



Effects of ultra-long fermentation time on the microbial community and flavor components of light-flavor *Xiaoqu Baijiu* based on fermentation tanks

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Abstract

Microbial structure and succession of fermented grains play a significant role in *Baijiu*'s flavor and quality. In this study, high-throughput sequencing (HTS) coupled with headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) were used to analyze the microbial community structures and flavor components in the fermented grains at the end of fermentation from different fermentation time of light-flavor *Xiaoqu Baijiu*. HTS results showed that *Lactobacillus acetotolerans*, *Lactobacillus helveticus*, *Lactobacillus buchneri*, *Wickerhamomyces*, *Saccharomyces*, and *Condenascus* were identified as the dominant microbes, but *Lactobacillus* (96.28%) exhibited obvious advantages at the end of ultra-long fermentation time (day 98). HS-SPME-GC-MS analysis revealed that esters and alcohols had the most abundance in fermented grains of day 98, containing high concentrations of ethyl acetate, diethyl succinate, phenylethyl alcohol, isoamyl alcohol, and n-propanol, which were related to the succession of *Lactobacillus* and yeast communities. Interestingly, the content of n-propanol in the ultra-long fermentation time samples (day 98) was 6 times of that in normal fermented grains (day 14), which may be caused by higher abundance of *Lactobacillus* in day 98 samples. Monte Carlo permutation test showed residual starch, acidity, and amino nitrogen ($p < 0.05$) were important factors affecting the microbial community. Together, these results shed light on the physicochemical changes, microbial dynamics, and key flavor components of fermented grains at the end of fermentation from different fermentation time and provide a strategy for further improvement of *Baijiu* quality.

Keywords *Baijiu* · Fermented grains · Microbial community · Physicochemical indexes · Volatile compounds

Introduction

Baijiu, as the national liquor of China, with a 2000-year history, is a type of traditional distilled spirit drink produced by spontaneous mixed solid-state fermentation. *Baijiu* is the world's most-consumed spirit, and the gross sales of *Baijiu* in 2019 were more than \$80 billion (Ye et al. 2021). The traditional *Baijiu* process mainly includes material preparation, *Jiuqu* (starter) preparation, alcoholic fermentation, solid state distillation, aging and blending (Jin et al. 2017). As

Jiuqu preparation and alcoholic fermentation are carried out under partially controlled conditions, the specific microflora has been well enriched with a unique ecological environment and production procedures through repeated practices over a long time. The specific microbiota along with different materials and processing procedures have created different types of *Baijiu*. Based on the flavor characteristics, *Baijiu* fell into three major types, sauce-flavor, strong-flavor, and light-flavor (Zheng and Han 2016). The light-flavor *Baijiu* brewing process takes place in ceramic jars, not the mud used in the other two types of *Baijiu*. The importance of fermentation pits and clay jars have been recognized by practice for thousands of years (Ye et al. 2021). *Jiuqu*, the fermentation starter of *Baijiu*, plays an important role in the fermentation process. According to the production process, *Jiuqu* includes *Daqu* (Made with raw wheat, barley and/or peas), *Xiaoqu* (Made with rice or rice bran), and *Fuqu* (Made with bran) (Jin et al. 2017). Light-flavor *Xiaoqu Baijiu* is produced by solid-state fermentation of sorghum with *Xiaoqu* (starter)

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containing various microbes and their complex interactions, which is a representative product made by Jing Brand Co., Ltd., one of the largest and most influential manufacturers of *Xiaoqu Baijiu* based on mechanized technology in China (Hu et al. 2021b). The quality evaluation standard of light-flavor *Xiaoqu Baijiu* mainly depends on sensory indexes and varieties and contents of core flavor compounds, which are associated with microbial metabolism during brewing process. Therefore, profiling the composition and metabolic activities of microbial community in fermented grains during the mechanized production is necessary to improve the quality of light-flavor *Xiaoqu Baijiu*.

Like all naturally fermented foods, the change in microbial composition and succession during the brewing process is critical to the development and final quality of *Baijiu* (Huang et al. 2020; Zhao et al. 2019; Tan et al. 2019). During the natural brewing process of various types of *Baijiu*, the microbial community diversity and structure are affected by some environmental factors, such as acidity, moisture, temperature, substrates, and fermentation time (Zhang et al. 2021; Hao et al. 2021). For instance, Zhang et al. (2021) found that altering the initial temperature of fermentation process led to differences in microbial succession rates and volatile flavors in the fermented grains of sauce-flavor *Baijiu*. Guan et al. (2020) revealed that changes in starch, moisture, and pH had significant effects on bacterial composition in strong-flavor *Baijiu* brewing process. Fan et al. (2020) expounded that the physicochemical properties changed continuously during aging of *Daqu* to provide a high esterifying activity and helped to stabilize the flavor compounds in mature *Daqu*. Thus microorganisms and environmental

factors are important factors affecting the formation of flavor components in *Baijiu*. Hu et al. (2021c) and Dong et al. (2020) studied the microbial communities and identified the core microorganisms related to higher alcohol formation in two kinds of light-flavor *Xiaoqu Baijiu* brewing process (traditional and new mechanical technologies). However, there are few research to reveal the relationship between microbial composition, physicochemical indexes, and volatile components of light-flavor *Xiaoqu Baijiu* brewing process.

Light-flavor *Xiaoqu Baijiu* produced based on mechanized technology is fermented in a truck-shaped tank (1.7 m × 1.0 m × 1.0 m) for 14 days with sorghum as the raw material and *Xiaoqu* as the starter (Jiang et al. 2019). Figure 1 shows the process flow chart of the detailed mechanized technology for the brewing process. The fermentation time was usually extended to 27 days due to vacations during the Chinese New Year. But due to COVID-19, people's work and life were severely affected and had to stay at home, which led to the delay in resuming work and production. So the fermentation period had been extended by 68–84 days, which was rare in a century. And so far, there are almost no research on the effects of ultra-long fermentation time on microbial composition, physicochemical factors, and flavor components in fermented grains. Lee et al. (2019) revealed the effect of long-term fermentation on volatile compounds in soy sauce and indicated that the contents of most compounds, such as acids, aldehydes, esters, lactones, and pyrazines, showed a tendency to increase, whereas those of alcohols and ketones decreased according to long-term fermentation. But it is unclear to what extent ultra-long

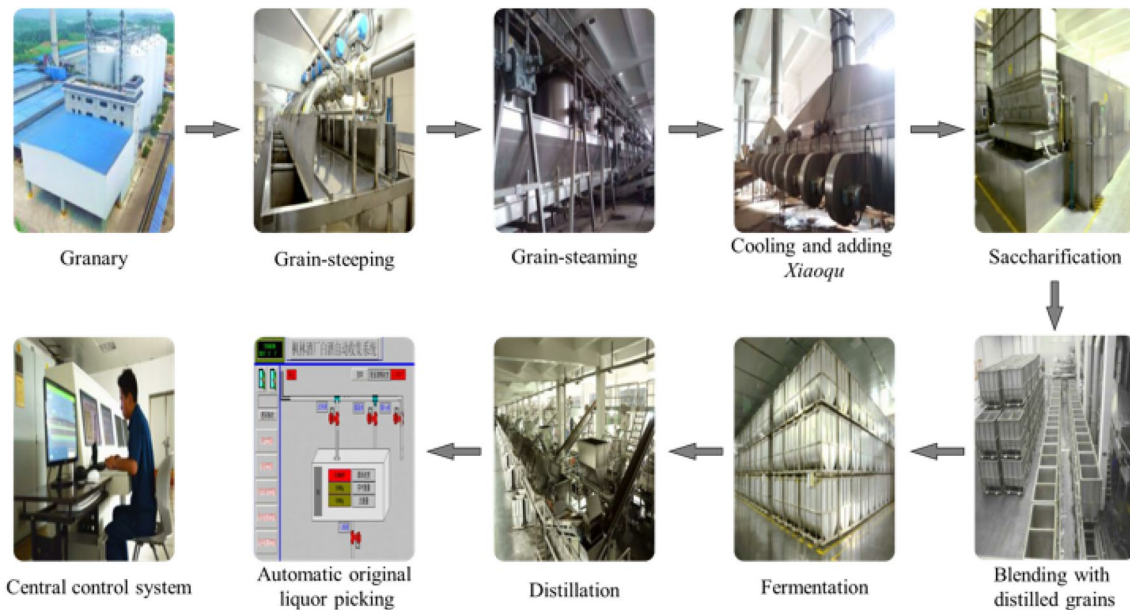


Fig. 1 Schematic of the mechanized brewing process of light-flavor *Xiaoqu Baijiu*

fermentation time affects the microbial community in the grains and quality of *Baijiu* fermentation.

Therefore, the aim of this work was to determine the effects of ultra-long fermentation time on microbial community, physicochemical factors, and volatile components profiles during the light-flavor *Xiaoqu Baijiu* fermentation. And three typical fermented grains selected from the production process of light-flavor *Xiaoqu Baijiu* based on mechanized technology with different fermentation time was subjected to investigation by high-throughput sequencing (HTS) and HP-SPME-GC-MS. To our knowledge, this is the first comprehensive study to report the effects of ultra-long fermentation time on its physicochemical properties, flavor components, and microbial community during the light-flavor *Xiaoqu Baijiu* fermentation. Together, our results reveal how ultra-long fermentation time affects the quality of *Baijiu* and provide a resource for further breeding of superior strains.

Materials and methods

Sample collection and pretreatment

Fermented grain samples were collected from a famous mechanized *Baijiu* factory (Hubei, China) in 2020. Samples (250 g) were collected from fermentation tanks in the middle layer (the depth of 0.5 m) at the end of the fermentation period of 14, 87, and 98 days, and labeled as D14, D87, and D98, respectively. Each sample had three biological replicates. These samples were stored at -20°C for DNA extraction and volatile components analysis, and 4°C for physicochemical properties analysis. Three independent batches were sampled for adequate representation.

Microbiological and physicochemical analysis

Changes in the physicochemical properties (moisture, residual starch, reducing sugar, acidity, and amino nitrogen) were measured as previous study described by Guan et al. (2020) and Fan et al. (2020). Moisture was determined by gravimetric method by drying fermented grain samples to a constant weight at 105°C . 10 g of fermented grain samples were suspended with 90 mL ultrapure water and ultrasonically treated at 0°C for 30 min. The suspension was centrifuged at 4°C for 10 min and the supernatant was pipetted to analyze the contents of acidity, amino nitrogen and reducing sugar. Acidity was determined by titration with 0.02 M NaOH yielding a titration endpoint of pH 8.2. Amino nitrogen was determined by titration with 0.05 mol/L NaOH yielding a titration endpoint of pH 9.2 by adding 10 mL formaldehyde solution. Reducing sugar was analyzed using DNS method. Residual starch

was calculated by calculating the difference between the total reducing sugar and the original reducing sugar after hydrolysis with HCl (20%, v/v) for 30 min.

Fermented grain samples (10 g) were put into an appropriate physiological salt solution to determine the population of microorganisms using selective media. Lactic acid bacteria (LAB) counts were performed on De Man, Rogosa, Sharpe Agar (MRSA) with 500 $\mu\text{g}/\text{mL}$ of natamycin at 37°C for 48 h. Yeasts and molds were enumerated on Rose Bengal Chloramphenicol Agar with 100 mg/L of chloramphenicol at 30°C for 48–72 h (Huang et al. 2020). All physicochemical analyses were conducted in triplicate.

DNA extraction, amplification, and sequencing

DNA extraction, PCR amplification, and amplicon sequencing were conducted at Biomarker Bioinformatics Technology Co., Ltd (Nanjing, China). The total genomic DNA was extracted from 5 g of each sample according to previously published protocols (Amend et al. 2020) and purified using the Powersoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) following the manual. DNA concentration was quantified on a NanoDrop spectrophotometer (Thermo Scientific). The V3–V4 regions of the bacterial 16S rDNA genes were amplified with the universal primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The internal transcribed spacer 1 (ITS1) region of the fungi was amplified with the forward primer ITS1F (5'-CTTGGTCATTTA GAGGAAGTAA-3') and the reverse primer ITS1R (5'-GCTGCGTTCTTCATCGATGC-3') (Zang et al. 2018).

PCR was carried out in 50 μL reaction system and thermal cycling conditions was as follows: initial denaturation at 95°C for 5 min, followed by 28 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 50 s, and extension at 72°C for 45 s, and a final extension at 72°C for 10 min. Three separate reactions were conducted to account for potentially heterogeneous amplification from the environmental template for each sample. PCR products were purified using AXYGEN Gel Extraction Kit (QIAGEN), and the concentrations were carefully assessed by Thermo Scientific NanoDrop 8000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The 16S rDNA gene amplicons using PacBio sequencing platform (third-generation amplicon technology) and ITS1 gene amplicons based on Illumina HiSeq deep sequencing platform (second-generation amplicon technology) were sequenced at Biomarker Bioinformatics Technology Co., Ltd., Nanjing, China. All sequencing data have been submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) with accession number PRJNA732254.

Volatile components analysis

The volatile components in fermented grains were determined by HS-SPME-GC-MS conducted on an Agilent 7890B GC system coupled with an Agilent 5977B MSD (CA, USA). The 10 g of the fermented grain sample was added to 25 mL of sterilized ultrapure water and soaked overnight at 4 °C. After ultrasonic treatment for 30 min, the sample was centrifuged at 6500×g at 4 °C for 10 min. The supernatant (8 mL) was transferred to a 20 mL vial containing 10 µL of 2-ethyl hexanol (internal standard) and 5 g of sodium chloride. Volatile compounds were collected for 45 min at 50 °C, followed by GC-MS analysis (Wang et al. 2020b). Volatile compounds were identified by matching with the NIST14.L spectrum database and their quantitative analysis was calculated by comparison with the area of the internal standard.

Bioinformatics and statistical analysis

The raw sequences were quality-filtered to obtain specific sequences with the Quantitative Insights Into Microbial Ecology (QIIME) software package. Sequences with $\geq 97\%$ similarities were clustered into operational taxonomic units (OTUs) according to UCLUST (Bokulich et al. 2013) and the bacterial taxonomic information was assigned to all OTUs by searching against the Silva databases using the Ribosomal Database Project (RDP) classifier within QIIME (Quast et al. 2012). The fungal sequences were queried against the GenBank non-redundant nucleotide database (nt) in NCBI using local BLASTn. The MEGAN program was used to assign BLAST hits to taxa of the NCBI taxonomy. After removing non-fungal sequence reads, the fungal sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level using Uclust (Huson et al. 2007). Alpha diversity indices (Chao1 richness and ACE richness estimators, Simpson and Shannon diversity indexes) reflecting the richness and the diversity of the microbial community were evaluated using the OTUs table in QIIME (Ramette 2007). Relative abundance of bacteria and fungi and proportions of volatile components in the samples were plotted using Origin 2018. To associate the correlations between the dominant microbiota and important flavor components, Spearman's correlation coefficient was calculated between the microbial genera and flavor components via IBM SPSS Statistics (version 19.0), and visualized as a correlation network with $|\rho| > 0.8$ and $p < 0.05$ in Cytoscape (v3.6.1) (Shannon et al. 2003; Liu et al. 2019). Redundancy analysis (RDA) and Monte Carlo permutation test were performed using the vegan packages in R to confirm the correlations between microbial community structures and physicochemical properties.

Results

General investigation of microbiota and physicochemical properties

The physicochemical properties and cultivable microbiota abundance of fermented grains characterized the process of fermentation and reflected the quality of the fermented grains, and provided the first insights into the microbial composition and metabolic changes, which offered a basis for further amplicon sequencing analysis (Huang et al. 2020). As shown in Fig. 2a, the moisture, acidity, and amino nitrogen contents increased, while the residual starch and reducing sugar contents decreased with the extension of fermentation time. The moisture of the fermented grains at the end of the normal fermentation time (day 14) remained around 71.81%, and increased steadily to 73.00% in the sample of D98. The titratable acidity and amino nitrogen content increased from 0.91 to 1.49 mmol/10 g and from 0.38 to 0.51 g/kg from the samples D14 to D98, respectively. Both of them showed a quick increasing tendency during the extension of fermentation time from D14 sample to D87 sample, followed by a substantially increasing from D87 sample to D98 sample. Conversely, a significant decrease of the reducing sugar and residual starch contents could be observed from D14 sample to D98 sample. Moreover, the reducing sugar decreased rapidly from 2 to 0.83 g/100 g, and the residual starch contents maintained a stable range from D87 sample to D98 sample. The more thorough fermented grains caused by the extended fermentation time resulted in the changes of these physicochemical indicators.

The total numbers of fungi, yeasts, and LAB were 5.92, 4.65 and 7.36 lg CFU/g in D14 sample, respectively (Fig. 2b). No molds were detected and the total numbers of yeasts declined with the extension of fermentation time in D87 and D98 samples. The total population of LAB was higher than that of fungi, and LAB had a relatively stable growth in D87 and D98 samples. The oxygen in the fermented grains was almost exhausted, and the acidity and alcohol content increased followed the extension of fermentation time, which led to the death of aerobic microbiota (molds, most types of yeasts) and survival of anaerobes and facultative anaerobic microorganisms (LAB).

Microbial richness and diversity

After filtering the low-quality reads and chimeras, there were a total of 33,087 effective sequences of 16S rDNA gene and 325,516 effective sequences of ITS1 rDNA gene to be obtained from all fermented grain samples (Table 1). Chao 1 and ACE indexes were used to estimate the microbial community richness, and Shannon index and Simpson index

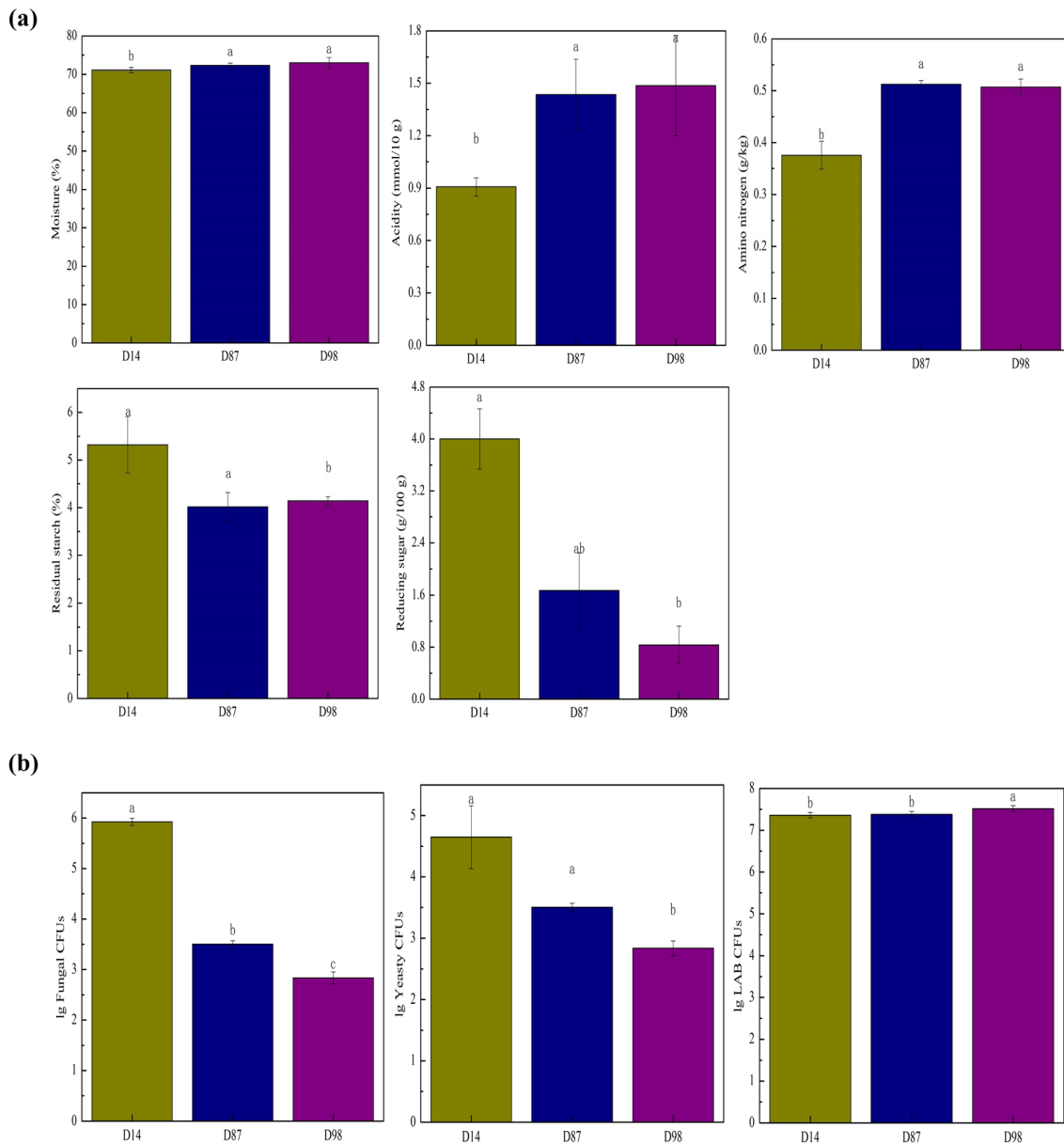


Fig. 2 Dynamics of physicochemical characteristics and the viable microbial counts of the fermented grains at the end of different fermentation time (a Changes in the physicochemical characteristics of fermented grains; b Changes in the viable microbial counts of

fermented grains. Different letters obtained by one-way ANOVA followed by the Duncan’s test indicated significant differences at $p < 0.05$)

Table 1 Reads, OTUs and alpha-diversity indexes

Sample ID	Reads	OTUs	Alpha-diversity indexes				
			Simpson	Shannon	Chao1	ACE	
16S	D14	10,876	61	0.34 ± 0.24	1.20 ± 0.70	42.63 ± 5.38	43.83 ± 4.34
	D87	11,303	49	0.54 ± 0.09	1.99 ± 0.29	43.22 ± 1.89	52.62 ± 3.28
	D98	10,908	37	0.72 ± 0.01	2.49 ± 0.10	32.55 ± 6.05	41.24 ± 11.57
ITS	D14	98,454	346	0.82 ± 0.02	3.89 ± 0.20	329.09 ± 11.97	397.98 ± 4.52
	D87	124,100	342	0.92 ± 0.03	5.12 ± 0.44	328.12 ± 17.38	372.95 ± 9.28
	D98	102,962	368	0.94 ± 0.04	5.41 ± 0.50	366.93 ± 68.68	372.87 ± 29.22

were used to evaluate the microbial community diversity. For bacterial communities, the richness estimators (Chao 1 and ACE) firstly increased from D14 to D87 and then decreased from D87 to D98 samples. But the microbial diversity (Simpson and Shannon) were observed to increase substantially with the extending fermentation time from D14, D87 to D98 samples. For fungal communities, the shift patterns of microbial richness and diversity were consistent with the OTUs indexes, both of which increased from D14, D87 to D98 samples.

Microbial composition and dynamics

The relative abundance of different bacterial microorganisms at the phylum level in all samples were shown in Fig. 3a. According to the sequences obtained from all samples, ten bacterial phyla were identified, and Firmicutes and Proteobacteria were the major phyla. There was a dramatic

increase (from 62.5 to 96.4%) in the proportion of Firmicutes during the extension of fermentation time from D14 to D87 samples, followed by a little decline from D87 to D98 samples, while the proportion of Proteobacteria was converse. Figure 3b showed the relative abundance of fungi in all samples at the phylum level. All of the analyzed sequences were classified into 7 fungal phyla, mainly containing Ascomycota, Basidiomycota, Glomeromycota, and Mucoromycota. Among them, Ascomycota dominated and represented the major fungal phylum in all samples, which slightly decreased (88.9–82%) from D14 to D98 samples. Ascomycota was prevalent in the production of fermented alcoholic beverages (Li et al. 2011). In general, the bacterial and fungal diversities at the phylum level were relatively simple (Zang et al. 2018).

Bacterial compositions at the species level and fungi at the genus level were determined to get a more detailed analysis of the bacterial and fungal dynamics associated

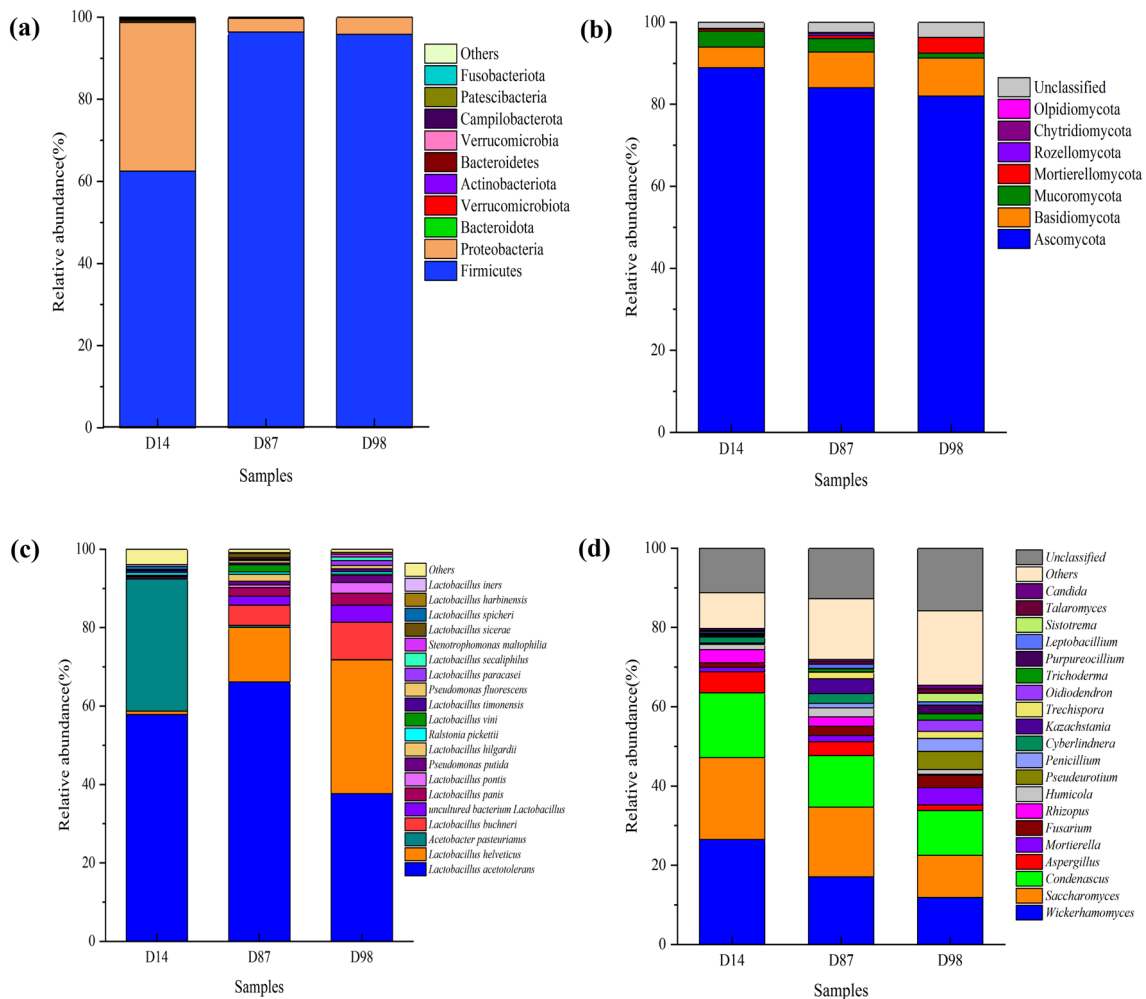


Fig. 3 Relative abundance of bacteria (a) and fungi (b) at the phylum level and bacteria (c) at the species and fungi (d) at the genus level in the samples

with fermented grain samples. At the species level, the top 20 species were shown in Fig. 3c. *Lactobacillus acetotolerans* and *Acetobacter pasteurianus* were the predominant microorganisms in the fermented grains at the end of the normal fermentation time (D14). With the continuation of fermentation, *A. pasteurianus* disappeared and the abundance of *L. acetotolerans* (66.2%) was the largest in D87 sample. In addition, the proportion of *Lactobacillus helveticus* and *Lactobacillus buchneri* gradually increased in D87 sample. Furthermore, *Lactobacillus* dominated at the end of the fermentation period (96.28%), which suggested that members of *Lactobacillus* probably played key roles in the late fermentation of *Baijiu*. Some researchers have reported that *Lactobacillus* could produce several antimicrobial metabolites, including organic acids, which was related to the metabolism of amino acids during food fermentation processes and improved texture and flavor of fermented food (Quijada et al. 2017; Sudhanshu et al. 2018).

For fungal community structure, the top 20 genera were showed in Fig. 3d. The fungal community was dominated by these genera *Wickerhamomyces* (26.5%), *Saccharomyces* (20.6%), and *Condenascus* (16.3%) in D14 sample, then decreased gradually with the fermentation period going on. Similarly, the relative abundance of *Aspergillus*, *Rhizopus* decreased from 5.3 to 1.4% and from 3.3 to 0.2% (from D14 to D98), respectively. Whereas the opposite dynamics of *Fusarium*, *Pseudeurotium*, *Penicillium*, *Trichoderma*, *Purpureocillium*, *Talaromyces*, and others were observed, which increased along with the fermentation time. Some studies have reported that yeast commonly contributes to the flavor and aroma of fermented products, such as *Wickerhamomyces* is conducive to the synthesis of ester compounds in the Chinese *Baijiu* brewing process (Wang et al. 2020a), while *Saccharomyces* is the most efficient ethanol producer during the *Baijiu* fermentation stage. Based on sensory evaluation, *Baijiu* produced by *Saccharomyces* is characterized by high scores for harmony and the highest values of aroma compared with other yeasts (Liu et al. 2017; Cai et al. 2018). *Condenascus*, a newly discovered genus in the fermented grains of *Baijiu* was identified, formerly belonging to the classification of *Thielavia* (Wang et al. 2019a).

Analysis of volatile components in fermented grains

About 64 major volatile compounds in fermented grains were identified by HS-SPME-GC-MS, and 31, 48, and 55 major components were found in D14, D87, and D98 samples, respectively. With the extension of the fermentation time, the types of volatile substances increased, especially from D14 sample to D87 sample. All volatile components were composed of six categories, including esters (25), alcohols (17, except ethanol), acids (7), phenols (6), aldehydes and ketones (4), and others (5). Figure 4 showed that esters accounted for

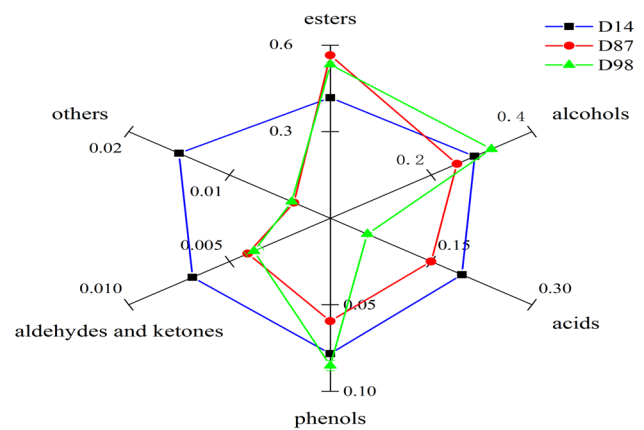


Fig. 4 Proportions of volatile components (esters, alcohols, acids, phenols, aldehydes, and ketones, and others) in the fermented grains of D14, D87, and D98 samples, respectively

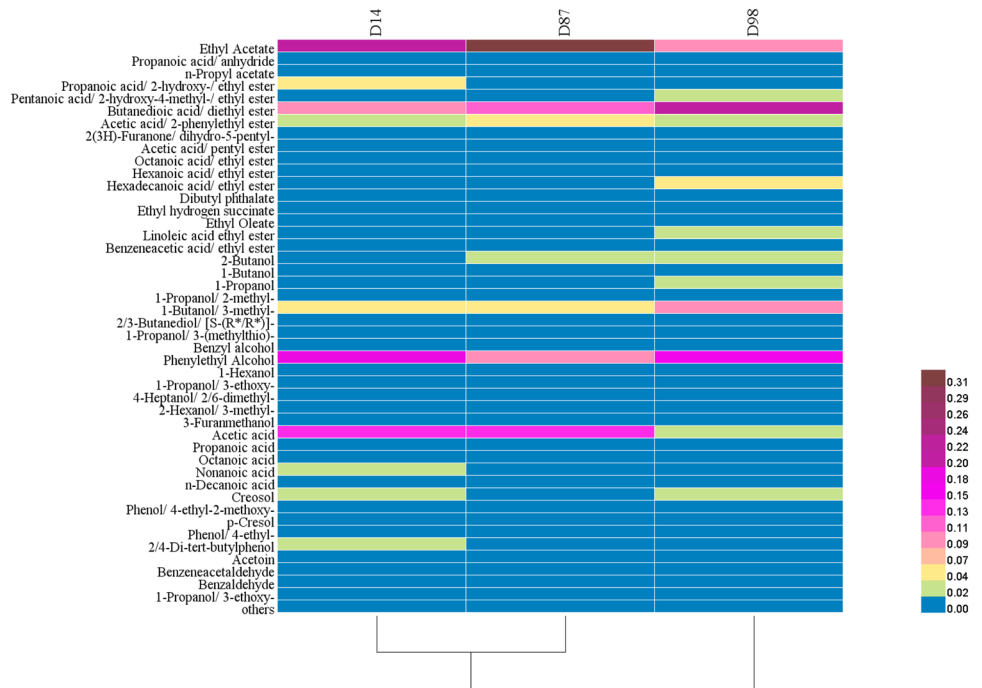
40–57% of all flavor components, which were dominant, followed by alcohols (25–32%) and acids (5–20%). The contents of esters and alcohols increased, while a noteworthy decrease of the acids, aldehydes and ketones, and others contents was observed along with the fermentation time.

Heatmap analysis of flavor components in fermented grains suggested that D98 sample fell into an alone cluster, while D14 and D87 samples were classified into another cluster (Fig. 5). Ethyl acetate (21.49%), ethyl acetate (32.96%), and diethyl succinate (21.74%) were the most abundant ester in D14, D87, and D98 samples, respectively. The ethyl acetate content increased along with the extension of fermentation time from D14 to D87 samples, followed by a significant decline (32.96–9.36%) from D87 to D98 samples. While the content of diethyl succinate had a marked increase (9.19–21.74%) from D14 to D98 samples. Except ethanol, the most abundant alcohol was phenylethyl alcohol accounting for 19.98%, 10.47%, and 16.60% in the fermented grains of D14, D87, and D98 samples, respectively, while isoamyl alcohol as the second most abundant alcohol, which accounted for 6.03%, 4.5%, and 9.51% in these above-mentioned corresponding samples, respectively. The most abundant acid in fermented grains was acetic acid accounting for 14.34%, 13.50%, and 2.54% in these aforementioned samples, respectively, which obviously decreased with the extended fermentation time. In addition, the hexadecanoic acid ethyl ester, linoleic acid ethyl ester, 2-butanol, and n-propanol contents increased with the extension of fermentation time.

Correlation analysis between dominant microbes and volatile components

The correlation network analysis indicated that 23 different volatile compounds were correlated with 14 dominant

Fig. 5 Heatmap of flavor components in the fermented grains of three typical samples



fungal genera (Fig. 6a) and 28 different volatile substances were correlated to 15 dominant bacterial species (Fig. 6b). Ethyl acetate, as the most abundant ester, displayed positive correlation with *Cyberlindnera*, *Kazachstania*, *Candida*, *Lactobacillus sicerae*, and *Lactobacillus vini*. The second most abundant diethyl succinate had positive correlation with *Lactobacillus panis*, *Lactobacillus secaliphilus* and negative correlation with *Aspergillus*. Phenylethyl alcohol showed negative correlation with *L. vini* and *Lactobacillus hilgardii*. Acetic acid with the most abundant in acids indicated positive correlation with *Aspergillus* and negative correlation with *Trichoderma*, *Lactobacillus pontis*, and *L. helveticus*.

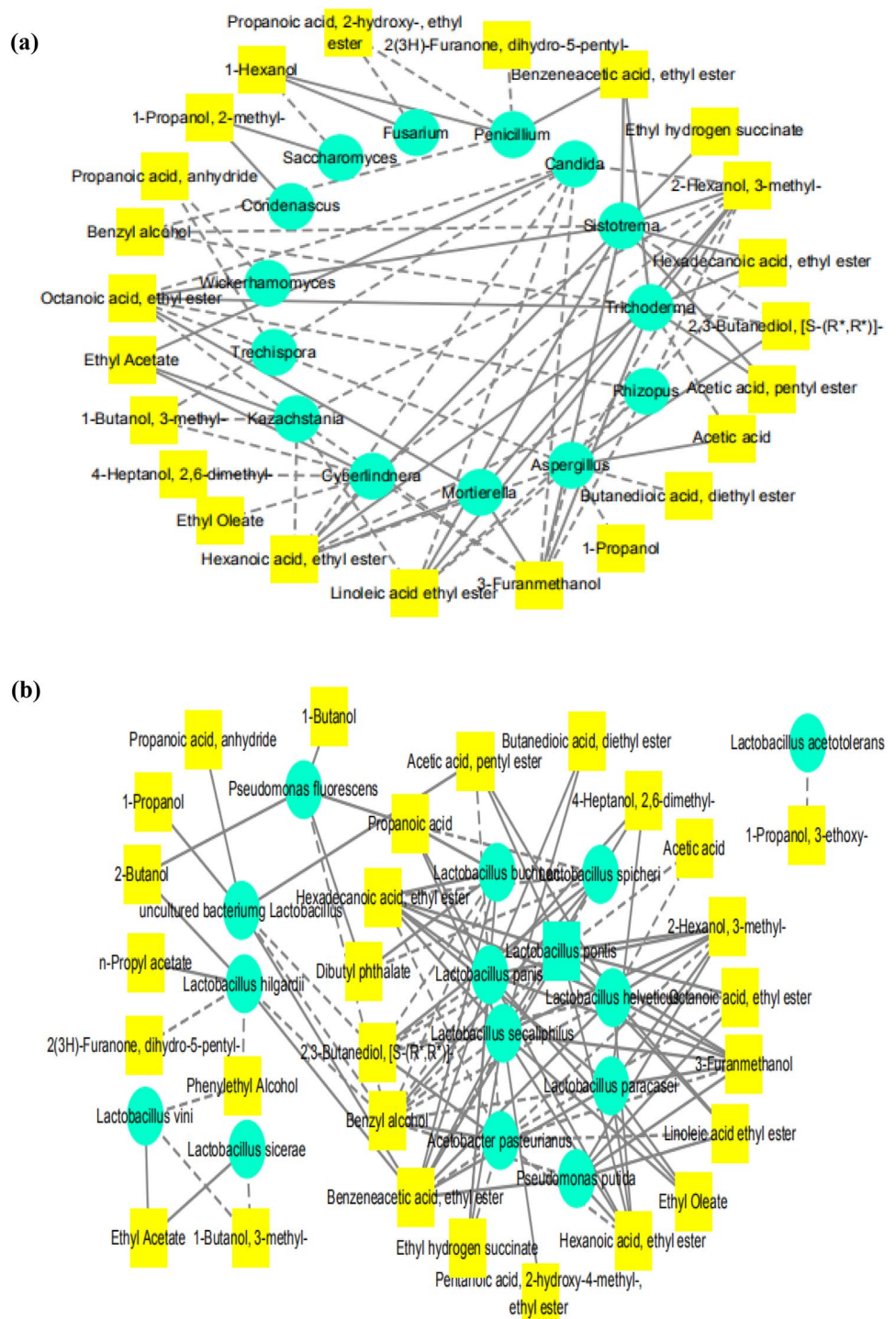
Higher alcohols, mainly including n-propanol, isobutyl alcohol, and isoamyl alcohol, were one class of the main flavor components in *Baijiu* and the proper contents contributed to the formation of the rich flavor of *Baijiu* (Hu et al. 2021c). Isobutyl alcohol displayed positive correlation with *Saccharomyces* and *Condenascus*. Isoamyl alcohol, the second most abundant alcohols, had negative correlation with *Kazachstania*, *Candida*, *Cyberlindnera*, *L. sicerae*, and *L. vini*, and these microbes were positively correlated with ethyl acetate. The n-propanol, as the significantly increased substance, displayed negative correlation with *Aspergillus* and positive correlation with an uncultured *Lactobacillus* sp. As illustrated in Fig. 3, the decrease in the relative content of *Aspergillus* and the increase in the relative content of an uncultured *Lactobacillus* sp. maybe resulted in a sharp increase of n-propanol content in the fermented grains with the extended fermentation time.

Relationships between physicochemical properties and microbial communities

It is well known that physicochemical properties (moisture content, acidity, amino nitrogen, residual starch, and sugar contents) are responsible for the microbial community changes that occur in microbial ecosystems (Jung et al. 2014). Therefore, RDA was used to further analyze the relationship between the microbial communities and physicochemical properties mentioned above. An acute angle represents a positive correlation, while an obtuse angle indicates a negative correlation between the species and physicochemical properties. Overall, the two axes explained 93.62% and 83.98% of the total variance for the bacterial (Fig. 7a, DCA = 1.93) and fungal (Fig. 7b, DCA = 2.11) communities differentiation, respectively, and it suggested remarkable correlations between the microbial communities and physicochemical properties. RDA showed residual starch and reducing sugar content had positive correlations with *A. pasteurianus*, *L. acetotolerans*, *Wickerhamomyces*, *Saccharomyces*, and *Condenascus*, and negative correlations with *L. helveticus*, *L. buchneri*, *Pseudeurotium*, *Oidiodendron*, and *Sistotrema*. While moisture, acidity, and amino nitrogen had the opposite correlations with these microbes.

Monte Carlo permutation test showed residual starch, acidity, and amino nitrogen contents ($p < 0.05$) were important factors affecting the microbial community (Table 2). Specifically, residual starch had significant positive correlation with *A. pasteurianus*, *L. acetotolerans*, *Wickerhamomyces*, *Saccharomyces*, and *Condenascus*.

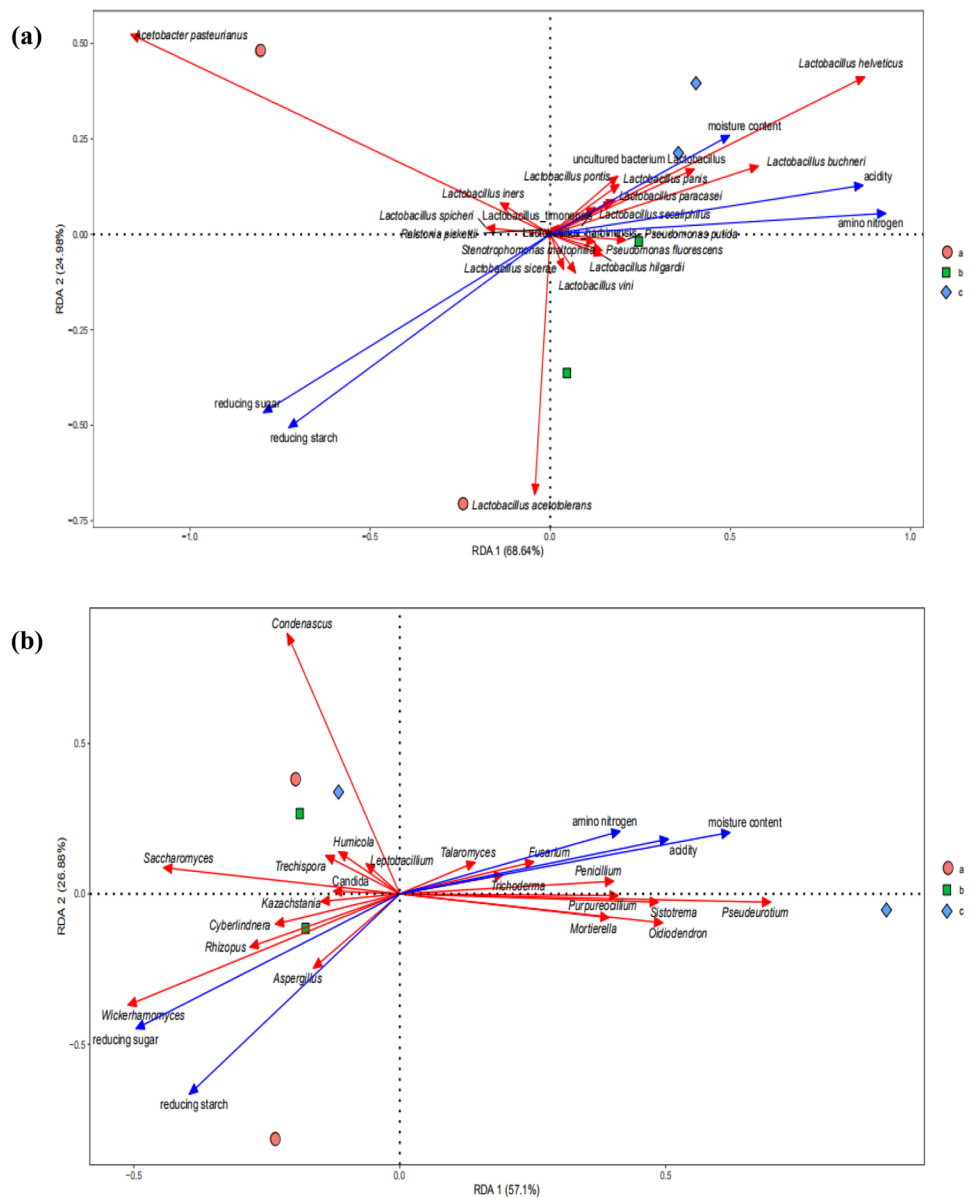
Fig. 6 Correlation network diagram among dominant fungal genera (a), bacterial species (b), and volatiles (Note: Microbes and volatiles are represented by green circle and yellow square modules, respectively; Positive and negative correlations among dominant genera are represented by solid and dotted edges, respectively)



Acidity and amino nitrogen content showed significant positive correlations with *L. helveticus*, *L. buchneri*, uncultured *Lactobacillus* sp., *Pseudeurotium*, *Oidiodendron*, and *Sistotrema*. As illustrated in Fig. 2, the increase of acidity and amino nitrogen content and decrease of the

residual starch content along with the extended fermentation time were related to the increase of *L. helveticus*, *L. buchneri*, uncultured *Lactobacillus* sp. and the reduction of *A. pasteurianus*, *Wickerhamomyces*, *Saccharomyces*, and *Condenascus*.

Fig. 7 Redundancy analysis (RDA) of bacterial communities at species level (a) and fungal communities at genus level (b), and physicochemical properties (Note: blue arrows represent different physicochemical properties, red arrows represent different microbes; the symbols filled circle, filled square and filled diamond represent samples D14, D87, and D98, respectively)



Discussion

The brewing process of light-flavor *Xiaoqu Baijiu* is composed of some basic steps, including raw materials handling, grains steeping, grains steaming, starter preparation, cooling, grains blending, fermentation and distillation, etc. (Wang et al. 2020b). As one of the most important brewing processes, fermentation is performed by the synergistic effect of various microorganisms present in the fermented grains ecosystem, consisting of molds, yeasts, and bacteria (Jung et al. 2014). Up till now, the characterization of the microbial communities of fermented grains is being elucidated with the development of next-generation high-throughput sequencing technology (Huang et al. 2020; Guan et al. 2020; Liu and Miao 2020). But there are relatively few

Table 2 Monte Carlo permutation test of the microbiota with physicochemical properties

Monte Carlo permutation test	r^2	p
Moisture	0.6948	0.15833
Acidity	0.8398	0.04167*
Amino nitrogen	0.8949	0.01528*
Residual starch	0.8974	0.02917*
Reducing sugar	0.6576	0.26667

* $p < 0.05$

studies on the relationship between environmental factors and microbial community composition in fermented grains due to the limitation of sampling from the closed fermentation process, especially the effects of ultra-long fermentation time on the microbial community, physicochemical factors, and flavor components in fermented grains are rarely studied. To the best of our knowledge, this study, for the first time, analyzed the relationships between microbes and physicochemical properties or flavor components of fermented grains at the end of light-flavor *Xiaoqu Baijiu* fermentation from different fermentation periods.

In general, the coupling of HTS and plate counting technology provides a clear overview of microbial population (Huang et al. 2020). In our study, second-generation and third-generation amplicon technologies were used to describe the fungal and bacterial diversity of light-flavor *Xiaoqu Baijiu*, respectively. Figure 3 showed that dominant bacterial at the species level included *L. acetotolerans*, *A. pasteurianus*, *L. helveticus*, and *L. buchneri*, and dominant fungi at the genus level were *Wickerhamomyces*, *Saccharomyces*, *Condenascus*, *Aspergillus*, and *Rhizopus* in D14, D87, and D98 samples. Wang and Xu (2019), and Huang et al. (2020) revealed *Bacillus* and yeast were the most influential microorganisms in the early stage of fermentation, and lactic acid bacteria predominated in the late fermentation phase of light-flavor *Baijiu*. Hu et al. (2021b) used HTS to investigate the structure of the microbial community and indicated that the core microbes were *Lactobacillus*, *A. pasteurianus*, *S. cerevisiae*, *W. anomalus*, *Aspergillus*, and *R. oryzae* at the end of fermentation in the mechanized light-flavor *Xiaoqu Baijiu* production, which were basically in line with our results. But we newly found a fungus *Condenascus* that has not been reported in *Baijiu*, which formerly belonged to the classification of *Thielavia* (Wang et al. 2019a).

Compared to common fermentation time (day 14), the abundance of fungi (*Wickerhamomyces*, *Saccharomyces*, *Condenascus*, *Aspergillus*, and *Rhizopus*) decreased, and the abundance of lactic acid bacteria (*L. acetotolerans*, *L. helveticus*, *L. buchneri*, and uncultured *Lactobacillus* sp.) increased to be dominant in the samples of ultra-long fermentation time (Fig. 3c, d). This was consistent with the results of culture-dependent analyses (Fig. 2). Previous research revealed that yeast population accounted for 45% of fungal abundance, followed by the LAB in the sample of normal fermentation time (day 14) (Hu et al. 2021c). But ultra-long fermentation time resulted in the increase of LAB and the decrease of yeasts, which were due to the changes of environmental factors, including nutrient limitation, high ethanol concentration, and low pH value (As showed in Fig. 1 and Fig. 2). For instance, *A. pasteurianus* was subdominant in D14 sample, but its relative abundance was less than 1% in D87 sample. And *Lactobacillus* dominated in

D98 (96.28%) sample. The proliferation of LAB and production of lactic acid may inhibit the growth of *A. pasteurianus*.

In this study, RDA was used to analyze the correlation between physicochemical properties and microbial community of light-flavor *Xiaoqu Baijiu*. The results revealed that residual starch and reducing sugar content had positive correlations with *A. pasteurianus*, *L. acetotolerans*, *Wickerhamomyces*, *Saccharomyces*, and *Condenascus*, and negative correlations with *L. helveticus*, *L. buchneri*, *Pseudotrematium*, *Oidiodendron*, and *Sistotrema*. While moisture, acidity, and amino nitrogen had the opposite correlations with these microbes. Furthermore, mantel tests showed that changes in residual starch, acidity, amino nitrogen had significant effects on microbial composition. Previous studies demonstrated high concentrations of water, ethanol as well as high acidity could facilitate the growth of *Lactobacillus* in the brewing process (Liu and Miao 2020). Therefore, the high acid environment in the ultra-long fermentation sample (D98) formed a micro-environment that was conducive to the growth of *Lactobacillus*.

Flavor components are very important indicators for *Baijiu* quality, which are largely affected by the microbes in fermentation micro-ecosystem (Wang et al. 2019b). The content of esters (such as ethyl acetate, diethyl succinate, phenylethyl acetate, ethyl palmitate, and ethyl linoleate) and alcohols (isoamyl alcohol and n-propanol) increased and the acids content (acetic acid) reduced significantly along with the fermentation progressed (Fig. 4). The later stage of fermentation process in fermented grains (25–60 days) could be described as the period of esterification and acetic acid is a precursor substance of synthetic esters, which led to a rise in the content of esters and a reduction in acids (Hu et al. 2021a). Ethyl acetate as main ester, is recognized as a key compound in light-flavor *Baijiu*. Although prolonging the fermentation time was beneficial to the accumulation of ethyl acetate, ultra-long fermentation time will cause its content to decrease, and other esters, such as diethyl succinate, accounted for the most part of esters in D98 sample (Fig. 5). Previous studies indicated that yeasts, bacteria, and molds took part in the synthesis of ethyl acetate (Guo and Jia 2014). In this study, ethyl acetate displayed positive correlation with *Cyberlindnera*, *Kazachstania*, *Candida*, *L. sicerae*, and *L. vini*, and diethyl succinate showed negative correlation with *Aspergillus* and positive correlation with *L. panis*, *L. secaliphilus* (Fig. 6). It indicated *Lactobacillus* contributed much in esters metabolism in light-flavor *Baijiu* fermentation, and Pang et al. (2021) revealed that inoculation with indigenous LAB changed the flavor profile of *Baijiu* significantly, especially promoting the formation of ethyl acetate. Besides, acetic acid and phenylethyl alcohol were the main aromatic compounds of *Baijiu* (Wang and Xu 2019). Acetic acid reduced significantly, and phenylethyl alcohol kept a relatively stable content along with the extension of

fermentation time from 14 to 98 days. Correlation analysis showed that acetic acid positively correlated with *Aspergillus* and negatively correlated with *Trichoderma*, *L. pontis*, and *L. helveticus* (Fig. 6). In the late phase of fermentation, LAB became the dominant species under anaerobic condition, and restrained the reproduction of *Acetobacter* by niche occupation. Thus, acetic acid was observed to decrease significantly in the long-time fermentation progress.

Higher alcohols, mainly including n-propanol, isobutyl alcohol, and isoamyl alcohol, are produced as the secondary metabolites of yeast during alcoholic fermentation (Wang et al. 2020c). At moderate level, these flavor components contribute to the flavor of many alcoholic beverages, whereas the excess level may be a health hazard (Hu et al. 2021c). Therefore, researchers have studied how to control the production of higher alcohols by process control and microbial regulation (Jiang et al. 2019; Pang et al. 2021; Zhong et al. 2019). In this study, the content of higher alcohols increased rapidly along with the extension of fermentation time, especially the n-propanol content increased by 6 times in fermented grains of D98 sample, compared to that in the D14 sample. Correlation network showed that n-propanol was positive correlation with an uncultured *Lactobacillus* sp., which was regard as one of major microbes for n-propanol production in *Baijiu* fermentation (Lu et al. 2019; Tian et al. 2020). Overall, microbial community had a significant variation induced by the ultra-long time fermentation, especially bacterial community. *Lactobacillus* accounted for the most part of microbes in fermented grains at the end of fermentation, which played a crucial role in the synthesis of key aroma compounds in fermented grains, such as ester, acid, alcohols, and so on.

Prolonging the fermentation time can improve the diversity of flavor components in *Baijiu*. For example, the fermentation time of strong-flavor *Baijiu* and sauce-flavor *Baijiu* is as long as 30 to 60 days, which is more likely to produce flavor components with diverse structural features and interesting biological activities, such as esters, acids, phenols, and pyrazines, which make *Baijiu* possesses the features of elegant and soft and endless aftertaste. However, ultra-long fermentation time also results in several potential side effects. First, it will lead to low liquor yield, this was encountered in strong-flavor *Baijiu* and sauce-flavor *Baijiu*. Another potential risk of ultra-long fermentation time is yielding more undesired components. In our case, ultra-long fermentation time is often accompanied by an increase in higher alcohols, especially n-propanol. Since the increase of LAB and ultra-long fermentation provide the possibility for the synthesis of n-propanol, which contributes negatively to the flavor of *Baijiu* when it is present in high concentrations. It is important to avoid the increase of the content of some components that cause human discomfort. In our study, the overall quality of *Baijiu* produced by 87 and 98 days fermentation was not as good as that of the normal

fermentation (D14). For this reason, they were used as flavoring liquor to produce different styles of *Baijiu*. Therefore, in the future, designing the appropriate fermentation time is not only conducive to brewing the original *Baijiu* containing different flavor components, but also balances the flavor components and yield of liquor.

Conclusion

Our results provide an overview of effect of ultra-long fermentation time on microbial community, physicochemical properties, and flavor components of light-flavor *Xiaoqu Baijiu* based on the fermentation tanks. Long-time fermentation significantly changes microbial community and improves the diversity of volatile components via adjusting physicochemical parameters, compared to the normal fermentation. *L. acetotolerans*, *L. helveticus*, *L. buchneri*, *Wickerhamomyces*, *Saccharomyces*, and *Condenascus* were identified as dominant microbes, and *Lactobacillus* absolutely dominated at the end of ultra-long fermented grains, which accounted for 96.28% of the total bacteria in fermented grains of D98 sample. Volatile components were mainly influenced by the microbial succession during ultra-long fermentation process, such as the massive production of n-propanol may be related to the proliferation of an uncultured *Lactobacillus* sp.. Residual starch, acidity, and amino nitrogen played important roles in promoting the formation of functional microbes. In a word, ultra-long fermentation time changes the overall flavor balance of *Baijiu* and improve the yielding of esters and alcohols of light-flavor *Xiaoqu Baijiu*, which are conducive to the production of special flavoring liquor. These results not only expanded our understanding on the factors that influenced the *Baijiu* quality, they may also provide a new strategy to breed new superior strains. We believe the quality of *Baijiu* can be stabilized by controlling the physicochemical changes influencing the succession of microbial communities and related microorganisms producing key flavor components well.

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Declarations

Conflict of interest The authors have no conflicts of interest to report.

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