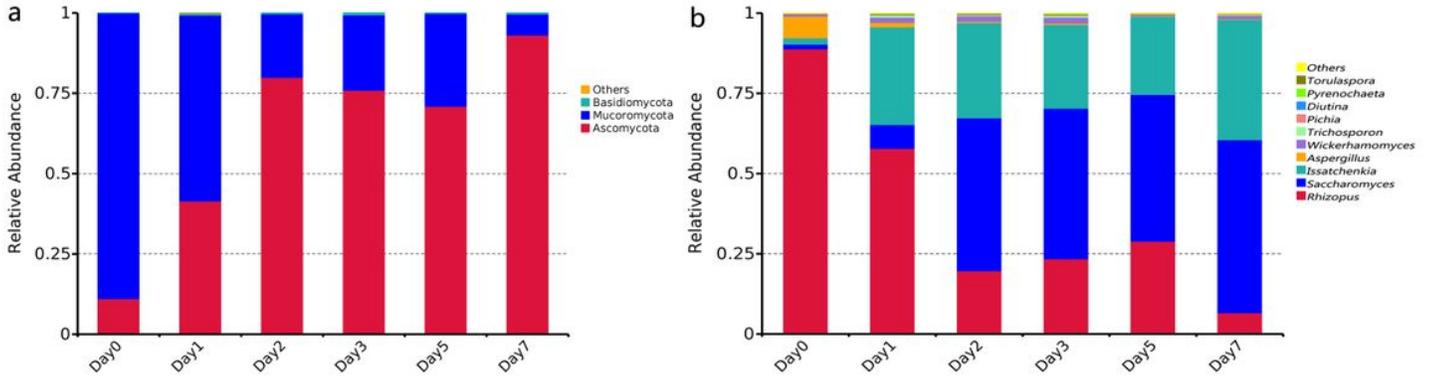


Figure 4

At the phylum level, 4 species were identified in samples. As shown in Fig. 4, the dominant fungi are Ascomycota and Mucoromycota at phylum level.

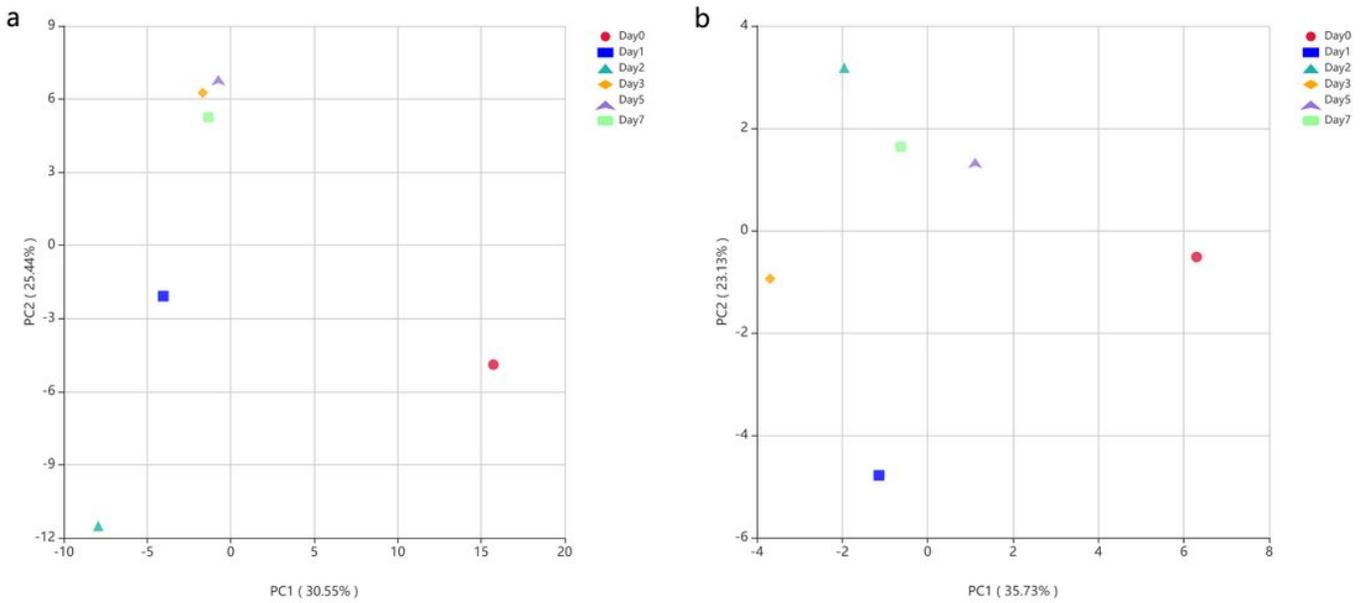


Figure 5

Principal coordinate analysis (PCA) was used to elucidate the abundance and microbial community. The maximum variations of bacterial communities were 30.55% (PC1) and 25.44% (PC2). The PCA showed that there were apparent differences in the bacterial community structure among the xiaoqu samples (Fig. 5a). PCA revealed that no obvious difference was found among the samples after third day for bacterial community structure. In fungal communities, the maximum variations were 35.73% (PC1) and 23.13% (PC2) as shown in Fig. 5b.