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# Microbial Diversity in Daqu During Production of Luzhou-Flavored Liquor

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## ABSTRACT

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Yeast, bacterial, and mold strains in different stages during the daqu-based production of Luzhou-flavored liquor were isolated and identified by analyzing the 26S rRNA, 16S rRNA, and internal transcribed spacer region sequences, respectively. In total, 113 yeast, 63 bacteria, and 128 mold strains were identified, with the majority identified to the species level and the remaining strains identified to the genus level. In the final daqu product (day 90), the dominant bacteria, yeast, and mold strains were *Bacillus subtilis* subsp. *inaquosorum*, *Saccharomyces fibuligera*, and *Lichtheimia ramosa*, respectively. Thus, in the finished product, the daqu microflora mainly comprised bacteria and molds, mostly consisting of heat-resistant spore-forming *Bacillus* spp. and filamentous fungi. Corresponding changes in the enzyme activity patterns during daqu production revealed that *Mucor circinelloides* f. *circinelloides* was strongly correlated with protease, whereas saccharifying enzyme activity was mainly correlated with *Rhizopus oryzae*. A relatively strong correlation was found between saccharifying and liquefied enzyme activities and *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Bacillus tequilensis*, *Weissella cibaria*, and *Enterobacter mori*. These results indicate the benefits of molecular methods for detailed microbial isolation and identification and provide basic information for subsequent studies to reveal the functional microorganisms of daqu during the production of Luzhou-flavored liquor.

**Keywords:** Bacteria, Daqu, Enzyme, Fungi, Luzhou-flavored liquor, Microbial diversity

The traditional Chinese baijiu (liquor) has a long history spanning thousands of years. However, the traditional Chinese baijiu production technology has obvious gaps in knowledge compared with other brewing technologies, mainly embodied by the fact that the functional microorganisms are not clearly elucidated, and therefore the specific brewing mechanism remains unknown. Indeed, there has been no establishment of a modern brewing science theory or technology in China, and it has thus far been difficult to update the operation mode from the status quo. In addition, the Chinese baijiu industry has a low degree of industrialization and modernization, which causes difficulty in quality control. The unique brewing process characteristics and complexity have further contributed to the difficulty in the transformation and upgrading of the industry (15).

\*The e-Xtra logo stands for “electronic extra” and indicates that Figure 3 appears in color online.

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Luzhou-flavored liquor (also known as strong-flavored liquor) is one of the three basic flavor types (Maotai, Luzhou, and Fen) of traditional Chinese baijiu brands, including Luzhou laojiao daqu liquor, Wuliangye liquor, Jiannanchun liquor, Quanxing daqu liquor, Tuopai liquor, Gujinggong liquor, Yanghe daqu liquor, Shuanggou daqu liquor, and Songheliangye. The sales of Luzhou-flavored liquor account for over 50% of all traditional Chinese liquors available in the market. Luzhou-flavored liquor uses grains and daqu as the raw materials, and it is produced by solid-state natural fermentation (2–3 months) and solid-state distillation under atmospheric conditions (22).

Daqu plays an important role during the production of traditional Chinese baijiu, similar to koji for Japanese sake production. According to the highest temperature used in the daqu production cycle, daqu can be divided into three main types in China: high-temperature daqu, medium-temperature daqu, and low-temperature daqu. In high-temperature daqu, the maximum temperature during production is 60–65°C, and it requires 60 days with 3–6 months storage, accounting for 50% of the raw material in a 10 month brewing cycle of Maotai-flavored liquor. For medium-temperature daqu, the highest temperature during production is 55–60°C, requiring approximately 30 days with 2–3 months storage, accounting for 20% of the raw material in a 2–3-month brewing cycle of Luzhou-flavored liquor. The low-temperature daqu is produced with a maximum temperature of 45–48°C, requiring 28 days with 1–2 months storage, accounting for 10% of Fen-flavored liquor with a fermentation period of 1–2 months. To brew Luzhou-flavored liquor with daqu, wheat is commonly used as the raw material (as well as barley, peas, sorghum, etc.) for daqu production. As shown in Figure 1, the daqu-making process includes embellishment of the material, crushing, adjusting the water absorption to 40%, natural inoculation or addition of old daqu, mechanical or manual molding (brick shape, 34 × 21 × 6.5 cm), raising the temperature by the accumulation of bricks in a room for one week and then maintaining the temperature at 55–60°C by turning over the bricks, or covering with straw or a sprinkler for one week, followed by drying for two weeks, and finally storing for 2–3 months until use. After crushing, the daqu and steamed sorghum and rice husk are mixed at a certain proportion and then fermented for 2–3 months under solid-state conditions in a mud pit, and the liquor is finally collected by solid-state distillation under atmospheric conditions (14,22).

To date, there have been numerous studies conducted to determine the functions of the various microbes in daqu and to reveal the brewing mechanism of Chinese traditional liquor (16). In contrast to koji (used in sake production), daqu is generally considered to comprise enriched wild microbial populations (mold, bacteria, and yeast) and therefore provides suitable microorganisms

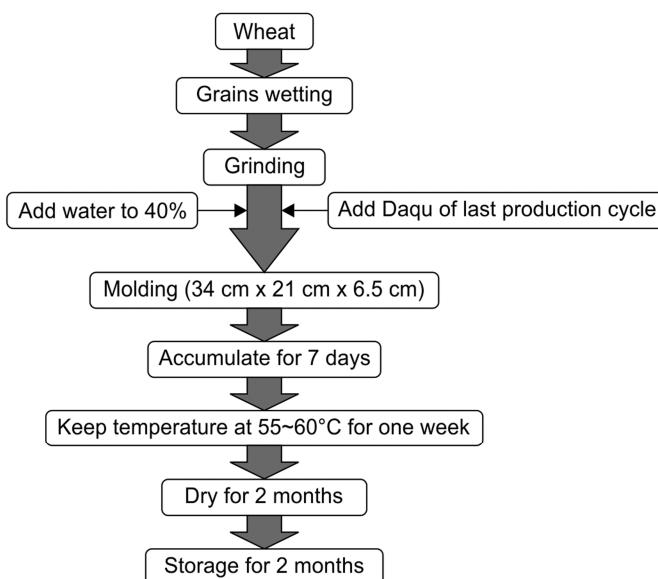
and microbial enzymes as a saccharifying and fermentation starter, such as saccharifying enzyme, liquefied starch enzyme protease required for grain fermentation, and the flavor precursor materials (19). Although the microbial populations change over the different periods of the daqu-making process, there are few reports on the correlations between the activities of important enzymes such as saccharifying enzyme, liquefying enzyme, and acid protease and the culturable microorganisms during the daqu-making process for Luzhou-flavored liquor production.

Therefore, in this study, we explored the changes in the microbial population and patterns of microbial composition at different stages during the medium-temperature daqu production process. Specifically, the 26S rRNA gene D1/D2 domain in yeast, 16S rRNA gene in bacteria, and internal transcribed spacer (ITS) region sequences in molds isolated at different stages during the daqu production process were examined, and a sequence homology search (BLAST) was performed against the GenBank database to compare the homology of tested strains with known strains. Furthermore, the corresponding changes in the activities of the liquefying enzyme, saccharifying enzyme, and protease in daqu at different production stages of Luzhou-flavored liquor, as well as the correlations between the enzyme activities and microorganism compositions, were analyzed to provide basic information regarding the functional microbes present in daqu during the production of Luzhou-flavored liquor.

## EXPERIMENTAL

### Samples

Daqu samples were respectively collected from Luzhou Laojiao Daqu Production Ecological Park of Luzhou Laojiao Co. Ltd., Luzhou, China. Sampling was carried out on days 5, 7, 10, 25, and 90 for the same sample throughout the production process. These time points were chosen based on the typical temperature profile during the daqu-making cycle of Luzhou-flavored liquor (Fig. 2). Specifically, the temperature of daqu increases rapidly from day 0 to day 5; the highest temperature is observed at day 7 and is maintained at this peak for about one week, and then it decreases to room temperature. The collected samples were stored at  $-4^{\circ}\text{C}$  and used as soon as possible.



**Fig. 1.** Flow diagram for the daqu production process of Luzhou-flavored liquor.

### Isolation of Yeast Strains

Yeast strains were isolated according to previously published methods (7). Each daqu sample collected on the same day was crushed and evenly mixed, and 50 g of crushed daqu was added to 450 mL of sterile water and evenly mixed. Serial dilutions of  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  were prepared by pipetting 1 mL of daqu suspension into a test tube containing 9 mL of sterile water. Yeasts were cultured and isolated on agar plates with yeast/peptone/dextrose medium (10 g of yeast powder, 20 g/L of dextrose, 20 g/L of peptone, and 20 g/L of agar) and potato/dextrose/agar (PDA) medium (200 g/L of potato, 20 g/L of dextrose, and 20 g/L of agar). The strains were cultured at room temperature for 12 h.

### Isolation of Mold Strains

Mold strains were isolated according to the same isolation method described above for yeast strains, except that the culture media were PDA and Rose Bengal agar base (20 g/L of dextrose, 20 g/L of peptone, 1 g/L of potassium dihydrogen phosphate, 0.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 mL/L of Rose Bengal stain, 1.0 mL/L of Pichloran, and 15 g/L of agar). The strains were cultured for 24 h at  $30^{\circ}\text{C}$  for normal strains and at  $50^{\circ}\text{C}$  for high-temperature-resistant strains.

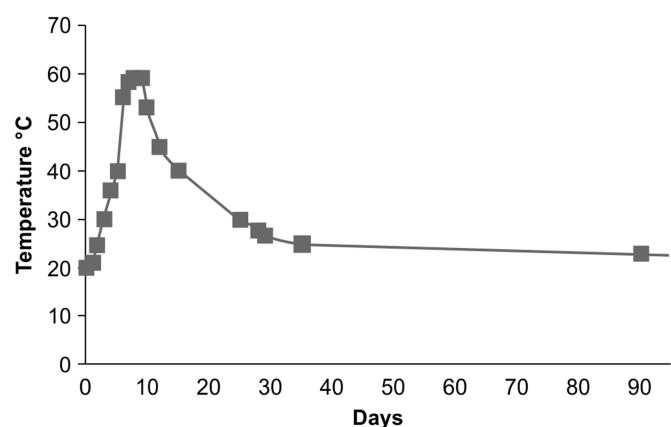
### Isolation of Bacterial Strains

Each sample was divided into two aliquots for separation: one aliquot was incubated in a water bath at  $80^{\circ}\text{C}$  for 10 min for the isolation of heat-resistant *Bacillus*, and the other was not incubated for the isolation of other bacterial strains. Bacterial strains were isolated following the same isolation method as described for yeast strains, except that the culture media were nutrient broth (10 g/L of peptone, 3 g/L of beef extract, 5 g/L of NaCl, and 15 g/L of agar) and Luria-Bertani broth (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, and 15 g/L of agar, pH 7.0). The strains were cultured at  $30^{\circ}\text{C}$  for 24–72 h.

### DNA Extraction

DNA was extracted using quartz sand-based cell wall disruption (24) and heating extraction (2). In brief, each colony with a 1 mm diameter was directly streaked, suspended in 5  $\mu\text{L}$  of water, incubated at  $95^{\circ}\text{C}$  for 5 min, and amplified by polymerase chain reaction (PCR). This extraction method was mainly used for partial yeast strains and bacterial strains.

The sequences of the 26S rRNA D1/D2 gene region in yeast strains were amplified using the primers NL1 and NL4 (6). The near-complete 16S rRNA gene from individual bacterial strains was amplified by PCR with the universal primers 27F (5'-AGAG



**Fig. 2.** Typical temperature profile during the daqu production cycle of Luzhou-flavored liquor.

TTTGATCCTGGCTCAG-3') and 1492R (5'-AAGGAGGTGATC CAG-3'). The ITS sequences from individual mold strains were amplified by PCR with the universal primers ITS4 (5'-TCCTCC GCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAGTCGT AACAGG-3'). Taq PCR Master Mix and the primers NL1, NL4, ITS4, ITS5, 27F, and 1492R were supplied by Biomed Biotech (Beijing, China). The PCR system (30  $\mu$ L) for amplification of the 26S rRNA gene, 16S rRNA gene, and ITS sequences included 15.0  $\mu$ L of 2 $\times$ Taq PCR Master Mix, followed by the addition of 1.0  $\mu$ L of 10  $\mu$ M forward primer, 1.0  $\mu$ L of 10  $\mu$ M reverse primer, template DNA, and finally filled up to 30  $\mu$ L with double distilled H<sub>2</sub>O. The PCR system (GT9612) was purchased from Bio-Gener Technology (Hangzhou, China), and amplified PCR products were sequenced by Biomed Biotech. Sequencing results were obtained by searching the GenBank database, and sequence similarity was compared using the BLAST online tool. The centrifuge (CF15R) was supplied by Hitachi (Tokyo, Japan), the electrophoresis system (JK600C) was purchased from JUNYI Electrophoresis Co. (Beijing, China), and the Alpha Innotech gel imaging system was supplied by Alpha (Shanxi, China).

#### Determination of the Activity of Liquefying Enzyme

To prepare the test enzyme solution, 1 g of daqu powder was dissolved in 0.02M pH 6.0 hydrogen phosphate salt/citric acid buffer in a small beaker, and then placed in a volumetric flask and diluted with the buffer solution. The solution was filtered with four layers of cotton gauze, and the filtrate was collected for determination of enzyme activity. A mixture of 2 mL of 2% soluble starch solution and 5 mL of buffer solution was placed in a 50 mL triangle bottle and set in a water bath at 60°C for 4–5 min; 0.5 mL of liquid enzyme was added, the time was immediately recorded, and the mixture was shaken vigorously. Approximately 0.25 mL of the reaction liquid was diluted with iodine drops (0.75 mL) in a color-mixing cavity, and the time (min) was recorded at the point at which the color changed from purple to red brown, matching the standard hue, to indicate the reaction end point. The enzyme activity was calculated according to the following formula: Activity unit =  $60/T \times 2\% \times n \times 1/0.5 \times 1/m$ , where  $n$  is the dilution ratio, 60 refers to a 60 min reference period, 0.5 is the amount of enzyme fluid tested (mL),  $2\% \times n$  is the amount of soluble starch (g),  $T$  is the response time (min), and  $m$  is the sampling weight (g).

#### Determination of Saccharifying Enzyme Activity

To prepare the enzyme solution, 1 g of daqu powder was weighed to an accuracy of 0.0002 g, dissolved in a small amount of acetic acid buffer, and stirred with a glass rod, and the supernatant fluid was carefully transferred to a volumetric flask. The solution was filtered by four layers of cotton gauze, and the filtrate was used for determination of enzyme activity. To two 50 mL colorimetric tubes (tube A and tube B), 25 mL of soluble starch and 5 mL of buffer were respectively added, shaken well, and preheated for 5 min in a 40°C constant-temperature water bath. Enzyme liquid (2 mL) was added to tube A (the sample tube), immediately shaken well, and the response was monitored for 30 min. Immediately following this step, 0.2 mL of sodium hydroxide solution was added, shaken well, and the two tubes were removed from the water bath to cool rapidly; 2 mL of the test enzyme fluid was added to tube B (the blank). Five milliliters of the reaction liquid or liquid blank was respectively pipetted and placed in an iodine flask, to which 10 mL of iodine solution and 15 mL of sodium hydroxide solution were added. The flasks were shaken well and plugged, and they were left to react for 15 min in the dark. Subsequently, 2 mL of sulfuric acid solution was added with sodium thiosulfate standard solution titration, until the blue color disappeared, indicating the reaction end point. Enzyme ac-

tivity was calculated according to the following formula:  $X = (A - B) \times c \times 90.05 \times 32.2/5 \times 1/2 \times n \times 2 = 579.9 \times (A - B) c \times n$ , where  $X$  is the enzymatic activity of the sample (g),  $A$  is the consumption volume of the sodium thiosulfate solution of the blank (mL),  $B$  is the consumption volume of the sodium thiosulfate solution of the sample (mL),  $c$  is the concentration of the sodium thiosulfate solution (mol/L), and  $n$  is the dilution ratio.

#### Determination of Protease Activity

To prepare the enzyme solution for the protease activity measurements, 1 g of daqu powder was weighed (accurate to 0.0002 g), dissolved in a small amount of acetic acid buffer, stirred with a glass rod, and the supernatant fluid was carefully placed in a volumetric flask. Casein solution was added to the flask and preheated for 5 min in a 40°C constant temperature water bath. Enzyme liquid (1 mL) was respectively placed in four tubes: one was the blank tube, to which 2 mL of trichloroacetic acid was added, and the other three were sample tubes containing 1 mL of casein. The tubes were shaken well and maintained at 40°C for 10 min. The tubes were removed, and 2 mL of trichloroacetic acid was added to the sample tubes, 1 mL of casein was added to the blank tube, and after 10 min, the precipitate was filtered. To 1 mL of filtrate, 5 mL of 0.4M Na<sub>2</sub>CO<sub>3</sub> and 1 mL of Folin reagent were added. After reacting for 20 min at 40°C, the optical density value was obtained at 680 nm; the blank tube was set to zero for calibration. Enzyme activity was calculated by the following formula:  $X = A \times K \times 4/10 \times n$ , where  $X$  is the enzymatic activity of the sample (g),  $A$  is the average absorbance of the parallel test samples,  $K$  is the constant suction light, and  $n$  is the dilution ratio.

The determination of the enzymatic activities of daqu at different stages was conducted essentially following the methods described by Shen (14). The correlations between bacteria, molds, and the activities of saccharifying enzyme, liquefied enzyme, and protease in daqu of Luzhou-flavored liquor were analyzed using constrained correspondence analysis with the R vegan software package (11).

## RESULTS

#### Isolation of Strains

Yeast strains in daqu of different stages were first determined using the plate counting method. As shown in Figure 3A, the yeast strains of daqu at the peak-temperature period (day 7) were dominated by *Wickerhamomyces anomalus*, followed by *Candida metapsilosis*, and their counts gradually decreased after day 7. *Saccharomyces cerevisiae* showed an evident increase on day 10 and was the dominant yeast strain in the finished daqu product (after 90 days).

Figure 3B shows that there were remarkable increases in the bacteria with respect to both overall counts and species numbers between days 5 and 7, and large numbers of bacteria were dead between days 7 and 10, except for *Bacillus* spp., during which the temperature of the daqu also reached its peak value of the cultivation period. On day 90, *Bacillus subtilis* subsp. *inaquosorum* became the primary dominant bacterial strain in daqu.

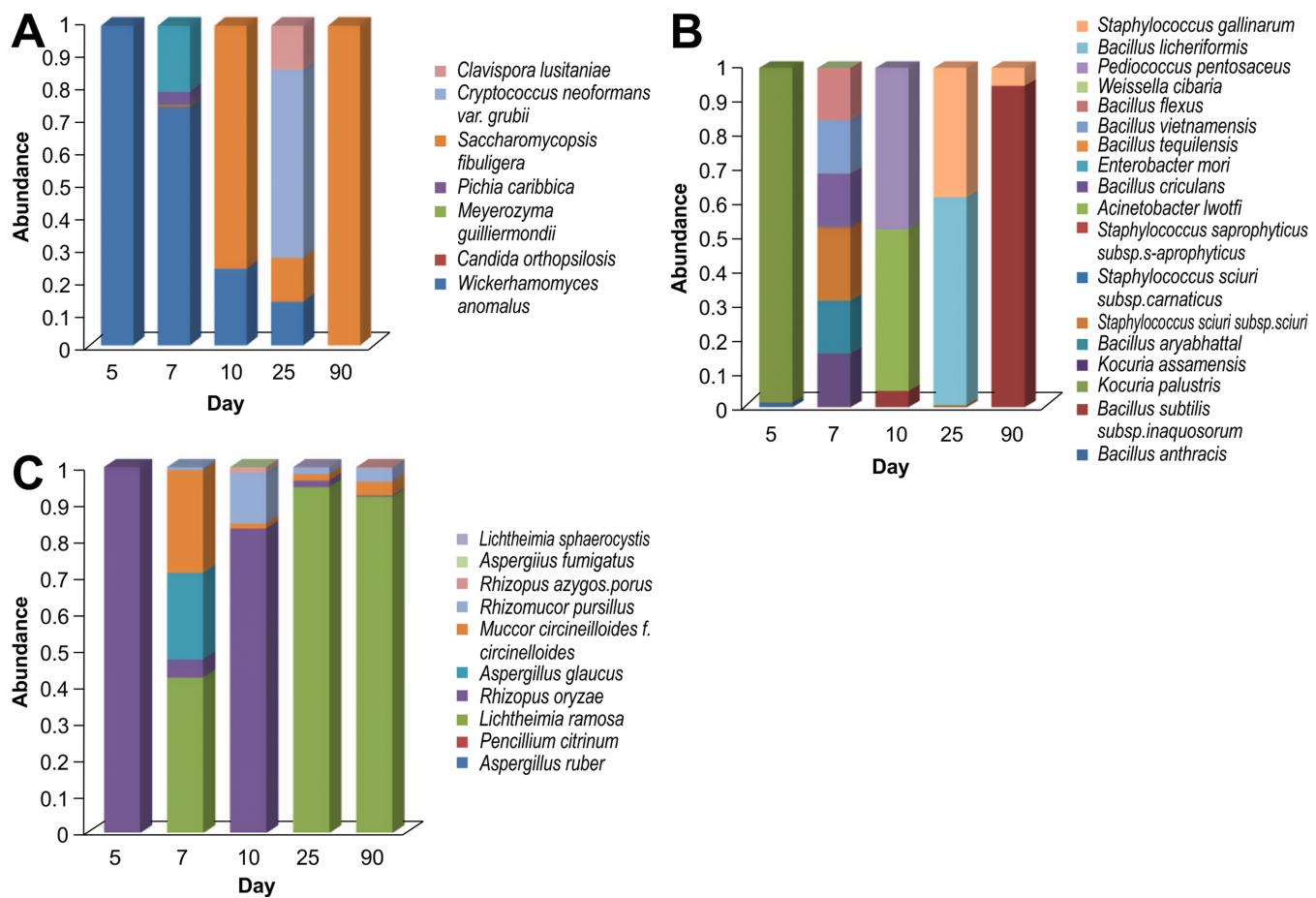
Figure 3C shows that large numbers of molds were present in daqu on day 5, with *Rhizopus oryzae* as the dominant mold strain. Subsequently, the mold count showed an initial reduction, followed by a slight increase as the temperature of daqu increased, and *Lichtheimia ramosa* was the dominant mold strain in the finished daqu product (day 90).

#### Amplification and Alignment of the Microbial Sequences

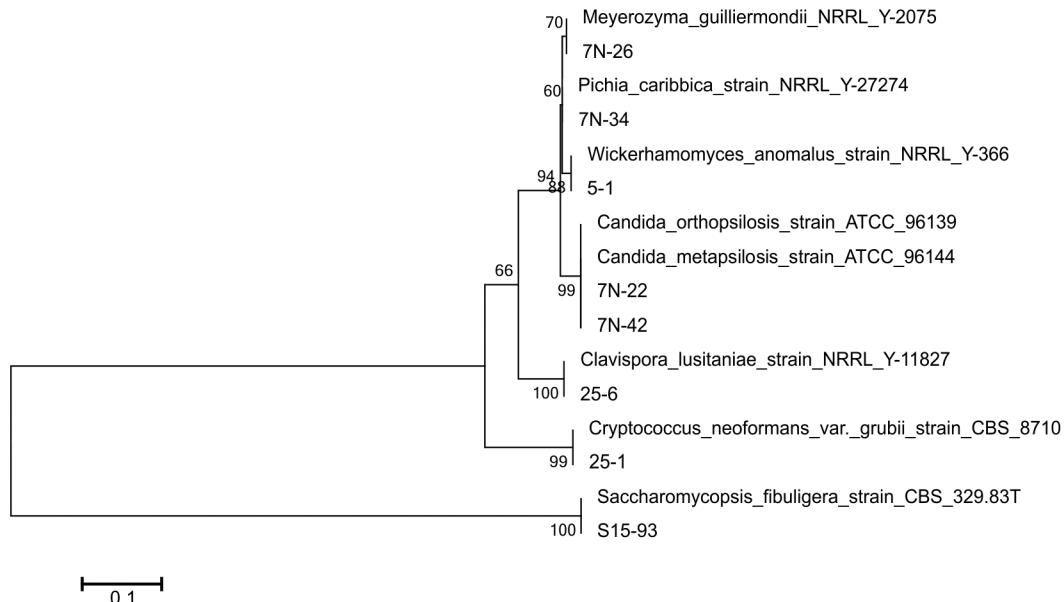
The 26S rRNA gene D1/D2 sequences of 113 isolated yeast strains, 16S rRNA gene sequences of 63 isolated bacterial strains, and ITS sequences of 128 isolated mold strains were searched for

sequence similarity in the GenBank database using BLAST. Strains with >99% similarity were defined as belonging to the same species, and strains with >95–99% similarity were defined as belonging to the same genus. The results showed that 112 of 113 yeast strains could be identified to the species level, belong-

ing to six genera, including *W. anomalus*, *S. fibuligera*, *Meyerozyma guilliermondii*, *Candida orthopsis*, *Pichia caribbica*, and *Cryptococcus neoformans* var. *grubii*. The remaining strain, 25-6, had the highest degree of homology with the type strain *Clavispora lusitaniae*, but the similarity was only 96%, indicating that



**Fig. 3.** Distributions of yeast (A), bacteria (B), and mold (C) strains in daqu at different stages of production.



**Fig. 4.** Phylogenetic tree of isolated yeasts and type strains.

this could be a new species in the genus that needs further identification. Among the 63 bacterial strains, 32 could be identified to the genus level, including *Bacillus*, *Kocuria*, *Staphylococcus*, and *Acinetobacter*, and the remaining 31 strains could be identified to the species level, comprising six genera, including *B. subtilis* subsp. *inaquosorum*, *Kocuria palustris*, *Bacillus aryabhattai*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Enterobacter mori*, *Weissella cibaria*, *Pediococcus pentosaceus*, *Bacillus licheniformis*, and *Staphylococcus gallinarum*. Of the 128 mold strains, 34 could be identified to the genus level (*Lichtheimia*), and 94 strains could be identified to the species level belonging to six genera, including *Aspergillus ruber*, *Penicillium citrinum*, *R. oryzae*, *Aspergillus glaucus*, *Rhizomucor pusillus*, *Mucor circinelloides* f. *circinelloides*, *Rhizopus azygiosporus*, *Aspergillus fumigates*, and *Lichtheimia sphaerocystis*.

#### Sequence Analysis and Phylogenetic Tree Construction for the Isolated Microbial Strains

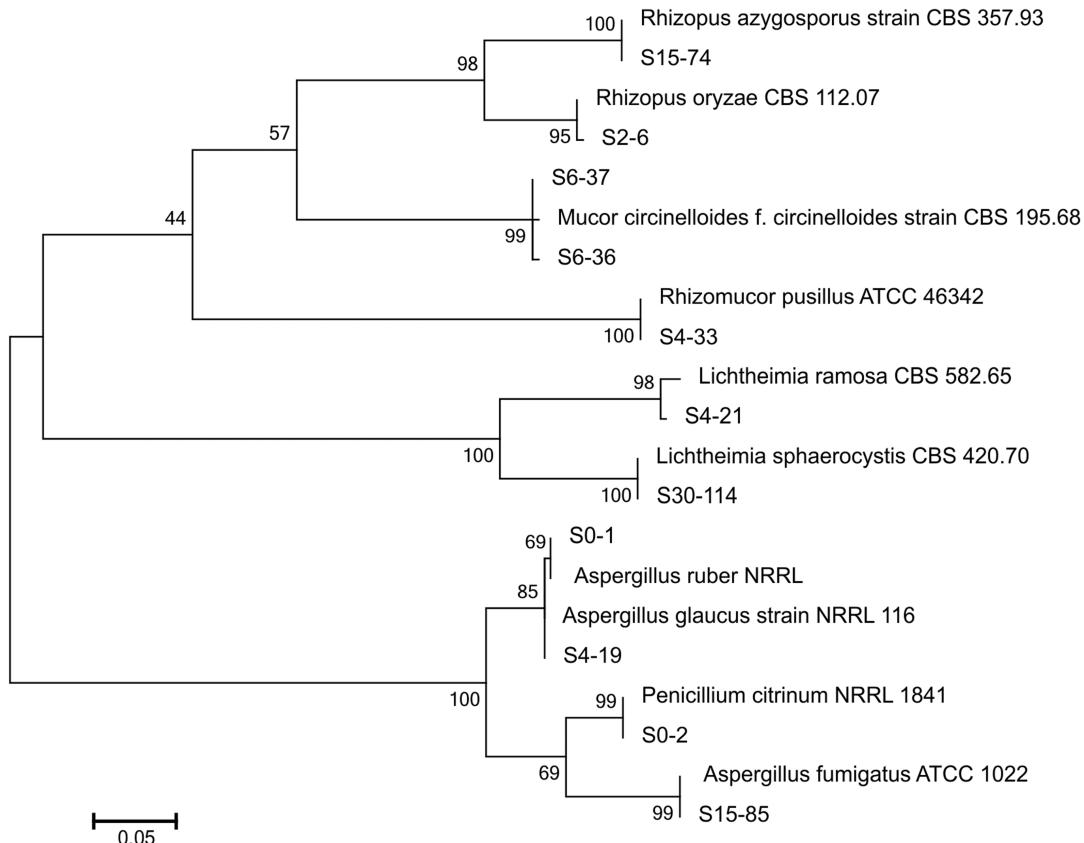
Phylogenetic trees were constructed for different kinds of yeasts, molds, and bacteria found in daqu and similar type strains (Figs. 4–6), and microbial strains showing the highest similarity with the isolated strains were selected as type strains. Because several microbes could not be identified to the species level, the confidence level of the classification in the phylogenetic trees was not high. Microbial strains with a similarity below 95% are most likely new species not listed in GenBank and should thus be further identified. These trees demonstrate a clear genetic relationship between different strains, and the diversity of bacteria and molds in daqu was higher than that of yeasts. The strains of the genus *Bacillus* were dominant among bacteria. Mold strains were mainly dominated by *Rhizopus* and *Aspergillus*. All yeast strains, except for *S. fibuligera*, were closely related.

#### Population Succession of Microorganisms During the Daqu-Making Cycle of Luzhou-Flavored Liquor

Figure 7 shows the population succession of yeasts, molds, and bacteria during the daqu-making cycle of Luzhou-flavored liquor. As shown in Figure 4, the microorganisms were mainly constituted by molds with a small amount of bacteria at day 5. However, at day 7, the majority of the daqu microbes were bacteria, followed by molds, and then yeast. At the peak-temperature period (day 10), the main microbes in the daqu were yeasts, followed by molds, with a small amount of bacteria. At the end of the daqu-making cycle, the microbes in daqu were mainly yeasts, followed by molds. As shown in Figure 7, the proportion of molds gradually reduced until the highest temperature was reached and then gradually increased until just before use (day 90). The proportions of yeasts and bacteria were relatively lower at the early stage; in particular, the proportion of yeasts increased to reach the maximum at the peak-temperature state and then reduced gradually until day 90, at which point it represented the lowest proportion among the three kinds of microbes. The proportion of bacteria was lowest at the peak-temperature stage, but then it increased along with the drop in daqu temperature. In the final product of daqu (day 90), the microbes mainly consisted of bacteria and molds.

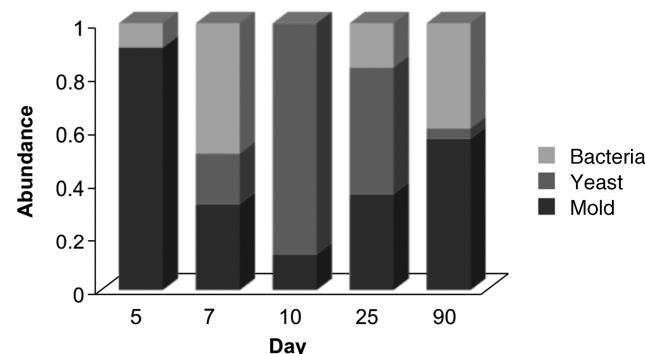
#### Activities of Liquefying Enzyme, Saccharifying Enzyme, and Protease in Daqu at Different Stages

Figure 8 shows that the activity of the liquefying enzyme in daqu was maintained at 0.9–1.1 U/g with little variation across the different stages of production. The activity of the saccharifying enzyme was high at days 7 and 25 and slightly decreased at days 10 and 90. The activity of the protease exhibited an initial increase followed by a slight decrease, and the peak value appeared to emerge between days 25 and 90.

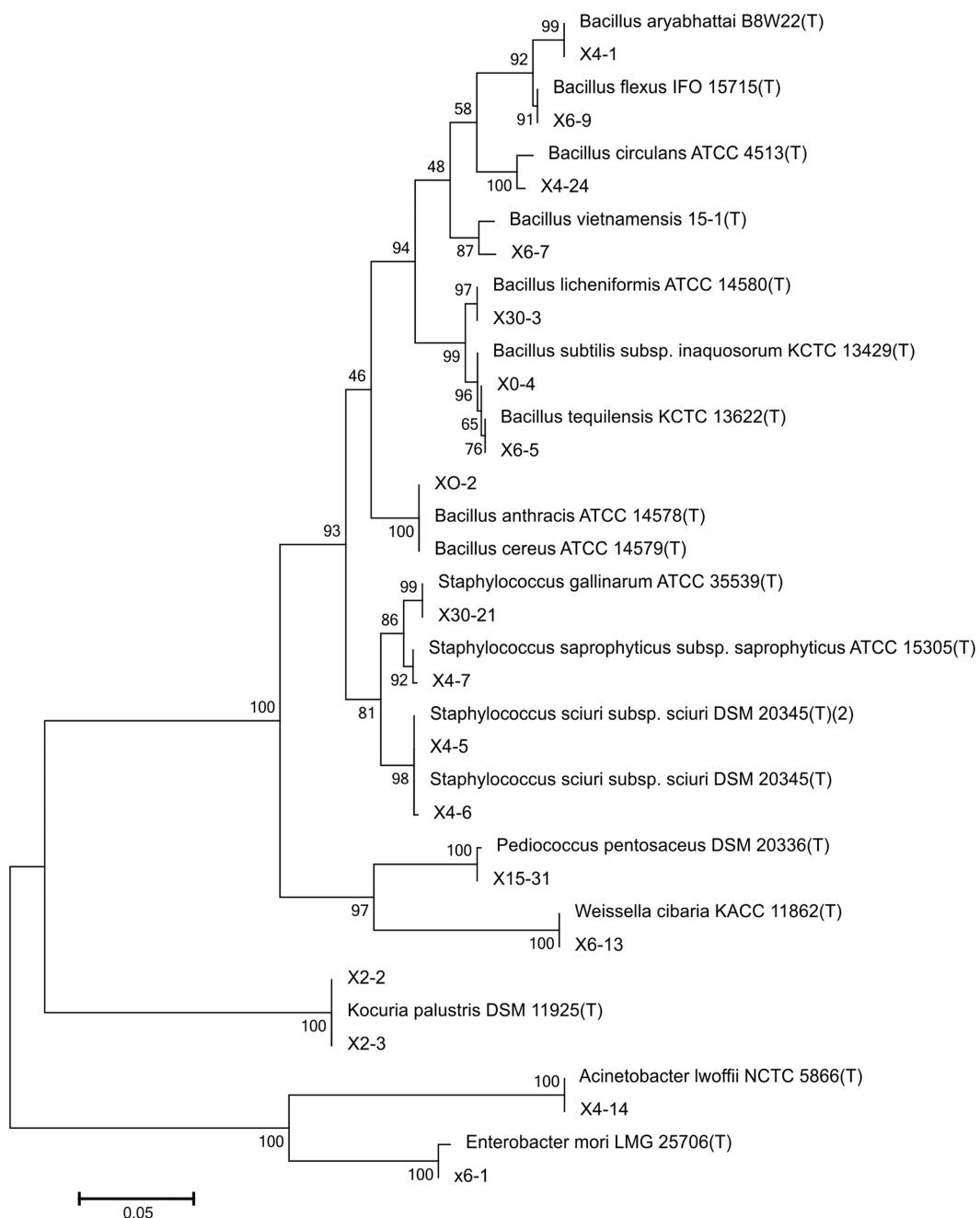


## **Correlations Between Bacteria, Molds, and Enzyme Activities in Daqu of Luzhou-Flavored Liquor**

The correlations between bacteria, molds, and the activities of saccharifying enzyme, liquefied enzyme, and protease in daqu of Lu-zhou-flavored liquor were analyzed using constrained correspondence analysis with an R package. As shown in Figure 9A, there were relatively strong correlations between the saccharifying enzyme and liquefied enzyme and *S. saprophyticus* subsp. *saprophyticus*, *Bacillus tequilensis*, *W. cibaria*, and *E. mori*. In addition, there were strong correlations between the activity of protease and *S. gallinarum*. As shown in Figure 9B, *M. circinelloides* f. *circinelloides* was strongly correlated with protease activity, whereas the activity of saccharifying enzyme was mainly correlated with *R. oryzae*, and *L. ramosa* was predominantly associated with the activity of liquefied enzyme, although this correlation was relatively weak.



**Fig. 7.** Population succession of microorganisms during the daqu production cycle of Luzhou-flavored liquor.



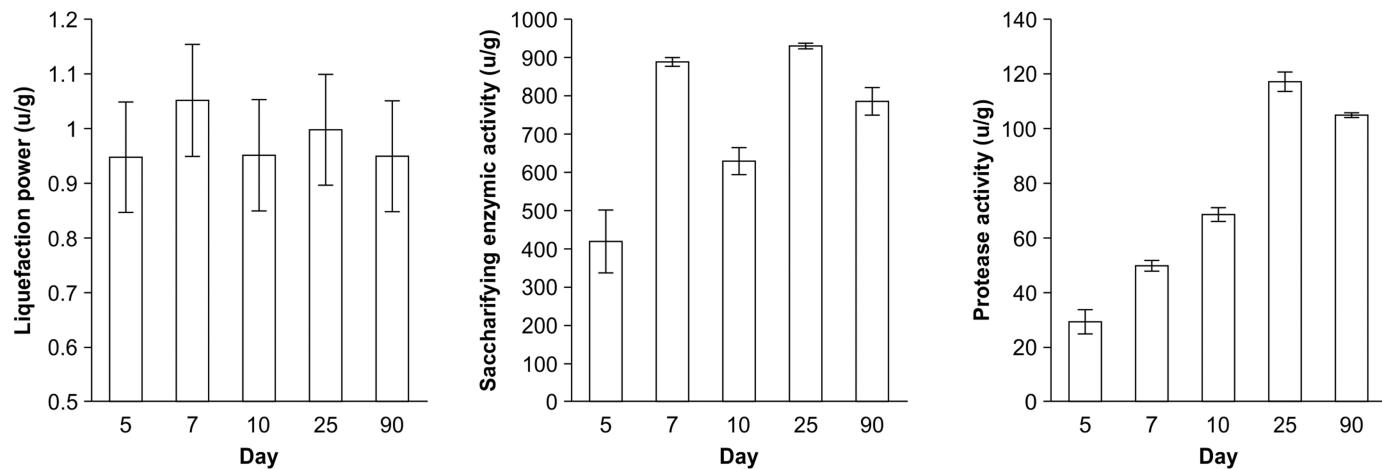
**Fig. 6.** Phylogenetic tree of isolated bacteria and type strains.

## DISCUSSION

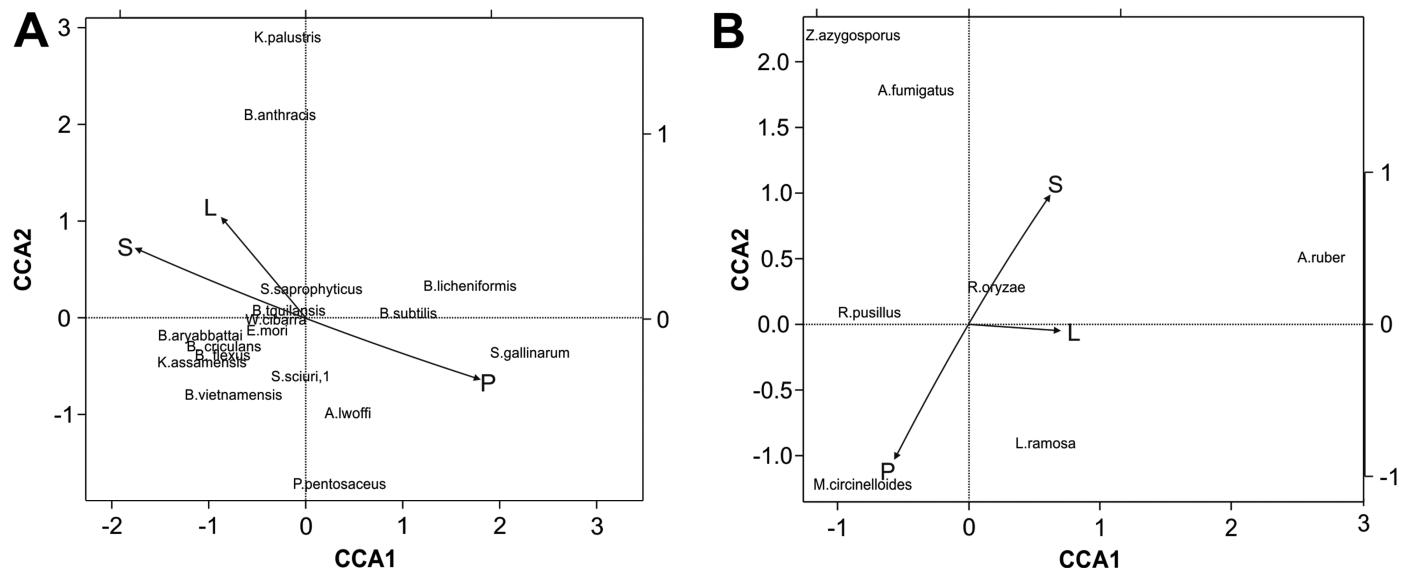
In contrast to malt-based culture systems for liquor production in Western countries, traditional Eastern liquor-making procedures involve the use of a fermentation starter from grains, such as koji for the production of Japanese sake and daqu for Chinese baijiu. The molds such as *Aspergillus oryzae*, *Rhizopus*, and *Mucor* are good functional microorganisms for saccharification during liquor production using starch as the raw material. Koji mainly provides enzymes for the saccharification of rice starch, whereas daqu not only provides saccharifying enzymes but also provides functional microorganisms such as yeasts. However, the functional microorganism compositions and their roles in traditional Chinese baijiu remain unclear.

*A. oryzae* is the unique microorganism in koji, whereas *Rhizopus* is found in traditional Chinese daqu, as well as in typical fermentation starters used in Vietnam, Burma, and Korea. *Rhizopus* can generate a variety of organic acids such as lactic

acid, fumaric acid, and succinic acid and thus plays an important role in establishing the liquor flavor profile. Bacteria such as *Lactobacillus*, propionic acid bacteria, and *Bacillus* are common among traditional Chinese liquor microorganisms. For example, thermophilic *Bacillus* is common in high-temperature daqu, and it influences the Maotai flavor at the high-temperature stage. In addition, because the bacteria secrete extracellular protease, they can increase the contents of total free amino acids, especially aromatic amino acids with a benzene ring (9). *Bacillus* in the daqu of Fen-flavored liquor is considered to play an important role in the flavor profile and to improve the aftertaste (17). Yeast is a key microorganism for the brewing process. *Saccharomyces cerevisiae* converts glucose to alcohol during the liquor production process, resulting in the production of esters, organic acids, and higher alcohols such as flavor substances. Among the yeast species in daqu, *Saccharomyces carlsbergensis* has strong fermenting power and *Hansenula anomala* has strong esterifying power. Usually, the number of



**Fig. 8.** Activities of saccharifying enzyme, liquefying enzyme, and protease in daqu at different stages of production. Error bars indicate standard deviations.



**Fig. 9.** Correlations between bacteria (A) and molds (B) and the enzyme activities in daqu of Luzhou-flavored liquor analyzed through constrained correspondence analysis with the R language package. The lengths of the arrows represent the degrees of relatedness between enzyme factors and the strains, for which a longer line indicates greater relevance. The angle between an arrow and shaft represents the extent of the correlation between enzyme activity and the mold or bacterial abundance, in which a smaller angle indicates a higher positive correlation. S = saccharifying enzyme; L = liquefied enzyme; and P = protease.

yeasts in daqu of Fen-flavored liquor is also greater than those of daqu in Luzhou- and Maotai-flavored liquors (19).

In the present study, the overall microbial count exhibited an initial increase, subsequent decrease, and eventual increase in general, and the microflora in daqu mainly consisted of molds and bacteria by day 5. The microbial count in daqu reached its peak value at the peak-temperature period (day 7). On day 90, the microflora in daqu mainly comprised bacteria and molds, mostly consisting of heat-resistant spore-forming *Bacillus* spp. and filamentous fungi.

Owing to inherent restrictions, previous studies using traditional isolation methods could not reveal the dominant strains at different stages of daqu production in the systematic and visible manner made possible with modern molecular biological technologies such as denaturing gradient gel electrophoresis (DGGE) and high-throughput methods. Ye et al. (20) analyzed the succession pattern of a fungal community during the fermentation and storage process of Luzhou-flavored daqu with PCR-DGGE technology. This technology intuitively demonstrated the changes in fungal diversity at different stages of the daqu production process but could only qualitatively identify microbes without isolating or identifying the microbes at different stages, thereby hindering subsequent intensive studies. Zheng et al. (23) performed a systematic analysis of the microbial diversity of 30- and 300-year pit muds of Chinese Luzhou-flavored liquor using high-throughput sequencing technology. These technologies show considerable advantages over traditional isolation and identification methods and can simply and intuitively reflect the microbial composition in a certain mass at a certain stage. However, compared with modern molecular biological technologies, traditional isolation methods have unique features and can obtain viable strains, providing the foundation for subsequent studies. Therefore, the future development trend will be to use modern molecular biological technologies as a guideline for traditional isolation methods. The methods used in this study are based on the traditional separation idea, using modern molecular biology to identify separated strains. Therefore, the combination of the advantages of traditional separation and molecular methods could improve the precision of identification. In this study, the correlation between these culturable strains and activity of enzymes was analyzed; however, the functions of these isolated strains require further investigation through experiments of single strains and mixed strains cultured under the same conditions used for daqu production.

With increasingly mature and simplified DNA sequencing technologies, sequencing methods have become widely used in microbial classification, identification, and systematic studies. A common practice in sequence analysis is to compare the 26S rRNA gene D1/D2 domain sequences of yeasts, 16S rRNA gene sequences of bacteria, and ITS sequences of molds with those registered in nucleotide sequence databases such as GenBank, EMBL, and DDBJ, which has considerably facilitated the isolation and identification of yeasts (18). The approximately 600 bp D1/D2 domain of 26S rRNA is located at the 5' end of the large subunit. This domain shows high variability and can be used for classification studies on microbial strains with a close genetic relationship. A complete analysis database has been established for the sequences of this domain. This database enables more convenient and accurate identification of yeast strains and is thus the most widely used database (3,13). Peterson and Kurtzman (12) compared the homology and DNA-DNA relatedness of the D1/D2 domain sequences of the 26S rRNA gene in partial yeasts and found a divergence rate of less than 1%. Kurtzman and Robnett (8) investigated the systematics of the D1/D2 domain of the 26S rRNA gene in 50 species of ascomycetous yeasts and extended this concept to all yeast species, and they proposed that 55 currently recognized yeast taxa are synonyms of earlier described

species. Accordingly, the D1/D2 domain of 26S rRNA has become the main criterion for yeast classification (10).

There are no general operating guidelines for bacterial identification based on the 16S rRNA gene sequencing method, and sequence analysis mainly relies on sequence similarity. Bosshard et al. (1) used a >99% similarity cut-off to define a species, >95–99% to define a genus, and <95% to define a family. Fox et al. (5) proposed that strains with a 5–15 bp difference in their complete 16S rRNA sequences do not belong to the same species. Some researchers have used >99.8 and 98.1% similarity cut-offs to define a species and a genus, respectively (4). In this study, sequence homology analysis was performed on microbes isolated from samples collected at different stages during the daqu production process following the method of Bosshard et al. (1). A total of 113 yeast strains, 63 bacterial strains, and 128 mold strains were identified, and most of the microbial strains could be identified to the species level, whereas the remaining strains were identified to the genus level. However, these results of the classification of isolated strains obtained in this study should be further confirmed using a confidence-based classifier such as the Ribosomal Database Project Classifier.

Furthermore, the activities of the liquefying enzyme, saccharifying enzyme, and protease in daqu of different stages were investigated, and the activity of the liquefying enzyme was stably maintained at 0.9–1.1 U/g. However, the activity of the saccharifying enzyme was high at days 7 and 25 and slightly decreased at days 10 and 90, indicating that the temperature changes during the daqu production and storage processes had a certain effect on the saccharifying power of daqu. Protease activity initially increased and then slightly decreased, with a peak value detected between days 25 and 90. Protease can break down proteins to generate amino acids, which can then participate in the Maillard reaction that facilitates the formation of flavor precursors. Owing to its high protease activity, daqu exhibits a rich flavor, which reflects the flavor evaluation criteria in sensory evaluations.

## CONCLUSIONS

As the fermentation starter for Chinese liquors, daqu provides the microbes, flavor precursors, and enzymes for the fermentation process. Given that microbes also play a fundamental role in the formation of enzymes and flavor precursors, it is necessary to optimize the enzymatic changes in daqu at different stages of production with the succession patterns of microbes to achieve the best flavor of daqu. Zhang (21) screened the aroma-producing microbes from daqu and studied the relationship between the aroma formation in daqu and the change of aroma-producing microbes isolated. Through the present study, we have provided comprehensive information on the patterns of changes in the microbial composition during the manufacturing of daqu and the corresponding changes of the activities of three kinds of enzymes. Overall, we found a stronger correlation between the saccharifying enzyme and liquefied enzyme and *S. saprophyticus* subsp. *saprophyticus*, *B. tequilensis*, *W. cibaria*, and *E. mori*, whereas protease activity was most strongly correlated with *S. gallinarum*. Furthermore, *M. circinelloides* f. *circinelloides* showed a strong correlation with protease activity, whereas *R. oryzae* was more strongly correlated with the activity of saccharifying enzyme. These findings provide insight into the succession of microbial and enzyme activity changes at different days during daqu production. Considerable research is required to clearly identify the correlations between microbes and enzymes, and we are planning to tackle these questions in our future work to reveal the functional microorganisms of daqu during the production of Luzhou-flavored liquor.

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