Microbial community changes during the mechanized production of light aroma Xiaoqu baijiu

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ABSTRACT
Multi-microorganisms mixed fermentation is the main characteristic of Chinese baijiu brewing. In this study, plate culture and high-throughput sequencing were used to investigate the changes in microbial community during baijiu brewing. The results revealed that fungi accounted for the largest number of culturable microorganisms, followed by bacteria and molds. The diversity of bacteria and fungi first increased and then decreased, before increasing slightly at the late stage. Most microbial species came from the environment or raw materials, and the microbial community changed greatly during fermentation; thus, the dominant microorganisms in the starter might not be the dominant organisms during fermentation. The bacterial composition changed in a certain pattern, and the core microbes were lactic acid bacteria and Acetobacter pasteurianus. The composition of each genus was relatively uniform at the early stage, while Lactobacillus spp. were dominant at the late stage. Among them, Lactobacillus sp., which ranked first, had low initial abundance, before reaching 63.7% at the end. Four fungi, Saccharomyces cerevisiae, Wickerhamomyces anomalus, Aspergillus sp. and Rhizopus oryzae, were dominant, accounting for 74.6%–93.6% of the species present, among which the first two accounted for 50.3%–81.2%. This study provides a basis for the mechanized production of Xiaoqu baijiu.

Introduction
Chinese baijiu is a traditional alcoholic beverage made from grains by natural fermentation [1], and is one of the six major distilled liquors in the world. The most prominent feature of Chinese baijiu brewing is the use of a mixture of various microorganisms, which allows simultaneous saccharification and fermentation [2]. Different types of baijiu are brewed using different types of microbial starter through different production processes. Currently, 12 types of baijiu with different styles and characteristics have been created. Among them, strong aroma, light aroma and sauce aroma are the mainstream aroma profiles. Light aroma baijiu has a long history, simple production process, high yield and short cycle; thus, it has become an important raw material for the production of medicinal and health-promoting liquors [3].

Light-aroma Xiaoqu (XQ) liquors are formed by solid or semi-solid fermentation with sorghum as the main raw material and XQ (microbial starter in small balls or cubes) as the starter, followed by distillation and storage [4]. Furthermore, in general, the microbial starter used in the production of Luzhou-flavor baijiu, Fen-flavor baijiu and Maotai-flavor baijiu is Daqu. The main microorganisms in Daqu are Aspergillus, Acetobacter, Lactobacillus and yeast, while they include Rhizopus, Mucor, and a small amount of yeast in XQ [5–7]. XQ not only provides abundant enzymes for degrading the substrates and synthesizing flavor substances [7, 8], but also provides a large volume of microorganisms and alcohol produced by their metabolism for baijiu fermentation [9]. Among many natural microbial starters, only a few types of microorganisms can drive the fermentation process. Not only do they produce various flavor substances, but more importantly, they maintain the interactions among microorganisms [10],...
which are known as the core microorganisms, and their composition and function determine the quality of the *baijiu* [11]. Although Chinese *baijiu* production has a long history, traditional manual production is labor-intensive, costly, and inefficient [12], and the open environment can easily cause problems, such as difficulty in controlling the fermentation temperature and susceptibility to contamination by undesirable microorganisms, resulting in variable quality of *baijiu* [1]. Jing Brand Co., Ltd. is the largest producer of XQ *baijiu* in China, and the *baijiu* produced by it is a typical representative of XQ *baijiu* in China. The construction of its mechanized brewing base was completed in 2013, providing an annual output of 50,000 tons of *baijiu*. The mechanized production workshop changed the fermentation temperature from natural room temperature to a constant temperature of 21–23°C all year round, and the raw materials no longer touch the ground or come into direct contact with humans.

During *baijiu* fermentation, microorganisms play a pivotal role in flavor development and aroma profile. Studying the composition, diversity and variation patterns of microorganisms is of great significance in *baijiu* production. In our previous study, metagenomic and metatranscriptomic analyses were used to investigate the microbial communities in Xiaoqu *baijiu* brewing produced by different technologies, and 5 core microbes (*Saccharomyces cerevisiae*, *Rhizopus delemar*, *Pichia kudriavzevii*, *Lactobacillus helveticus*, and *R. oryzae*) were found in the fermentation [13]. However, because of the small sample size, there is currently no exact blueprint for the main sources of microorganisms, their changing patterns, or the composition of core microorganisms at every stage of fermentation of XQ *baijiu* produced by a mechanized process. In this study, the composition and changes of bacteria and fungi during the mechanized brewing process of XQ *baijiu* are expounded by using high-throughput sequencing and culturable techniques to provide a theoretical basis for *baijiu* production and process improvement.

**Materials and methods**

**Experimental design and sampling**

The fermented grain sample was obtained from the Fenglin Winery of the Jing Brand Co., Ltd. The fermentation period lasted for 15 days. On day 0 (d0; beginning of fermentation), 1 (d1), 3 (d3), 5 (d5), 7 (d7), 10 (d10) and 15 (d15; the end of fermentation), samples were collected via the five-point sampling method, i.e. five sub-samples were collected at different locations of the fermenter and mixed evenly into one sample. Each sample was divided into two parts, one of which was stored at −80°C for DNA extraction, and the other was stored at 4°C for the immediate detection of culturable microorganisms and physicochemical indices.

**Physicochemical index determination**

The water content was determined by the weighing method. Specifically, 20.00 g of sample was placed in a crucible to be dried in an oven at 105°C for 3–5 h, and the water content was calculated according to the weight difference before and after drying. The reducing sugar content was determined by Fehling’s method [14]. The process of starch detection was as follows: 5.00 g of sample was weighed and placed into a 250 mL conical flask, added to 100 mL of 5% HCl solution, bathed in boiling water for 30 min, quickly cooled, added to 20% NaOH for neutralization until the solution became slightly acidic, filtered, added to distilled water to a volume of 250 mL, and shaken well. Then, the same method as that for reducing sugar detection was applied. Acidity was determined by titration method with 0.1 mol/L NaOH solution [15]. Detection of alcohol by volume; 100.00 g of fermented grains were added to 200 mL of water, from which 100 mL of solution was distilled with a 500 mL all-glass distiller and the alcohol content was measured with a DMA5000 densimeter.

**Plate counting**

First, 25.00 g of fermented grain sample was placed in a conical flask, added to 225 mL of distilled water, and shaken on a shaker at 120 r/min for 30 min. Then, 1 mL of the suspension was pipetted and diluted to an appropriate concentration successively by 10-times gradients. The bacteria were cultured in Luria–Bertani medium (LB) at 37°C for two days [16], the molds were cultured in Czapek Dox medium [17] containing 100 μg/mL ampicillin at 30°C for three days, and the yeasts were cultured in YPD medium (1% of yeast extract, 2% of peptone, and 2% of glucose) containing 100 μg/mL ampicillin at 30°C for three days [18], after which they were counted separately.

**High throughput sequencing and analysis**

Genomic DNA was extracted from the samples using reagent kits, and DNA quality was detected by 1%
agarose gel electrophoresis. High-throughput sequencing was completed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd., and the libraries were built according to Illumina’s standard process. The libraries of bacteria were built with primers targeting the V3-V4 region, namely, 341 F: 5′-CCTAYGGGRBGCASCAG-3′ and 806 R: 5′-GGACTACNNGGGTATCTAAT-3′, and those of fungi were built with primers targeting the ITS1 region, namely, ITS5-1737F: 5′-GGAAGTAAAAGTCGTAACAAGG-3′ and ITS2-2043R: 5′-GCTGCGTTCTTCATCGATGC-3′. PCR reaction system (50 μL); 25 μL of 2× Premix Taq, 3 μL of template (20 ng/μL), 1 μL of each primer F and R (10 μmol/L), and 20 μL of nuclease-free water. PCR reaction conditions; 94°C for 5 min, 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s, for a total of 30 cycles; 72°C for 10 min (PCR Amplifier: ABI GeneAmp® 9700; ABI, CA, USA). The constructed amplicon libraries were subjected to PE300 paired-end sequencing on the Illumina Hiseq3000 platform. The PE reads obtained by sequencing were spliced according to the overlap relationship among PE reads, and the sequence quality was controlled and filtered at the same time. The samples were distinguished according to the barcodes at the beginning and end of the sequences as well as the primer information to obtain the effective sequences, the sequence directions were corrected, and the optimized sequences were obtained using FLASH and Trimmomatic software.

Operational taxonomic unit (OTU) cluster analysis was carried out using USEarch software. Statistical analysis of biological information was carried out for OTUs with similarity levels greater than 99%, and each OTU represented a species [19]. Based on OTU cluster analysis and species comparison results at different classification levels, the diversity index was analyzed based on OTUs using BIO-DAP (Fundy National Park, Canada) software. The V3-V4 regions of 16S rDNA genes of bacteria were compared using the Greengenes database for bacteria and the RDP Classifier. The ITS1 regions of fungi were compared using the UNITE database for fungi and the Basic Local Alignment Search Tool (BLAST). Using QIIME platform and KRONA software, species annotation and abundance analysis were carried out.

Results and discussion

**Changes in the physical and chemical index**

In *baijiu* brewing, temperature, water content and acidity are usually considered as key environmental variables, which not only jointly reflect the fermentation process with indexes, such as reducing sugar and starch [20], but are also closely related to the activities of microorganisms and play an important role in ensuring the quality of the base liquor [12, 21]. In the brewing process, the temperature first rose rapidly and reached the maximum on day 5, and then slowly decreased. The acidity was stable for the first 5 days, gradually increased, and was the highest at the end (Figure 1A). At the late stage of *baijiu* fermentation, *Lactobacilli* and other microorganisms generally produce a large amount of acidic substances [22]. Appropriate acidity is beneficial to the gelatinization and saccharification of fermented grains and can also suppress the growth of harmful microorganisms and contribute to the taste of *baijiu* [20].

As shown in Figure 1(B), the water content increased slowly at first and remained unaltered after the day 5.
Alcohol was mainly produced on day 1, and the alcohol by volume increased rapidly from 3.13 to 64.83 on day 1, reached 70.20 on day 3, and then remained stable. When the concentration of alcohol or acid was relatively high, the growth of yeast was inhibited [23]. Starch is the material basis for alcohol fermentation. The content of starch gradually decreased during fermentation, especially rapidly from day 1 to 3, and the growth of yeast was very active at this stage. Furthermore, the reducing sugar content basically stabilized to zero on day 3 of fermentation.

Changes in the numbers of culturable microorganisms

The yeast levels increased rapidly in the first 3 days, reaching the maximum of $1.26 \times 10^8$ CFU/g at day 3 (day 0, $2.9 \times 10^7$ CFU/g), and then decreased slowly. The bacteria levels increased slowly in the first 5 days, increased rapidly from d5 to d7, and then decreased slowly after reaching the maximum of $2.0 \times 10^7$ CFU/g at day 7. At the beginning, the bacteria levels were higher than those of yeasts, but at the end, the opposite trend was observed. The mold levels decreased continuously, with $3.9 \times 10^3$ CFU/g found at the start of fermentation and $1.3 \times 10^2$ CFU/g at the end of fermentation (Figure 1C).

The results of plate counting in this study indicated that yeast proliferated rapidly in from day 1 to 3, reaching the highest level, then gradually decreased. Bacteria grew slowly in between day 1 and 5 because the temperature at this stage was low and unsuitable for bacterial growth. On day 5, the fermentation temperature was above $30^\circ$C, and some alcohol-tolerant bacteria multiplied. At different stages, the types of microorganisms changed in certain patterns. At the late stage, constant-temperature fermentation can be modified to variable-temperature fermentation to optimize the fermentation conditions.

Microbial community based on high-throughput sequencing

In baijiu brewing, bacteria are mainly used to produce aroma components and their precursors and are an important source of the unique flavor of light-aroma XQ baijiu [24]. As shown in Figure 2, at the phylum level, Firmicutes and Proteobacteria were predominant, and the number of bacteria belonging to other phyla was very small. At the genus level, there were
15 main genera, including *Lactobacillus*, *Acetobacter*, *Weissella*, *Bacillus*, *Lactococcus*, and *Gluconobacter*. At the early stage of day 1 to 3, the composition of each genus was relatively uniform. During the brewing process, *Lactobacillus* showed an upward trend, with an absolute predominance from day 5, and its abundance was over 50%. Table 1 showed the top 30 most abundant species, collectively accounting for approximately more than 90% of all species present, among which *Lactobacillus* species accounted for the highest proportion with 10 species. The no. 1 *Lactobacillus* sp. had a low initial abundance but reached 63.7% by the end.

According to the previous study, it was most likely the *Lactobacillus helveticus* [13]. Other *Lactobacillus* species present include *Weissella paramesenteroides*, *Lactococcus lactis*, *Lactococcus piscium*, *Leuconostoc pseudomesenteroides*, and *Leuconostoc lactis*. *Acetobacter pasteurianus* ranked second, with an abundance of 0.62% at the beginning and 16.6% at the end. In the fermentation process, the bacterial community changed dramatically and the top 10 species in XQ were not dominant at the end of fermentation.

In this study, the results showed the core bacteria were *Lactobacillus* sp., *A. pasteurianus*, *W. paramesenteroides*, *Gluconobacter* sp., and *Bacillus* sp. (Table 1). It was different from *Daqu baijiu* production, in which *Bacillus* dominated the process of brewing [2, 9]. *Lactobacillus* sp. play a dominant role in the fermentation process of light-aroma *baijiu* [6, 25]. They can produce lactic acid and acetic acid via metabolism to maintain an acidic environment and reduce the diversity of microorganisms, thereby promoting fermentation of *baijiu* [26]. The lactic acid produced is conducive to the formation of ethyl lactate, which is an important basic substance for the formation of aroma components in *baijiu* [27]. The sour flavor substances in *baijiu* are mainly lactic acid and acetic acid. *A. pasteurianus* can convert ethanol to acetic acid, while *Gluconobacter* oxidizes ethanol into acetic acid. However, the difference is that *Gluconobacter* appears in sugar-rich environments, so its abundance is relatively high at the early stage of fermentation, while *A. pasteurianus* mostly appears in alcohol-rich environments, thus, has high abundance at the late stage of fermentation. *W. paramesenteroides* exists in soy sauce, pickles, sausages and other fermented foods, and plays an important role in the synthesis of organic acids, esters and short chain fatty acids in food [28]. *Bacillus* mainly exists in distiller’s yeast, and its abundance is relatively high when the temperature is high in the fermentation process. It enhances the secretion of amylase [29], provides amino acids, organic acids

### Table 1. Relative abundance of bacterial species based on high throughput sequencing (top 30, %).

| Taxonomy                        | XQ | Rank | d0   | d1   | d3   | d5   | d7   | d10  | d15  |
|---------------------------------|----|------|------|------|------|------|------|------|------|
| *Lactobacillus* sp.             | –  | –    | –    | 11.9 | 39.2 | 39.9 | 73.1 | 63.7 |
| *Acetobacter pasteurianus*      | 0.34 | 0.62 | 14.1 | 7.32 | 5.97 | 22.9 | 6.92 | 16.6 |
| *Lactobacillus* sp.             | –  | 4.54 | 5.05 | 14.3 | 5.37 | 4.91 | 2.78 | 0.51 |
| *Lactobacillus* sp.             | –  | 0.37 | 11.1 | 9.15 | 7.09 | 4.92 | 4.29 |
| *Weissella paramesenteroides*   | 0.22 | 7.50 | 14.4 | 4.55 | 3.10 | 1.70 | 0.72 | –    |
| *Gluconobacter* sp.             | 6.29 | 4    | 4.76 | 9.57 | 5.26 | 6.03 | 2.04 | 0.55 | 0.76 |
| *Bacillus* sp.                  | 4.07 | 0.20 | 6.15 | 9.10 | 6.19 | 3.82 | 1.57 | 0.33 |
| *Lactococcus lactis*            | 38.4 | 1    | 23.6 | 0.53 | 0.73 | 0.52 | 0.38 | 0.12 |
| *Lactococcus piscium*           | 0   | –    | 5.04 | 7.94 | 5.32 | 3.23 | 1.33 | 0.26 |
| *Lactobacillus* sp.             | 2.94 | 5    | 2.63 | 4.62 | 4.52 | 2.79 | 1.70 | 0.66 |
| *Bacillus delbrueckii*          | 0.19 | 9    | 11.0 | 0.16 | 0.21 | 0.28 | 0.56 | 0.77 | 0.28 |
| *Pantoaea* sp.                  | 7.29 | 3    | 3.86 | 7.58 | 0.90 | 0.30 | 0.24 | 0.13 |
| *Lactobacillus pontis*          | 0   | 0    | –    | 1.08 | 1.32 | 1.65 | 1.24 | 6.38 |
| *Lactobacillus* sp.             | 1.06 | 0.96 | 2.65 | 3.03 | 1.26 | 0.79 | 0.28 | –    |
| *Unclassified Bacteria*         | –   | 2.45 | 5.08 | 0.38 | –    | –    | –    | 0    |
| *Weissella* sp.                 | 2.29 | 6    | 1.67 | 3.29 | 1.61 | 0.86 | 0.40 | 0.18 |
| *Leuconostoc pseudomesenteroides* | 0   | 0.45 | 3.78 | 1.06 | 1.05 | 0.59 | 0.13 | –    |
| *Uncultured bacterium*          | 0.16 | 10   | 4.40 | 0.46 | 0.23 | 0.20 | 0.12 | –    |
| *Leuconostoc lactis*            | 1.04 | 7    | 2.23 | 1.28 | 0.52 | 0.52 | 0.37 | –    |
| *Lactobacillus acetoterarans*    | –   | –    | –    | 0.10 | 0.67 | 1.16 | 0.25 | 2.41 |
| *Pediococcus* sp.               | –   | 2.20 | 0.66 | 0.86 | 0.28 | 0.27 | 0.12 | –    |
| *Bacillus* sp.                  | 10.2 | 2    | 2.51 | 0.94 | 0.36 | 0.13 | –    | –    |
| *Enterobacter* sp.              | 16.3 | 0.95 | 0.74 | 0.62 | 0.66 | 0.36 | 0.12 | –    |
| *Unclassified Cyanobacteria*     | 6.49 | 2.56 | 0.55 | 0.29 | –    | –    | 0    | 0    |
| *Lactobacillus* sp.             | –   | 0.53 | 0    | 0.10 | 0.39 | 0.28 | 0.72 | 1.28 |
| *Gluconacetobacter sacchari*     | –   | 1.90 | 0.53 | 0.46 | 0.24 | –    | –    | –    |
| *Xanthomonas* sp.               | 0   | 0.33 | 1.13 | 0.95 | 0.34 | 0.17 | –    | –    |
| *Bacillus* oleronius*           | 0   | 1.96 | –    | –    | –    | –    | 0    | 0    |
| *Xanthomonadaceae*              | 0   | –    | –    | –    | 1.24 | –    | 0    | 0    |
| Others                          | 2.53 | 4.64 | 10.67 | 9.90 | 5.83 | 4.79 | 2.02 | 1.45 |

*–*,-, below 0.1; The “Rank” in the second column refers to the order of bacterial species in the Xiqqu (XQ); *–* refers to the next taxon was unclassified; n = 2.
and other compounds, and can interact with other microorganisms to form representative aroma components [10, 30].

At the phylum level, Basidiomycota, Ascomycota and Zygomycota were found. At the genus level, nine genera, including Saccharomyces, Wickerhamomyces, Aspergillus, Trichosporon, and Monascus, as well as two fungi of unknown genera were present. The proportion of the top 20 at the species level was generally over 97% (Table 2). The first two species were Saccharomycyes cerevisiae and Wickerhamomyces anomalus, with a proportion of 50.3–81.2%, followed by two types of molds, Aspergillus sp. and Rhizopus oryzae. The proportions of these four fungi were between 74.6% and 93.6%, indicating that several types of microorganisms play a major role in baijiu brewing. Trichosporon sp. ranked first among the yeasts in XQ but were not dominant in the fermentation process.

The results showed that the core fungi were S. cerevisiae, W. anomalus, Aspergillus sp., R. oryzae, etc. It was a little different from the previous study which showed that the 4 core fungi were S. cerevisiae, Rhizopus delemar, Pichia kudriavzevii, and R. oryzae [13]. Fungal communities mainly consisted of species of Saccharomycetaeae and Rhizopus [6]. S. cerevisiae is the most important microorganism in the process of alcohol fermentation, and is involved in the formation of alcohol and flavor substances [31–33]. W. anomalus is an aroma-producing yeast that can produce a large amount of ethyl acetate in the fermentation process [34]. S. cerevisiae has important contributions to the fermentation rate and ethanol production, but generally cannot directly convert starch to glucose [35]. Molds can produce hydrolases, such as amylase, protease and lipase, which are used for the saccharification of starch and decomposition of macromolecular substances. These enzymes also provide essential flavor compounds and enhance the formation of the aroma [36]. Aspergillus produces proteolytic enzymes and other lyases, which promote the saccharification of starch, hydrolysis of proteins, and formation of flavonoids. Rhizopus is the main saccharifying microorganism in the process of baijiu brewing. For example, the abundance of R. oryzae could reach 88% at the beginning of Sichuan Xiaoqu baijiu brewing [6]. Rhizopus widely exists in the starters and fermented grains of various liquors and can rapidly utilize various raw materials to produce glycerol, lactic acid, amylase and protease, among other hydrolases and volatile compounds [29, 37].

Baijiu is produced by multi-microbes mixed fermentation. The synergy among populations is closely related to the quality of baijiu [1, 28], and the uniformity of the community structure ensures its stability [38]. Lactobacillus sp. and S. cerevisiae widely coexist during natural fermentation. Lactobacillus spp. promote the growth of brewing yeasts and facilitate their metabolism, resulting in the production of acids, alcohols, esters and other flavor substances [39]. Some substances produced by the metabolism of S. cerevisiae can inhibit the synthesis of ethyl acetate by A. pasteurianus, and this inhibition is eliminated when there are live yeast in the fermentation system [40]. The growth of S. cerevisiae and W. anomalus influence one another, and this co-culturing produces more varieties and higher contents of acetic ester, ethyl ester, higher alcohols, aldehydes and ketones [41].

### Table 2. Relative abundance of fungal species based on high throughput sequencing (top 20%).

| Taxonomy | XQ | Rank | d0  | d1  | d3  | d5  | d7  | d10 | d15 |
|----------|----|------|-----|-----|-----|-----|-----|-----|-----|
| Saccharomyces cerevisiae | 0.88 | 6 | 48.2 | 29.7 | 59.6 | 52.9 | 38.6 | 16.6 | 45.4 |
| Wickerhamomyces anomalus | 18.4 | 2 | 2.08 | 29.2 | 21.6 | 18.0 | 26.7 | 39.2 | 18.5 |
| Aspergillus sp. | 2.08 | 5 | 14.8 | 21.5 | 12.1 | 20.8 | 25.0 | 37.2 | 10.2 |
| Rhizopus oryzae | 0.24 | 19.9 | 5.81 | 0.27 | 0.19 | 0.13 | 0.24 | 0.51 |
| Hypocreales sp. | 0.11 | 0.24 | 0.19 | 0.11 | 0.17 | 0.26 | 0.82 | 13.4 |
| Saccharomyces cereales | 7.26 | 3 | 5.93 | 5.91 | 1.12 | 0.88 | 0.43 | 0.12 | 0.29 |
| Trichosporon sp. | 64.8 | 1 | 4.15 | 3.28 | 0.91 | 0.44 | 0.72 | 0.20 | 0.36 |
| Saccharomyces cereales | 0.72 | 7 | 0.15 | 0.10 | 0.14 | 0.16 | 0.23 | 0.27 | 0.42 |
| Monascus purpureus | 3.39 | 4 | 0.45 | 0.74 | 0.62 | 1.23 | 0.82 | 1.72 | 1.22 |
| Meyerozyma guilliermondii | 0.00 | 2 | – | – | – | – | – | – | – |
| Candida humilis | 0.00 | 1 | – | – | – | – | – | – | – |
| Trichosporon asahii | 0.59 | 8 | 0.40 | 0.61 | 0.19 | 0.27 | 0.42 | 0.27 | 0.43 |
| Trichosporon ovales | 0.44 | 9 | 0.75 | 0.51 | 0.13 | 0.23 | 0.34 | – | 0.17 |
| Candida tropicalis | 0.12 | 2.26 | 0.11 | 1.19 | 1.54 | 0.87 | 0.58 | 0.13 |
| Wickerhamomyces sp. | 0.18 | 0.14 | 0.22 | 0.75 | 0.66 | 0.44 | 0.16 |
| Gibberella zeae | 0.00 | 0 | – | – | – | 0.52 | – | – | 0.20 |
| Aspergillus flavus | 0.00 | 0 | – | – | – | – | 0.11 | 0.20 | 0.17 |
| Ascomycota | 0.00 | 0 | – | – | – | 0.10 | – | – | 0.32 |
| Gibberella intricans | 0.00 | 0 | – | – | – | – | 0.18 | 0.25 | – |
| Kluyveromyces marxianus | 0.00 | 0.72 | 0.15 | 0.10 | – | 0.13 | – | 0 | – |
| Others | 1.06 | 0.49 | 0.82 | 0.65 | 0.82 | 2.34 | 1.30 | 4.13 | 0.14 |

*–*, below 0.1; The “Rank” in the second column refers to the order of fungal species in the Xiaoqu (XQ); * Asterisks indicate that the next taxon was unclassified; n = 2.
**Sample relationship**

Non-metric multidimensional scaling (NMDS) analysis showed that the reproducibility between two replicate samples of bacteria composition was good, especially at d0, d1, d3, d10 and d15 (Figure 3). The bacterial composition changed in a certain pattern, with close relationships among samples at d0, d1 and d3, as well as at d3, d5, d7 and d10. The composition of the fungi also had good reproducibility, but the change pattern was not significant compared with the composition of the bacteria.

Chinese baijiu is a traditional fermented food, and much attention has been paid to the sources, composition and functions of microorganisms in its brewing process. Analysis of XQ and samples obtained on d0, d1 and d15 showed that there were 26 species of bacteria in the XQ sample, 51 in the d0 sample (19 from XQ), 74 in the d1 sample, and 20 in the d15 sample (Figure 4). In addition, 7 species of bacteria were found in all four samples. A total of 16 fungal species were found in the XQ sample, 15 in the d0 sample (11 species from XQ), 19 in the d1 sample and 34 in the d15 sample. Additionally, 7 species of fungi were found in all four samples. The Venn diagrams revealed that most species came from the environment or raw materials (Figure 4). These results indicate that the microorganisms not only came from the microbial starter, but also from the environment and raw materials.

**Diversity index**

As shown in Table 3, when the threshold was 0.01 to 0.05%, the number of OTUs was greatly reduced. The diversity index of d0 at the beginning of fermentation was higher than that of XQ. During the fermentation process, the bacterial diversity index first rose to the highest value on d1, then decreased to the minimum on d10, and finally increased slightly at the late stage. The diversity of fungi showed more complex variation than bacteria-first increasing to the maximum on d1, then rapidly decreasing until d3, remaining constant until d5, and finally increasing slightly.

The initial fermented grains were rich in nutrition, inhibition by alcohol was minor, and various microorganisms grew rapidly, and therefore, the diversity increased rapidly. When the inhibition of alcohol occurred, the growth of many microorganisms was inhibited and diversity decreased. The diversity increased slightly after day 10, and many alcohol-tolerant microorganisms began to grow. The changes in environment and nutrition during brewing explain the changes in community species diversity [42]. The diversity index reflects the changes in the microbial community, as confirmed in the present study.

**Table 3.** Microbial diversity index based on OTUs of high-throughput sequencing.

| Item      | XQ   | d0   | d1   | d3   | d5   | d7   | d10  | d15  |
|-----------|------|------|------|------|------|------|------|------|
| **Bacteria** |      |      |      |      |      |      |      |      |
| OTU (≥0.01%) | 55   | 151  | 172  | 172  | 152  | 143  | 126  | 96   |
| OTU (≥0.05%) | 26   | 51   | 74   | 66   | 54   | 58   | 29   | 20   |
| Simpson index (1/D) | 4.49 | 10.11| 13.66| 13.14| 5.51 | 4.48 | 1.84 | 2.27 |
| Shannon index (He’) | 2.02 | 2.88 | 3.16 | 3.09 | 2.55 | 2.23 | 1.31 | 1.36 |
| **Fungi** |      |      |      |      |      |      |      |      |
| OTU (≥0.01%) | 51   | 54   | 96   | 106  | 138  | 152  | 137  | 227  |
| OTU (≥0.05%) | 16   | 15   | 19   | 19   | 23   | 28   | 20   | 34   |
| Simpson index (1/D) | 2.34 | 3.33 | 4.4  | 2.4  | 2.36 | 3.53 | 3.12 | 3.71 |
| Shannon index (He’) | 1.28 | 1.58 | 1.77 | 1.25 | 1.25 | 1.62 | 1.43 | 1.89 |

1/D, reciprocal of Simpson’s diversity index; n = 2.
Conclusions
In this study, traditional culture method and modern high throughput sequencing technology were used to investigate the changes in microbial community at every stage of baijiu brewing. The results revealed that most species of microorganisms originated from the environment or raw materials, and the dominant microorganisms in Xiaoqqu are not necessarily dominant during the fermentation process. The diversity of bacteria and fungi increased initially, then decreased, and again increased slightly at the late stage of brewing process. The core species of bacteria were Lactobacillus spp., A. pasteurianus, W. paramesenteroides and L. lactis. The core species of fungi were S. cerevisiae, W. anomalous, Aspergillus sp. and R. oryzae. The microbial community changed greatly, with yeasts dominating the early stage and bacteria dominating the late stage. Thus, a temperature control process by stage could be adopted. This study provided fundamental understanding of the microbial communities in the mechanized production of light- aroma Xiaoqu baijiu.

Disclosure statement
The authors declare there are no competing interests. QY, SC, LZ are affiliated with Jing Brand Co., Ltd.

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Data availability statement
All data generated is included in this present study, and the data are available on Mendeley: http://dx.doi.org/10.17632/mds4wpcS3m.1. Moreover, the sequencing data has been submitted to the NCBI website with a BioProject number of PRJNA699760.

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