Journal of Bioscience and Bioengineering
VOL. 127 No. 3, 309–317, 2019

www.elsevier.com/locate/jbiosc

Roles of aging in the production of light-flavored *Daqu*

Guangsen Fan,[1,2] Zhilei Fu,[2] Baoguo Sun,[1,2] Yuhang Zhang,[3] Xinlei Wang,[3] Yanqiu Xia,[2] Mingquan Huang,[2] and Xiuting Li[1,2,*]

Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology and Business University (BTBU), Beijing 100048, China,[1] School of Food and Chemical Engineering, Beijing Technology and Business University (BTBU), Beijing 100048, China,[2] and Hengshui Laobaigan Co. Ltd, Hengshui, Heibei 053000, China[3]

Received 25 March 2018; accepted 9 August 2018
Available online 18 September 2018

Daqu*, a complex starting material used for *Baijiu* production, contains microorganisms, enzymes, and volatile compounds. An important part of *Daqu* production is aging, but the physicochemical and microbial changes during aging remain largely unknown. This study characterized aging according to physicochemical parameters, volatile compounds, and microbial communities. Aging was found to aid in the stabilization of the physicochemical parameters. Solid-phase microextraction-gas chromatography-mass spectrometry was used to detect 72 types of volatile compounds, which were predominantly alcohols, esters, aldehydes, alkenes, and alkanes. During aging, these compounds changed considerably, but their structures eventually stabilized. A high-throughput sequencing approach was used to analyze the changing composition of the microbial communities. In general, aging helped to enrich and stabilize the microbial population for making *Baijiu*. A total of 35 bacteria were detected as prokaryotic; among these, 15 had a diversity abundance ratio of more than 1%. The dominant bacteria were from the genus *Pantoea*, but these decreased with aging, while bacteria from *Lactobacillus* and *Weissella* increased. After aging for 2 months, *Pantoea*, *Lactobacillus* and *Weissella* accounted for 0.4%, 54.0%, and 18.9%, respectively. A total of 12 eukaryotic yeast and fungi were detected, the most abundant of which were Incertae_Sedis_incertae_sedis, *Saccharomycopsis*, Trichocomaceae_unclassified, *Pichia*, Tremellales_unclassified, and *Galactomyces*. During aging, the levels of Trichocomaceae_unclassified, *Saccharomycopsis*, and *Galactomyces* initially decreased but then increased. *Pichia* stayed unchanged as aging progressed. In conclusion, aging led to rebalanced interactions among *Daqu* microbes and was important in improving *Daqu* quality and ensuring its stability.

[**Key words:** Aging process; *Daqu*; Microbial communities; Physicochemical parameters; Volatile compounds]

Daqu is an essential fermentation starter culture for the production of *Baijiu* (1). It not only comprises important crude enzymes and complex microbial flora, but it is also a source of brewing and aroma precursors that determine the flavor characteristics of its products (2). *Daqu* is produced through solid-state fermentation of wheat, barley, and/or peas in a traditional four-stage process: (i) ingredients formulation, grinding, mixing and shaping; (ii) incubation of microbes in its natural surrounding; (iii) spontaneous solid-state fermentation for 1 month; and (iv) drying and ripening over a period of 2–6 months to reach maturity. This final stage is named aging (3). Thus, the production of *Daqu* is not a simple process. Each step in the production process affects the quality of *Daqu*, which in turn affects the quality of *Baijiu*.

In the four-step production process, it is obvious that aging is a time-consuming process (3). Although there is no clear explanation of the internal mechanism for the long-term aging process, it is a fact that the production of high-quality *Baijiu* requires the use of appropriately aged (mature) *Daqu* (4). Over the thousands of years that *Daqu* has been made, great care has been taken in the aging steps, but, to date, the production of *Daqu* is considered an art rather than a science (4). During aging, fermented *Daqu* is placed in an open well-ventilated area for a period of time, away from heat or moisture. Aging is the most complicated and critical step in *Daqu* manufacture. During this procedure, numerous microorganisms, enzymes, and volatile compounds are created and enriched. Upon maturity, *Daqu* is considered to have attained the effects of removing miscellaneous traits, pure character, and increased fragrance, according to perceptions of experienced production workers (5). This means that aging is not simply a storage process, but also involves microbial enrichment, primary and secondary metabolism processes, and changes in various microbes and other compounds found in the mix before the *Daqu* is mature (4,6). Moreover, aging can cause enriched microbes to interact further, leading to the mixed microbial communities to form balanced and stable *Baijiu*. Aging is a process of balancing various microbial and chemical functions. During this period, flavor substances in *Daqu* change because of microbial activity and tend to be flavored to suit the most popular *Baijiu* (4,6).

Only well-aged *Daqu* can be used in *Baijiu* production (4). Determining when to stop the aging of *Daqu* is an important control point in *Daqu* making (4). At present, whether *Daqu* will be used in production (that is, an assessment of its quality) depends mainly on the production experience and physicochemical parameters, which are subjective and uncertain. Therefore, these factors cause

* Corresponding author at: Beijing Technology and Business University, No. 11 Fucheng Street, Haidian District, Beijing 100084, China. Tel.: +86 10 68985342; fax: +86 10 68985252.
E-mail address: lixt@btbu.edu.cn (X. Li).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.08.005

inconsistency in the production of *Baijiu* (4). To date, the effects of aging in *Daqu* making have not been explained scientifically, and this problems to be solved must be addressed to allow the mechanization of *Daqu* production.

Although *Daqu* aging plays an important role in *Baijiu* manufacture, few studies have examined the dynamics of microbial diversity, volatile compounds, and physicochemical parameters during aging. In the present study, we investigated the changes in microbial communities using high-throughput sequencing, and the changes in flavor substances by solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) during aging. In addition, physicochemical analysis was conducted to analyze the potential role of microorganisms. Results from this study may help to provide a theoretical basis to scientifically determine when aging of *Daqu* should be stopped. To simplify the investigation, light-flavored *Daqu* was selected because the time required for aging can be reduced to 2–3 months. To the best of our knowledge, this is the first comprehensive assessment of *Daqu* aging using physicochemical parameters, flavor substances, and microbial flora.

MATERIALS AND METHODS

Sampling Three *Daqus* from the same production set were sampled at different times (sample A: beginning of aging, sampled on September 21, 2016; sample B: after 1 month of aging, sampled on October 21, 2016; sample C: after 2 months of aging, sampled on Novermber 21, 2016) up to maturity as judged by experienced workers, and then collected from the liquor (light-flavored) from Laobaigan Brewing (Hebei Hengshui Laobaigan Liquor, Hengshui, China). *Daqu* bricks were produced from wheat grains during the autumn of 2016. To obtain reliable samples, bricks from each stage were randomly selected from upper, middle, and lower locations in triplicate. These were then ground, mixed, and representative samples were collected for each *Daqu* by the quartering method (7). Samples were transferred to sterile bags, sealed, and stored at −80 °C until analysis.

Analysis of physicochemical properties The physicochemical properties of *Daqu* were measured in triplicate samples. The moisture of *Daqu* was measured as the ratio of dry weight to wet weight at 105°C (8). Protein and starch were determined by the Kjeldahl method and by Fehling's titration method, respectively (8,9). Acidity, pH, and reducing sugar were analyzed through the *Daqu* water extracts. *Daqu* (10 g) was soaked in 100 mL of distilled water for 30 min, and water extracts were collected after filtration. Acidity was evaluated by alkali titration (8). The pH was measured using a pH meter positioned in the slurry. Reducing sugar was determined by colorimetric analysis after reaction with 3,5-dinitrosalicyclic acid (10). To evaluate the activity and quality of *Daqu* samples, saccharifying activity (U), liquefying activity (U), esterifying activity (U), and fermenting activity (U) were determined according to the general technical standard methods (8).

Analysis of volatile compounds Volatile compounds of *Daqu* were determined as previously described by Fan et al. (11). In brief, 2-octanol (Sigma–Aldrich, St. Louis, MO, USA) was added into 500-mg *Daqu* samples as internal standard. First, the *Daqu* samples were suspended in 5 mL of Milli-Q water and tightly sealed in a 15-mL vial using a teflon/silicone septum. Second, the suspension was mixed, and allowed to equilibrate for 10 min at 50°C. Third, the analytes in the headspace of the suspension were extracted using the SPME fiber (50/30 μm divinylbenzene/carboxen on polydimethylsiloxane; Supelco, Bellefonte, PA, USA) for 30 min.

The volatile compounds were analyzed in the GC-MS system (Trace MS/GC; Thermo Quest Finnigan, Silicon Valley, CA, USA) equipped with a TG-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) using split-less injection. GC conditions were set as follows: the oven and injection temperature was 250°C; the carrier gas was helium at a constant rate of 0.8 mL/min; the oven temperature was 50°C for 2 min, then increased at 2°C/min up to 85°C, held for 0.1 min, and finally raised to 230°C at 5°C/min. The final temperature was maintained for 15 min. The vaporization chamber temperature was 250°C. The electron impact (EI) energy was 70 eV, and the ion source temperature was set at 230°C. EI mass spectra ranged from 30 to 550 amu.

The individual volatile compounds were identified by comparison with the mass spectral data of the NIST 05a library (Finnigan). Retention indices (RIs) were calculated in accordance with the modified Kovats method (12). Approximate quantities of each compound were determined by comparison of its peak area to the integrated peaks of the total ion chromatogram and calculated by comparing peak areas with that of the 2-octanol internal standard. The amounts of individual constituents present in the sample were calculated and expressed as milligrams per kilogram of *Daqu*. Triplicate analyses were performed.

DNA extraction and quantitation The *Daqu* samples were pretreated using a method described previously (13). The genomic DNA was extracted using the commercial PowerSoil DNA Isolation Kit (Mo-Bio, Carlsbad, CA, USA). The quality of extracted DNA was assessed through electrophoresis in 0.6% (w/v) agarose gels and spectrophotometry (ratio of optical density at 260 nm/280 nm). All extracted DNA samples were stored at −80°C and were used as templates for polymerase chain reaction (PCR) amplification.

Plate count method The quantity of microbes in *Daqu* was determined based on the plate count method according to the previous reports (14).

PCR amplification The V4-V5 region on 16S ribosomal RNA gene of bacteria and 18S ribosomal RNA gene of fungi were amplified by PCR. The V4-V5 region on 16S ribosomal RNA gene was amplified with forward primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse primers 806R (5′-GGACTACHVGGGTWTCTAAT-3′), while the fungal 18S ribosomal RNA gene was amplified with forward primer (SSU0817F) TTAGCATGGAATAATRRAATAGGA and reverse primer (SSU1196R) TCTGGACCTGGTGAGTTTCC. The reverse primer was modified by adding an eight-base error-correcting barcode, which is unique to each sample and serves as a multiplexing marker. PCR reactions were performed in triplicate in a 20-μL mixture, and the PCR reactions were as follows: 95°C for 3 min, followed by 32 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 10 min.

Illumina MiSeq sequencing Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor-ST (Promega, Madison, WI, USA). Purified amplicons were pooled in equimolar manner and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform according to the standard protocols.

Processing of sequencing data Raw FASTQ files were demultiplexed and quality-filtered using QIIME (version 1.17) with the following criteria: (i) The 300 bp reads were truncated at any site with an average quality score of < 20 bp over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp. (ii) Exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) Only sequences that overlapped by more than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded.

Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1, http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA/18S rRNA gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU115) 16S rRNA and 18S rRNA database using a confidence threshold of 70%.

Alpha diversity was evaluated through QIIME to generate rarefaction curves, Good's coverage, Chao1, ACE, and Shannon and Simpson diversity indices. Beta diversity was evaluated with the UniFrac method. In addition, the group_significance.py script of QIIME was run to compare the OTUs frequencies across the samples. Both weighted and unweighted Bray–Curtis calculations were performed for the principal coordinate analysis (PCoA).

Statistical analysis All statistical analyses were performed with SPSS16.0 (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was used to compare the means. Mean separations were performed using Tukey's test. Differences at $P < 0.05$ were considered significant.

Accession numbers The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (SRP109886).

RESULTS AND DISCUSSION

Aging, the last step of *Daqu* production, predominantly influences the quality of *Daqu*. This study analyzed the changes that occur in *Daqu* during aging from three perspectives: physicochemical parameters, flavor, and microbiota. In general, the results showed that the physicochemical parameters and volatile compounds as well as the microbial composition of *Daqu* varied as aging continued.

Physicochemical properties Table 1 shows the physicochemical parameters of *Daqu* as aging progressed. Some physicochemical parameters changed slightly during aging, and these were closely related to the changes and interactions within the microbial community structure of *Daqu* (Table 1). During aging, acidity increased and pH decreased (Table 1), which is related to the increase in the diversity of slightly acidic bacteria generated from *Daqu*, such as *Acetobacter* and *Lactobacillus*. *Acetobacter* has been shown to oxidize many types of sugars and alcohols to produce organic acids as end products, and *Lactobacillus* has been shown to utilize fermentable carbohydrates and free sugars to produce lactic acid as the major end product (15). It was observed that the protein content gradually increased

TABLE 1. Changes in physicochemical properties of light-flavored *Daqu* during aging.

Sample	Moisture (%)	Protein (%)	Starch (%)	Acidity (mmol g^{-1})	pH	RS (%)	Saccharifing activity (U)	Liquefying activity (U)	Esterifying activity (U)	Fermenting activity (U)
A	16.65 ± 0.33^{a}	15.28 ± 0.23^{a}	53.11 ± 0.21^{a}	0.027 ± 0.002^{a}	7.25 ± 0.00^{a}	0.30 ± 0.01^{a}	964.00 ± 5.50^{a}	2.18 ± 0.10^{a}	649.41 ± 5.89^{a}	3.10 ± 0.03^{a}
B	15.05 ± 0.10^{b}	15.70 ± 0.12^{b}	53.17 ± 0.12^{a}	0.030 ± 0.003^{a}	7.04 ± 0.00^{b}	0.35 ± 0.04^{a}	991.00 ± 7.01^{b}	2.18 ± 0.10^{a}	678.09 ± 5.22^{b}	3.42 ± 0.04^{b}
C	14.85 ± 0.38^{b}	16.11 ± 0.02^{c}	53.23 ± 0.07^{a}	0.040 ± 0.001^{b}	6.69 ± 0.01^{c}	0.33 ± 0.03^{a}	969.00 ± 4.11^{a}	2.22 ± 0.06^{a}	681.77 ± 1.48^{b}	3.28 ± 0.11^{b}

Samples: A, beginning of aging; B, after 1 month of aging; C, after 2 months of aging. RS, reducing sugar. Same letters in the column do not differ significantly at 5% probability by Tukey test.

with aging. Two possible explanations for protein changes were proposed: first, the moisture of *Daqu* after 2 months of aging was significantly lower than that at the beginning of aging ($P < 0.05$), which led to the numerical increase in protein content. Second, the enrichment of microbial communities was partially responsible for this increase. Esterifying activity and fermenting activity were found to increase during aging, particularly during the first month. The increased esterifying activity may be related to changes in *Weissella*, *Pediococcus*, and *Pichia* (1,16–18). After the first month of aging, the diversity of the above-mentioned bacteria increased and remained unchanged with further aging. The enhanced fermenting activity was mainly attributable to the enrichment of *Rhizopus oryzae* and *Pichia kudriavzevii*, which could convert starch into alcohol (18,19).

Volatile compounds In this study, 72 volatile compounds, including alcohols, esters, alkenes, nitrogen-containing compounds, aldehydes, alkanes, ketones, phenols, polyaromatic hydrocarbons (PAHs), benzene, furans, and pyrans were detected in *Daqu* (Table S1). Overall, the volatile substances were found to change during the aging of *Daqu*, which was associated with the changes in microbial communities and microbial metabolism over this period (1,20–22). In general, alcohols, esters, and nitrogen-containing compounds were slightly reduced in type and concentration. In contrast, olefinic compounds and PAHs increased. Moreover, aldehyde and alkyl compounds remained relatively stable. These trends may be caused by the combined effects of the volatilization of these compounds and the production and transformation of microorganisms during aging. In the process of aging, the moisture of *Daqu* continuously decreases, and *Daqu* is in contact with the external environment, resulting in a continuous change in the communities and metabolic activities of microorganisms. These gradual changes are expected to affect changes in contents of compounds with aging. Various flavor substances changed slightly but tended to be stable. These findings are similar to the results of Zhang et al. (23), with some differences; Zhang's study (23) showed that volatile compounds reduce slightly as aging increases, and then tend to stabilize. By comparing thresholds and the change in contents of various flavor substances, it can be found that aging may be a process of harmonization of *Daqu* flavor (Table S1) (4).

Alcohol compounds are important flavor substances. Alcohol content accounts for the highest proportion in all organic components of *Baijiu*. In this study, 16 alcohols were identified in *Daqu*, but only 4 out of 16 alcohols, 1-hexanol, 1-octen-3-ol, phenylethyl alcohol, and 1-nonanol, were detected in all samples. These compounds were formed mostly from sugars under aerobic conditions, by yeasts using amino acids under anaerobic conditions, or by yeasts through reduction of corresponding aldehydes (24,25). Esters often contribute rosy, honey, floral, and fruity odors and were identified previously in Chinese *Baijiu*; they may also be important contributors to the aroma of *Daqu* samples (25). Yeast and other microorganisms can synthesize esters during fermentation and aging (21). Table S1 shows that ethyl esters were the most abundant esters among 11 identified esters in *Daqu*, and these compounds were reported as major contributors to pleasant fruity, herbal, floral, rosy-, and honey-, pineapple-, apple-, and banana-like aroma in Chinese *Baijiu* (25).

Although alkene compounds are widely regarded as a vital odor component, information on alkene compounds in *Baijiu* is scarce. The present study identified seven alkene compounds in *Daqu*, and found that the concentrations of alkenes in sample B were 4.16-fold higher than those in sample A, suggesting that microbial metabolism is primarily responsible for the accumulation of alkenes in *Daqu*. It is noteworthy that only caryophyllene was found in all samples (Table S1). Caryophyllene has a mild clove aroma and gives a particular flowery smell to *Baijiu*. Studies have demonstrated that caryophyllene possesses anti-inflammatory, analgesic, and antibechic activities, eliminates phlegm, reduces anxiety and depression, and repels mosquitoes (26). Three pyrazines were identified; among them, tetramethyl-pyrazine was not only the most abundant, but also was the only pyrazine in all samples of *Daqu*. Tetramethyl-pyrazine is one of the most common pyrazines, and it is known to be responsible for the characteristic odor of oriental food (27). Eight aldehydes and four ketones, contributing honey and fruity odors, were identified in *Daqu* (28). Aldehydes and ketones are usually derived from lipid and amino acid degradation by yeasts (29). Formation of volatile compounds in *Daqu* results from numerous metabolic reactions and they are largely influenced by complex dynamics of microbial population during aging (1,22).

Overall prokaryotic communities structure and diversity In prokaryotic microbes, 80,824 valid sequences were obtained from all samples (Table S2). Rarefaction analysis indicated that all prokaryotic communities were well-represented, as rarefaction curves approached saturation plateau (Fig. S1A). A total of 67 OTUs that belonged to 35 genera and six phyla were obtained based on 3% dissimilarity in 16S rRNA sequences while considering that OTUs with $\geq$5 sequences were valid. Although the OTU number was similar to that in some reports, it was relatively lower than some other studies (7,14,30–32). Colony counting was added to further confirm the results. The microbe species were less diverse in the *Daqu* samples when compared with some reports, as verified by the above method (Fig. S2) (3). In fact, the OTU number was more than that in a few of the previous reports (1). Thus, the OTU number was different in different *Daqu* samples, which may be a result of the production process or environmental conditions (31). Among these OTUs, the relative abundances of 70% of OTUs were less than 1%. A total of 48 OTUs (71.6% of total OTUs) were shared by all samples (samples A–C, Fig. S3). In addition, ten (14.9%) unique phenotypes were observed in only one sample. Species richness and diversity were evaluated using Chao1, ACE, and Shannon and Simpson diversity indexes. The lowest diversity was observed in sample A (Table S2). Shannon's diversity index increased significantly at the onset of aging until after 1 month. Then, the index decreased slightly to 2.47 after 2 months (sample C). Richness estimators (Chao1 and ACE) were consistent with Shannon's diversity index. These data indicated that aging-time-related changes occurred at the OTU level. Based on the relative abundances of OTUs and hcluster_tree analysis, prokaryotic communities in three samples formed two clusters: (i) group I

was found in sample A, and (ii) group II was found in samples B and C (Fig. 1).

In this study, six bacterial phyla were identified in the samples: Firmicutes, Proteobacteria, Cyanobacteria, Actinobacteria, Bacteria_unclassified, and Chloroflexi. Among these, four phyla have sequences of more than 1% in the total prokaryotic sequences (Fig. 2). The predominant phylum was Firmicutes, accounting for 66.05% of total prokaryotic reads, which was in accordance with the previous reports (30). This phylum contributed to 19.3%, 89.3%, and 91.5% of prokaryotic sequence reads in samples A, B, and C, respectively. Proteobacteria was a subdominant phylum and contributed to 29.22% of the total prokaryotic sequences. The remaining phyla of Cyanobacteria (3.29%), Actinobacteria (1.01%), Bacteria_unclassified (0.42%), and Chloroflexi (0.01%), collectively made up 4.74% of the total prokaryotic sequences. Only phyla Firmicutes and Proteobacteria showed significant differences between samples A and B (or C). At the start of the aging step, *Daqu* was dominated by Proteobacteria, which accounts for 76.3%. As aging proceeded, from 1 month to 2 months, *Daqu* was dominated by Firmicutes, accounting for 89.3% and 91.5%, respectively (Fig. 2). The increase in Firmicutes was mainly driven by an increase in the abundance of *Lactobacillus*, corresponding to the increase acid concentration (Table 1). Summarizing the relevant research, it is found that Firmicutes always shows an increasing trend in the process of brewing *Baijiu*, and this change is more related to the increase in abundance of *Lactobacillus* (33).

At the genus level, in sample A, bacteria were dominated by *Pantoea* (70.7%), followed by other abundant OTUs (>1%) including *Lactobacillus*, *Weissella*, *Cyanobacteria*, *Erwinia*, *Staphylococcus*, and *Pseudomonas* (Figs. 1 and 2B). As aging proceeded, *Pantoea* was gradually reduced and accounted for 1.1% and 0.4% in 1 month and 2 month *Daqu*, respectively. In sample A, other dominant OTUs affiliated with *Erwinia* and *Pseudomonas* decreased to <0.1% of total

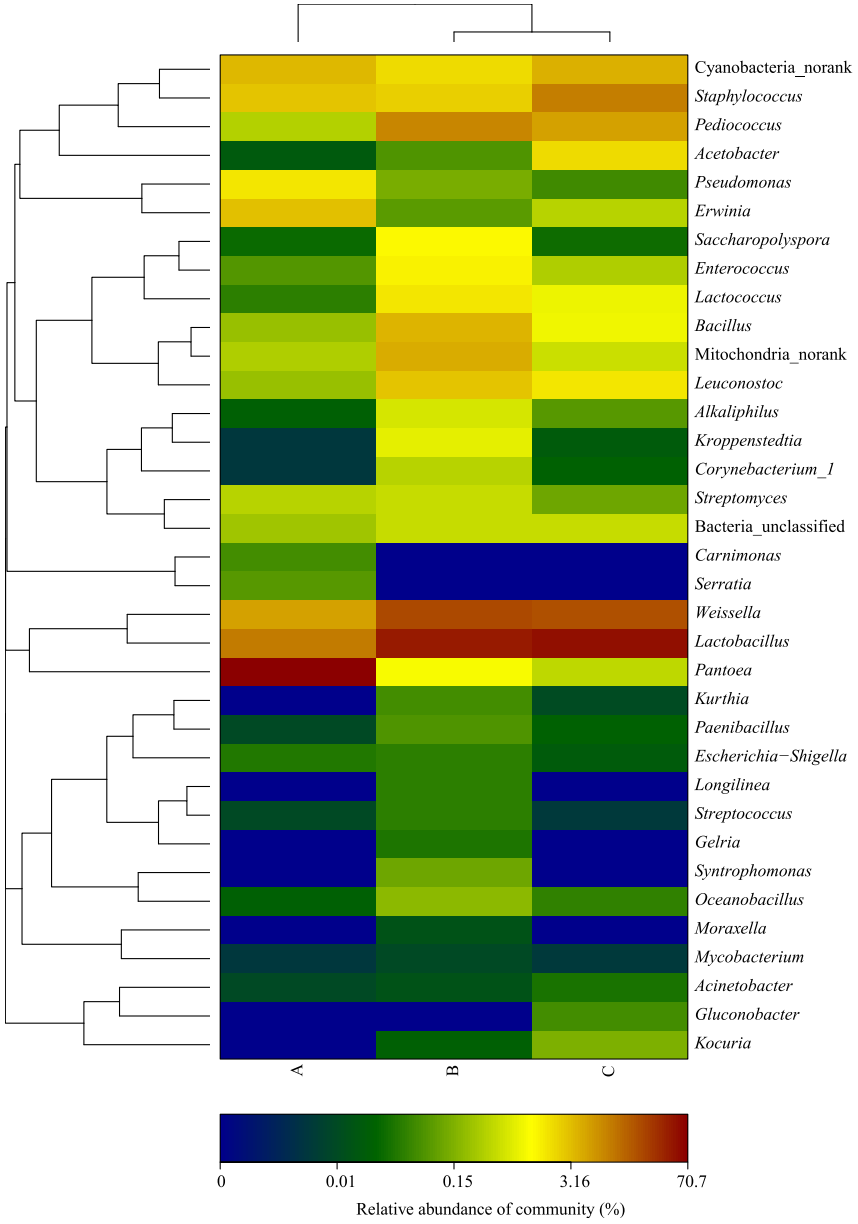

FIG. 1. Taxonomic classification of sequences from prokaryotic communities of three samples (sample A, beginning of aging; sample B, after 1 month of aging; sample C, after 2 months of aging) at genus level. The relative abundance was calculated by dividing the number of classified tags by the total tags number of each sample. All genera detected in light-flavored *Daqu* are shown. The relative abundance of each genus was indicated by color intensity in a heat map. The clustering analysis was performed using UPGMA, which is a type of hierarchical clustering method based on unweighted UniFrac distance metrics, and showed the relationship of the prokaryotic communities in the three samples.

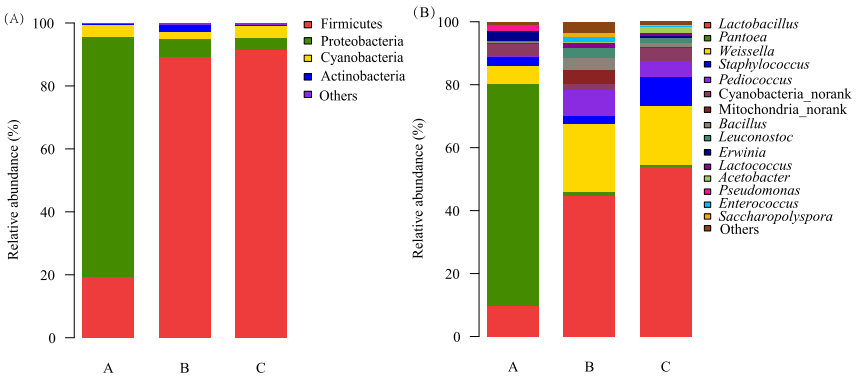

FIG. 2. Analysis of prokaryotic communities composition using high-throughput sequencing. Results of taxonomy at the phylum level (A) and genus level (B) are shown. The relative abundance defines sequence percentages in samples (sample A, beginning of aging; sample B, after 1 month of aging; sample C, after 2 months of aging) as depicted by the colors in the heat map. As there are only four phyla (A) and 15 genera (B) with a frequency greater than 1%, phyla and genera detected with less than 1% abundance in all samples are classified as others.

reads in samples B and C, respectively. By contrast, most abundant OTUs in sample B were mainly affiliated with *Lactobacillus*, *Weissella*, *Pediococcus*, *Alphaproteobacteria*, *Bacillus*, *Leuconostoc*, *Staphylococcus*, *Cyanobacteria*, *Lactococcus*, *Enterococcus*, *Saccharopolyspora*, and *Pantoea*; the main microbial species in sample C were *Lactobacillus*, *Weissella*, *Staphylococcus*, *Pediococcus*, *Cyanobacteria*, *Acetobacter*, and *Leuconostoc*. It is noteworthy that *Lactobacillus* and *Weissella* increased and accounted for 54.0% and 18.9%, respectively, in 2 month *Daqu*. Moreover, *Staphylococcus*, *Pediococcus*, Cyanobacteria_norank, *Acetobacter*, *Leuconostoc*, *Bacillus*, and *Lactococcus* accounted for relatively high proportions after 2 months of aging and were more abundant than at the beginning of aging. Some differences with the reports of Hu et al. (14) were noted, but these were attributed to different *Daqu* samples that had different production processes and environments.

Core microbiota comprised the dominant microbial genera. In this study, we identified ten genera in prokaryotic microbes, including *Lactobacillus*, *Weissella*, *Pantoea*, *Staphylococcus*, *Pediococcus*, Cyanobacteria_norank, *Acetobacter*, *Leuconostoc*, *Bacillus*, and *Lactococcus*, as core microbiota in mature *Daqu*. Core microbiota plays an important role in the maturation of *Daqu*. Five lactic acid bacteria (LAB) genera were identified in *Daqu*: *Lactobacillus*, *Weissella*, *Pediococcus*, *Leuconostoc*, and *Lactococcus*. LABs were one of dominant bacterial groups in *Daqu*, consistent with previous studies (13). LABs are known to play a significant and robust role in improving *Baijiu* taste; they not only produce lactic acid from glucose or starch, but also provide substrates for esterification by yeasts, and affect the microbial stability of final products through lactic acid production (34). Moreover, LABs can affect the growth of yeasts and mold; this influence significantly contributes to *Baijiu* quality. Similar to other fermentation processes, lactic acid produced by LABs inhibits the propagation of other spoilage bacteria (35). Tao et al. (36) reported that lactic acid and pH were most important factors influencing community structure. The pH decreased with aging. This phenomenon corresponded to the increased abundance of *Lactobacillus*, which produces lactic acid as a major end product in carbohydrate fermentation. Accumulation of lactic acid possibly lowered the pH. These findings and correlations are consistent with the finding that lactic acid accumulation decreased pit-mud pH (36). Decreased pH has been found to change the composition of microbial communities. Corona et al. (16) found that a 2x inoculum of *Weissellas* generated the highest percentage of esters. Matthews et al. (17) also reported the presence of esterase activity in *Pediococcus* isolates from wine.

Pantoea was the common and dominant bacterial genus found in *Daqu*, but its function in *Baijiu* production is still unknown (1). High-intensity *Staphylococcus* produces aromatic compounds, such as 3-methyl-1-butanol, diacetyl, 2-butanone, and acetoin, which may play important roles in *Baijiu* production (13). Cyanobacteria_norank were detected in our study; however, little information is available regarding its roles in fermented products. Cyanobacteria_norank metabolism may be related to the nitrogen cycle and produce organic nutrients (37). One type of acetic acid bacteria (AAB) species (*Acetobacter orientalis*) was detected in *Daqu*, and this bacterium ferments glucose to ethanol and transforms ethanol to acetic acid, which contributes positively to flavor through the production of esters, such as isoamyl acetate and 2-phenylethyl acetate (38). *Bacillus* species, which are important in *Daqu*, secrete various hydrolytic enzymes. Previous studies indicated that amylase, protease, lipase, cellulases, pectinases, glucanases, and other enzymes secreted by *Bacillus* convert starch and proteins into glucose and amino acids, thereby contributing to the development of volatile compound precursors or volatile compounds, such as pyrazines and aromatic and phenolic compounds, during fermentation (3). Furthermore, some metabolites from *Bacillus* species contain aromatic components, such as diacetyl, that facilitate subsequent reactions necessary for flavor production (38). The present study found additional microorganisms, *Alkaliphilus* oremlandii_OhILAs, *Syntrophomonas_wolfei*_subsp._*saponavida*, *Kocuria kristinae*, *Mycobacterium mucogenicum*, and *Moraxella osloensis*, which have not been identified in *Daqu* previously. This result should be followed up and possibly expanded in future studies.

Overall eukaryotic communities structure and diversity We recovered 97,702 high-quality 18S rDNA gene sequences (Table S2). As assessed by Good's coverage estimator, sampling completeness returned values above 99% in all cases. For all samples, the rarefaction curve and Shannon diversity curves leveled off strongly, suggesting that the majority of the eukaryotic communities for samples were captured in the current analysis (Fig. S1B). The diversity of eukaryotic microbes was lower than that of prokaryotic microbes. Based on 97% similarity, the obtained valid sequences were classified as 20 OTUs that belonged to 12 genera (Fig. 3). As for prokaryotic microbes, the OTU number of eukaryotes was also related to *Daqu* samples. Sample B showed a higher number of observed OTUs with ampler range than remaining samples; Chao1 and ACE indices also indicated higher eukaryotic microbial diversity in sample B. The eukaryotic communities in the three samples also formed two clusters, which were different from the prokaryotic communities; samples A and C were in one group, and sample B was in the other (Fig. 3).

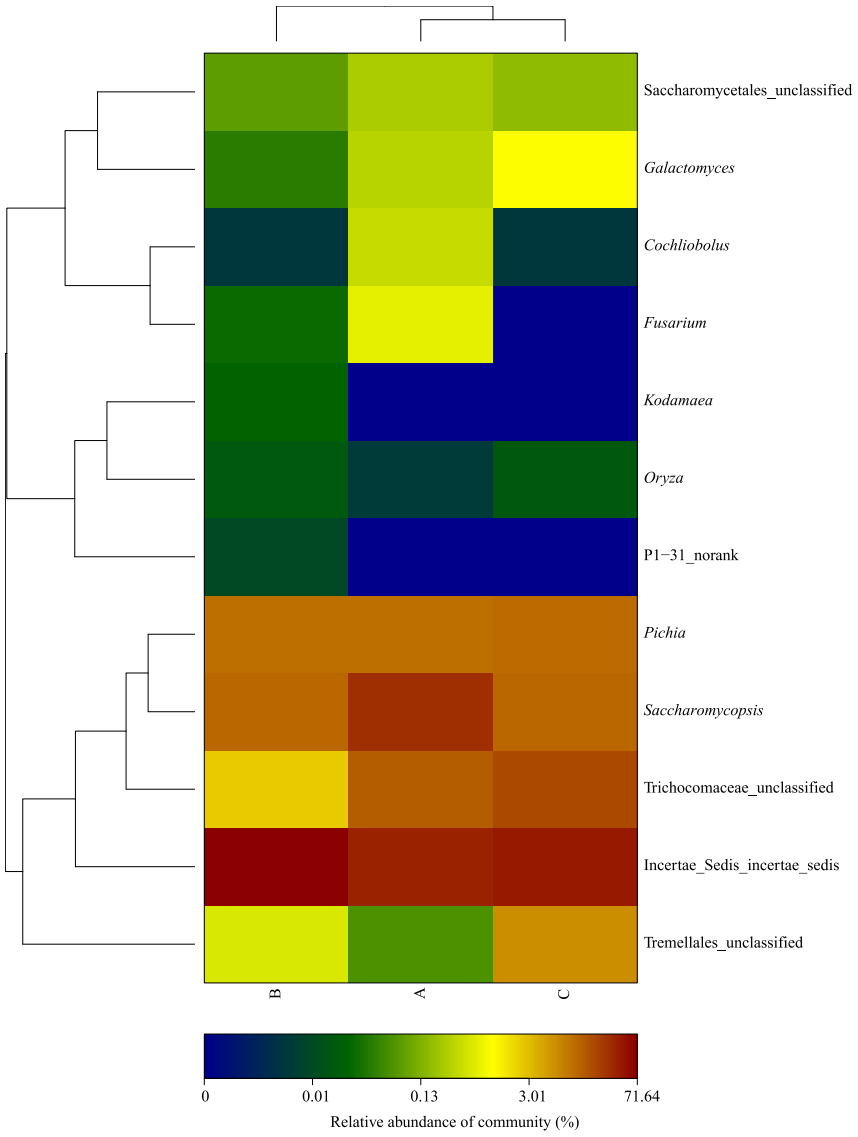

FIG. 3. Taxonomic classification of sequences from eukaryotic communities of three samples at genus level: sample A, beginning of aging; sample B, after 1 month of aging; sample C, after 2 months of aging. The relative abundance was calculated by dividing the number of classified tags by the total tags number of each sample. All genera detected in light-flavored *Daqu* are shown. The relative abundance of each genus is indicated by color intensity in a heat map. The clustering analysis was performed using UPGMA, which is a type of hierarchical clustering method based on unweighted UniFrac distance metrics, and shows the relationship of the eukaryotic communities in the three samples.

The microorganisms present in samples were from four phyla: Ascomycota, Zygomycota, Basidiomycota, and Phragmoplastopyta, which was consistent with other report (33). Ascomycota was the dominant phylum in samples A and C; Zygomycota was mainly observed in sample B (Fig. 4A). In other words, as aging proceeds, Ascomycota initially declined and later increased; Zygomycota showed the opposite trend (Fig. 4).

In this study, using 18S rDNA allowed eukaryotic identification at the genus level. Per abundance of reads, microbial population in three main categories were differentiated (Fig. 3). Incertae_Sedis_incertae_sedis, *Saccharomycopsis*, Trichocomaceae_unclassified, and *Pichia* made up the dominant genera group. In all samples, the relative abundance of at least one genera was between 2.56% and more than 70% of respective total reads. A second group contained sub-dominant genera corresponding to frequently encountered ones (0.01%–1% of total reads for each sample): Tremellales_unclassified, *Galactomyces*, and Saccharomycetales_unclassified. A third group consisted of rare sequences, which were detected occasionally (comprising 0.0001%–0.01% of total reads for each sample): *Fusarium*, *Cochliobolus*, *Kodamaea*, *Oryza*, and P1-31_norank. Four genera (Incertae_Sedis_incertae_sedis, *Saccharomycopsis*, Trichocomaceae_unclassified, and *Pichia*) constituted the largest group present in sample A. In addition to the above genera, Tremellales_unclassified was commonly present in sample B. Tremellales_unclassified and *Galactomyces* were identified in sample C (Fig. 3).

In sample B, percentages of Incertae_Sedis_incertae_sedis increased, although visible diversity was observed in sample A. However, this diversity reduced slightly after 2 months of aging (sample C). Considering the evolution of microbial communities, the number of Tremellales_unclassified reads significantly increased from sample A to C, and this number remained high until the end of the aging period. Furthermore, *Pichia* reads were constant from the beginning of aging. A reduction in reads was observed for *Saccharomycopsis* (Fig. 3).

Yeasts and molds are mainly responsible for saccharification and alcoholic fermentation to produce *Baijiu*. In this study, four non-Saccharomyces yeasts were detected: *P. kudriavzevii*, *Candida*

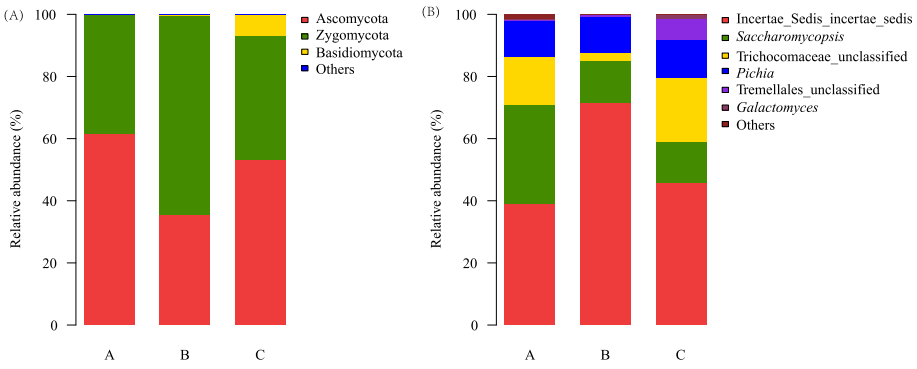

FIG. 4. Analysis of eukaryotic communities composition using high-throughput sequencing. Results of taxonomy at the phylum level (A) and genus level (B). The relative abundance defines sequence percentages in samples as depicted by colors in the heat map: sample A, beginning of aging; sample B, after 1 month of aging; sample C, after 2 months of aging. As there are only three phyla (A) and six genera (B) with a frequency greater than 1%, phyla and genera detected with less than 1% abundance in all samples are classified as others.

rugose, *Saccharomycopsis fibuligera*, and *Kodamaea ohmeri*. *P. kudriavzevii* is the most important fungi in *Daqu* production; in combination with LAB, this organism is associated with production of flavor and ethanol (18). *S. fibuligera*, which is the main producer of amylases, acid protease, and β-glucosidase, has strong saccharification capability and participates in converting starch into glucose during the initial stages of alcohol fermentation (20). In the present study, seven species of molds were obtained. *Absidia_idahoensis*_var._*thermophila* and *R. oryzae* were found to be dominant molds and this observation was inconsistent with previous results (13,39). It is likely that the molds imparted their characteristics to the *Daqu*. *Absidia* is a common species in some kinds of *Daqu*. This genus secretes hydrolyzing enzymes that decompose macromolecules and produce smaller metabolites, thereby affecting *Baijiu* flavor (13,39). During fermentation, *R. oryzae* is known to produce volatile compounds, such as ethanol, 2-methyl-1-butanol, and 3-methyl-1-butanol (19). *Rhizomucor pusillus* is a thermophilic fungus that is commonly present in cereals. When present in *Daqu*, it can affect *Baijiu* flavor by rapidly utilizing numerous carbon sources by inducing the synthesis of a variety of glucanases, phosphatases, acid proteinases, and alcohol dehydrogenases (40). *Galactomyces candidum* secretes proteases and lipases, which degrade proteins and lipids into precursors of various volatile compounds and may contribute to *Baijiu* flavor (41). In *Daqu*, few reports provided data on *Wallemia_sebi*, which is a halophilic fungal genus. The function of this fungus in fermentation starter cultures is still unknown and requires further investigation (13).

Relationships between volatile compounds and microbial communities Correlation analysis was used to evaluate the relationship between volatile compounds and microbial communities (Fig. 5). The analysis showed a significant correlation between various flavor substances and the microbial community structure through the clustering phenomenon (Fig. 5). Substances can be divided into three clusters: (i) flavor substance cluster A (V2, V3, V7, V9, V10, V11, V14, V19, V20, V22, V23, V28, V29, V37, V38, V40, V46, V49, V52, V56, V62, V64, and V68) and microbial communities *Erwinia*, *Pseudomonas*, *Pantoea*, *Serratia*, *Carnimonas*, *Fusarium*, *Saccharomycopsis*, *Cochliobolus*, and *Oryza*; (ii) flavor substance cluster B (V8, V16, V24, V35, V39, V41, V44, V53, V67, and V69) and microbial communities Mitochondria_norank, *Corynebacterium*_1, *Kroppenstedtia*, *Syntrophomonas*, *Gelria*, *Mycobacterium*, *Saccharopolyspora*, *Longilinea*, *Moraxella*, *Kodamaea*, and P1-31_norank; and (iii) flavor substance cluster C (V4, V13, V15, V30, V31, V34, V51, V54, V55, V60, V65, V70, V71, and V72) and microbial communities *Acetobacter*, *Gluconobacter*, and Tremellales_unclassified. Flavor substance cluster A mainly exists at the start of the aging process, and the corresponding microbial communities are mainly present at that time. Flavor substance cluster B and the corresponding microbial communities mainly existed in 1 month-old *Daqu*, and flavor substance cluster C and the corresponding microbial communities were mainly present in 2 month-old *Daqu*. The microbial activity is high during *Daqu* aging, as reflected in the metabolism and evolution of the microbial bacteria communities in the presence of flavor substances. The elevated microbial activity and changed flavor profile is the reason for aging *Daqu*. Similar to the pit used for *Baijiu* fermentation, the evolution of a bacterial communities is required to reach a balanced bacterial population to make good *Baijiu*. *Saccharomycopsis* was reported to contribute to the formation of flavor and taste in a previous study (42). *Acetobacter* can produce organic acids as final products with an obligate aerobic metabolism, and contributes positively to the flavor by yielding esters (31,43). *Gluconobacter* can produce acetic acid, which is the precursor of ethyl acetate (7). Moreover, correlations between *Lactobacillus* and caryophyllene, as well as between Saccharomycetales_unclassified and 1-octen-3-ol, are significant. However, to date, there is no report of *Lactobacillus* and Saccharomycetales_unclassified synthesizing or metabolizing the corresponding flavor substances. The correlation and possible metabolism require further verification and study. Ethyl caprylate is an important flavor substance in liquor and smells like brandy and pineapple. Moreover, ester synthesis has yet to be studied in *Daqu* aging. *Bacillus* has been found to utilize, metabolize and transform esters and a study of the mechanism of ethyl caprylate synthesis with *Bacillus* could be enlightening. Lipase from *Bacillus* is an efficient catalyst in the synthesis of ethyl caprylate, thus providing some insight into the mechanism of ethyl caprylate synthesis (44).

Starter cultures supply the microbial source and nutrients for *Baijiu* fermentation. In this study, traditional *Daqu* starter culture was characterized according to changes in physicochemical properties, flavor substances, and microbial communities as a function of aging time. The results demonstrated the importance of aging to transform and stabilize the natural microbial communities with volatile compounds originating from the raw materials to produce *Daqu*. Aging *Daqu* is a process whereby microbes interact and evolve to reach a steady-state balance. In addition, the aging process caused changes in physicochemical parameters and flavor substances, thereby forming special organoleptic properties in the mature *Daqu*. This study showed the importance of aging *Daqu*. Three parameters were used to comprehensively verify whether *Daqu* had reached the appropriate quality for use in *Baijiu* production. Moreover, further studies should be conducted on targeted microbes, physicochemical parameters, and flavor substances to scientifically measure when *Daqu* is mature (ready for use). The

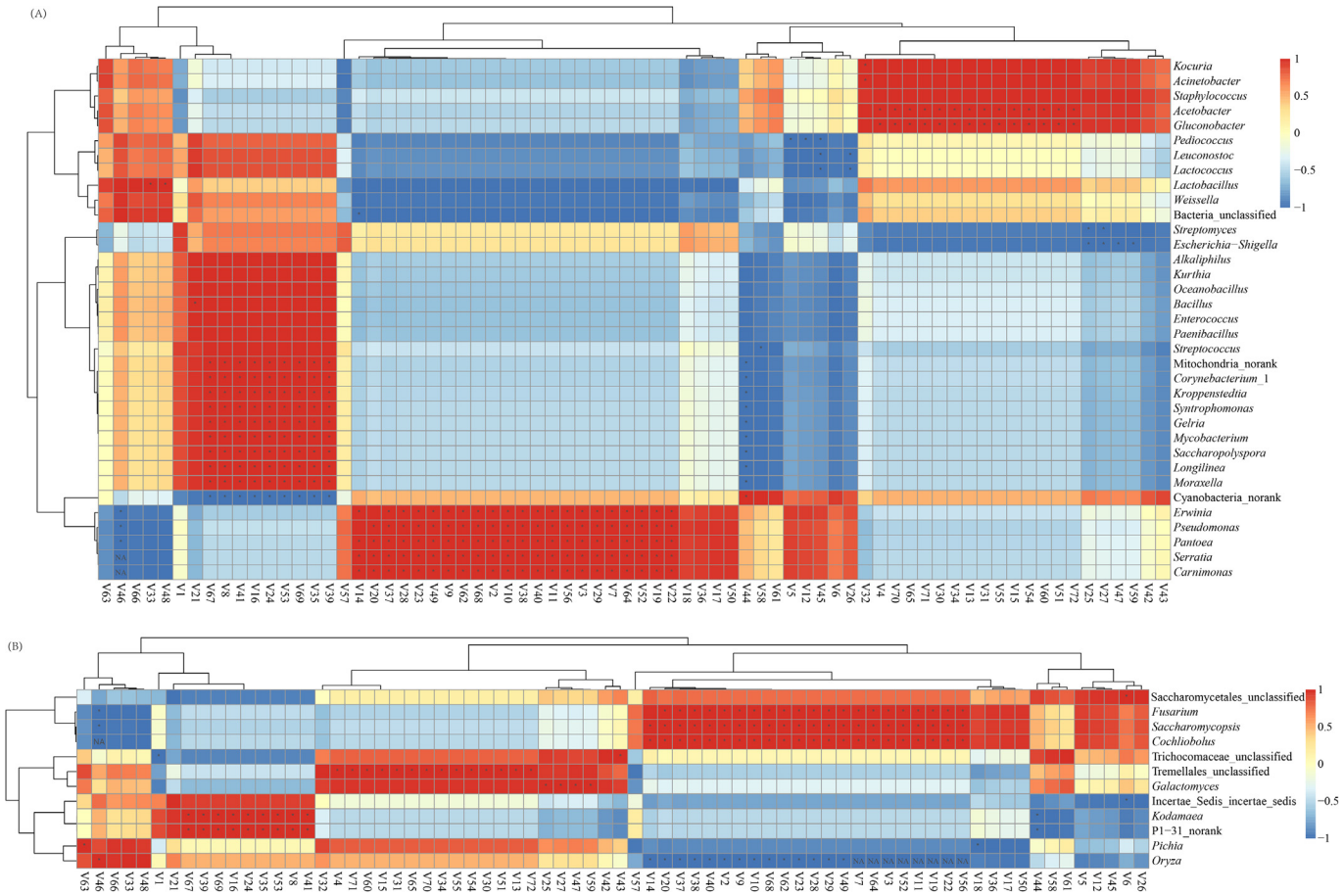

FIG. 5. Correlation between volatile compounds (V1−V72: different volatile compounds in Table S1) and prokaryotic communities (A) and eukaryotic communities (B) found in *Daqu*. Scale bar colors denote the nature of the correlation, with 1 indicating a perfectly positive correlation (red) and −1 indicating a perfectly negative correlation (blue) between volatile compounds and prokaryotic communities or eukaryotic communities. Asterisk shows significant correlations ($P < 0.05$), and NA shows no correlations.

ultimate goal is to produce a high-quality, consistent and reliable *Daqu* for use as a starter culture for a variety of products.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.08.005.

ACKNOWLEDGMENTS

We thank Prof. Sharon Shoemaker and Austin Schultz for insightful discussions and providing language help. This research was supported by the National Natural Science Foundation of China (No. 31671798 and 31701592), General Financial Grant from the China Postdoctoral Science Foundation (Grant No. 2016M590026) and the Foundation of Beijing Technology and Business University (No. LKJJ2017-10).

References

1. **Gou, M., Wang, H. Z., Yuan, H. W., Zhang, W. X., Tang, Y. Q., and Kida, K.:** Characterization of the microbial community in three types of fermentation starters used for Chinese liquor production, J. Inst. Brew., **121**, 620−627 (2015).
2. **Wu, X. H., Zheng, X. W., Han, B. Z., Vervoort, J., and Nout, M. J. R.:** Characterization of Chinese liquor starter, "Daqu", by flavor type with H-1 NMR-Based nontargeted analysis, J. Agric. Food Chem., **57**, 11354−11359 (2009).
3. **Zheng, X. W., Yan, Z., Han, B. Z., Zwietering, M. H., Samson, R. A., Boekhout, T., and Nout, M. J. R.:** Complex microbiota of a Chinese "Fen" liquor fermentation starter (Fen-*Daqu*), revealed by culture-dependent and culture-independent methods, Food Microbiol., **31**, 293−300 (2012).
4. **Liang, C., Du, H., and Xu, Y.:** The succession of procaryotic microbial community and the flavor components in the storage process of *Daqu*, Microbiol. Chin., **44**, 384−393 (2017) (in Chinese).
5. **Song, R. B., Shao, Z. L., and Song, J.:** Change in physiochemical indexes and microbial quantity of Nongxiang *Daqu* during the storage process, Liquor Mak. Sci. Technol., 59−61 (2017) (in Chinese).
6. **Zhang, L., Lu, H. M., Chen, L., Qiao, Y., He, X., and An, J. J.:** Study on changes in biochemistry of Maotai-flavor *Daqu* during storage, Mod. Food Sci. Technol., **33**, 1−7 (2017) (in Chinese).
7. **Wang, X. S., Du, H., and Xu, Y.:** Source tracking of prokaryotic communities in fermented grain of Chinese strong-flavor liquor, Int. J. Food Microbiol., **244**, 27−35 (2017).
8. **China Light Industry Press:** People's Republic of China professional standard. General methods of analysis for *Daqu* (QB/T 4257-2011), pp. 1−15. Ministry of Industry and Information Technology of the People's Republic of China (2011) (in Chinese).
9. **Nozawa, S., Hakoda, A., Sakaida, K., Suzuki, T., and Yasui, A.:** Method performance study of the determination of total nitrogen in soy sauce by the Kjeldahl method, Anal. Sci., **21**, 1129−1132 (2005).
10. **Miller, G. L.:** Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem., **31**, 426−428 (1959).
11. **Fan, G. S., Sun, B. G., Fu, Z. L., Xia, Y. Q., Huang, M. Q., Xu, C. Y., and Li, X. T.:** Analysis of physicochemical indices, volatile flavor components and microbial community of a light-flavor *Daqu*, J. Am. Soc. Brew. Chem., **76**, 209−218 (2018).
12. **Cates, V. E. and Meloan, C. E.:** Separation of sulfones by gas chromatography, J. Chromatogr., **11**, 472−478 (1963).
13. **Zhang, L. Q., Wu, C. D., Ding, X. F., Zheng, J., and Zhou, R. Q.:** Characterization of microbial communities in Chinese liquor fermentation starters *Daqu* using nested PCR-DGGE, World J. Microbiol. Biotechnol., **30**, 3055−3063 (2014).
14. **Hu, Y. L., Dun, Y. H., Li, S. A., Fu, B., Xiong, X. M., Peng, N., Liang, Y. X., and Zhao, S. M.:** Changes in microbial community during fermentation of high-temperature Daqu used in the production of Chinese "Baiyunbian" liquor, J. Inst. Brew., **123**, 594−599 (2017).
15. **Sengun, I. Y. and Karabiyikli, S.:** Importance of acetic acid bacteria in food industry, Food Control, **22**, 647−656 (2011).
16. **Corona, O., Alfonzo, A., Ventimiglia, G., Nasca, A., Francesca, N., Martorana, A., Moschetti, G., and Settanni, L.:** Industrial application of

selected lactic acid bacteria isolated from local semolinas for typical sourdough bread production, Food Microbiol., **59**, 43–56 (2016).
17. **Matthews, A., Grbin, P. R., and Jiranek, V.:** Biochemical characterisation of the esterase activities of wine lactic acid bacteria, Appl. Microbiol. Biotechnol., **77**, 329–337 (2007).
18. **Nout, M. J. R.:** Rich nutrition from the poorest - cereal fermentations in Africa and Asia, Food Microbiol., **26**, 685–692 (2009).
19. **Feng, X. M., Larsen, T. O., and Schnurer, J.:** Production of volatile compounds by *Rhizopus oligosporus* during soybean and barley tempeh fermentation, Int. J. Food Microbiol., **113**, 133–141 (2007).
20. **Chi, Z. M., Chi, Z., Liu, G. L., Wang, F., Ju, L., and Zhang, T.:** *Saccharomycopsis fibuligera* and its applications in biotechnology, Biotechnol. Adv., **27**, 423–431 (2009).
21. **Chen, S. and Xu, Y.:** The influence of yeast strains on the volatile flavour compounds of Chinese rice wine, J. Inst. Brew., **116**, 190–196 (2010).
22. **Ding, X. F., Wu, C. D., Huang, J., and Zhou, R. Q.:** Characterization of interphase volatile compounds in Chinese Luzhou-flavor liquor fermentation cellar analyzed by head space-solid phase micro extraction coupled with gas chromatography mass spectrometry (HS-SPME/GC/MS), LWT Food Sci. Technol., **66**, 124–133 (2016).
23. **Zhang, C. L., Ao, Z. H., Chui, W. Q., Shen, C. H., Tao, W. Y., Zhang, S. Y., and Wang, X. J.:** Changes in volatile compounds of Daqu during fermentation and storage by headspace solid phase microextraction combined with gas chromatography-mass spectrum, Food Ferment. Ind., **37**, 198–203 (2011) (in Chinese).
24. **Fan, W. L., Shen, H. Y., and Xu, Y.:** Quantification of volatile compounds in Chinese soy sauce aroma type liquor by stir bar sorptive extraction and gas chromatography-mass spectrometry, J. Sci. Food Agric., **91**, 1187–1198 (2011).
25. **Fan, W. L. and Qian, M. C.:** Characterization of aroma compounds of Chinese "Wuliangye" and "Jiannanchun" liquors by aroma extract dilution analysis, J. Agric. Food Chem., **54**, 2695–2704 (2006).
26. **Sabik, H., Fortin, J., and Martin, N.:** Identification of pyrazine derivatives in a typical maple syrup using headspace solid-phase microextraction with gas chromatography-mass spectrometry, Food Chem., **133**, 1006–1010 (2012).
27. **Zhu, B. F., Xu, Y., and Fan, W. L.:** High-yield fermentative preparation of tetramethylpyrazine by *Bacillus* sp. Using an endogenous precursor approach, J. Ind. Microbiol. Biotechnol., **37**, 179–186 (2010).
28. **Li, H. H., Liu, J. L., Liang, J. H., Gao, J. J., Sun, J. Y., Huang, M. Q., Zheng, F. P., and Sun, B. G.:** Research on volatile compounds in 2 kinds of Gujinggong liquor, J. Food Sci. Technol., **34**, 55–65 (2016) (in Chinese).
29. **Luo, T., Fan, W. L., and Xu, Y.:** Characterization of volatile and semi-volatile compounds in Chinese rice wines by headspace solid phase microextraction followed by gas chromatography-mass spectrometry, J. Inst. Brew., **114**, 172–179 (2008).
30. **Xiao, C., Lu, Z. M., Zhang, X. J., Wang, S. T., Ao, L., Shen, C. H., Shi, J. S., and Xu, Z. H.:** Bio-heat is a key environmental driver shaping the microbial community of medium-temperature Daqu, Appl. Environ. Microbiol., **83**, e01550-17 (2017).
31. **Wang, J., Zhong, Q. P., Yang, Y. Y., Li, H. R., Wang, L., Tong, Y. G., Fang, X., and Liao, Z. L.:** Comparison of bacterial diversity between two traditional starters and the round-koji-maker starter for traditional Cantonese Chi-flavor liquor brewing, Front. Microbiol., **9**, 1053 (2018).
32. **Wang, X. S., Du, H., Zhang, Y., and Xu, Y.:** Environmental microbiota drives microbial succession and metabolic profiles during Chinese liquor fermentation, Appl. Environ. Microbiol., **84**, AEM.02369-17 (2017).
33. **Zhang, Y. Y., Zhu, X. Y., Li, X. Z., Tao, Y., Jia, J., and He, X. H.:** The process-related dynamics of microbial community during a simulated fermentation of Chinese strong-flavored liquor, BMC Microbiol., **17**, 196 (2017).
34. **Yousif, N. M. K., Huch, M., Schuster, T., Cho, G., Dirar, H. A., Holzapfel, W. H., and Franz, C. M. A. P.:** Diversity of lactic acid bacteria from Hussuwa, a traditional African fermented sorghum food, Food Microbiol., **27**, 757–768 (2010).
35. **Lv, X. C., Weng, X., Zhang, W., Rao, P. F., and Ni, L.:** Microbial diversity of traditional fermentation starters for Hong Qu glutinous rice wine as determined by PCR-mediated DGGE, Food Control, **28**, 426–434 (2012).
36. **Tao, Y., Li, J. B., Rui, J. P., Xu, Z. C., Zhou, Y., Hu, X. H., Wang, X., Liu, M. H., Li, D. P., and Li, X. Z.:** Prokaryotic communities in pit mud from different-aged cellars used for the production of Chinese strong-flavored liquor, Appl. Environ. Microbiol., **80**, 2254–2260 (2014).
37. **Herrero, A., Muro-Pastor, A. M., and Flores, E.:** Nitrogen control in cyanobacteria, J. Bacteriol., **183**, 411–425 (2001).
38. **Wang, C. L., Shi, D. J., and Gong, G. L.:** Microorganisms in *Daqu*: a starter culture of Chinese Maotai-flavor liquor, World J. Microbiol. Biotechnol., **24**, 2183–2190 (2008).
39. **Wang, H. Y., Gao, Y. B., Fan, Q. W., and Xu, Y.:** Characterization and comparison of microbial community of different typical Chinese liquor *Daqus* by PCR-DGGE, Lett. Appl. Microbiol., **53**, 134–140 (2011).
40. **Li, P., Aflakpui, F. W. K., Yu, H., Luo, L. X., and Lin, W. T.:** Characterization of activity and microbial diversity of typical types of *Daqu* for traditional Chinese vinegar, Ann. Microbiol., **65**, 2019–2027 (2015).
41. **Sacristan, N., Gonzalez, L., Ma Castro, J., Ma Fresno, J., and Eugenia Tornadijo, M.:** Technological characterization of *Geotrichum candidum* strains isolated from a traditional Spanish goats' milk cheese, Food Microbiol., **30**, 260–266 (2012).
42. **Nie, Z. Q., Zheng, Y., Xie, S. K., Zhang, X. L., Song, J., Xia, M. L., and Wang, M.:** Unraveling the correlation between microbiota succession and metabolite changes in traditional Shanxi aged vinegar, Sci. Rep., **7**, 9240 (2017).
43. **Sun, W. N., Xiao, H. Z., Peng, Q., Zhang, Q. G., Li, X. X., and Han, Y.:** Analysis of bacterial diversity of Chinese Luzhou-flavor liquor brewed in different seasons by Illumina Miseq sequencing, Ann. Microbiol., **66**, 1293–1301 (2016).
44. **Ahmed, E. H., Raghavendra, T., and Madamwar, D.:** A thermostable alkaline lipase from a local isolate *Bacillus subtilis* EH 37: characterization, partial purification, and application in organic synthesis, Appl. Biochem. Biotechnol., **160**, 2102–2113 (2010).