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PCR-DGGE Analysis of the Microbial Communities in Three Different Chinese "Baiyunbian" Liquor Fermentation Starters

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A systematic investigation was performed on the bacterial, *Bacillus*, fungal, and yeast communities of the three types of *Daqu* (mechanically prepared, manually prepared, and mixed prepared) used in Baiyunbian Company by reconditioning PCR-denaturing gradient gel electrophoresis (PCR-DGGE). The DGGE results showed that the microbes in the three types of *Daqu* were mainly thermotolerant and thermophilic microbes, and the most dominant bacterial species were *Bacillus* and *Virgibacillus*, followed by *Lactobacillus* and *Trichococcus*. Furthermore, the dominant fungi were found to be molds, such as *Rasamsonia*, *Penicillium*, *Aspergillus*, and *Monascus*, and the dominant yeasts were *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera*, *Pichia anomala*, and *Debaryomyces hansenii*. In general, the three types of *Daqu* showed slight differences in microbial communities, and the Shannon indexes (H′) of the manually prepared and mechanically prepared *Daqu* were similar. The results suggest that mechanically prepared *Daqu* can replace manually prepared *Daqu* in liquor production, and this research provides useful information for liquor production and process improvement.

Keywords: *Bacillus*, *Daqu*, fermentation starter, microbial community, PCR-DGGE

Introduction

Chinese liquor is one of the six most well-known distilled spirits in the world, with a production of 12.26 million liters in 2013. Chinese liquor can be categorized into five aroma styles: soy sauce aroma, strong aroma, light aroma, sweet honey, and miscellaneous aroma [29]. The miscellaneous aroma style includes several types that are derived from the other four styles. "Baiyunbian" liquor, a major liquor in China (production of 0.2 million liters/year), is a typical representative of Maotai-Luzhou-flavor liquors. Its production cycle of 300 days yields a unique aroma style that is a combination of the soy sauce aroma (Maotai-flavor liquor) and the strong aroma (Luzhou-flavor liquor).

Daqu, an ancient fermentation starter, is produced by solid-state fermentation of wheat, barley, and/or peas, with the ingredients being formulated by grinding and mixing, shaping, incubation, and maturation [32, 36, 37]. *Daqu* is prepared through the natural inoculation of bacteria, molds, and yeasts derived from raw materials, air, ground, the human body, and other related materials in nature. *Daqu* is an important saccharifying and fermentation agent for the production of Chinese liquor, and provides nutrients for microbial growth. The numerous flavor compounds derived from the microbes present in *Daqu* influences the liquor quality during fermentation [30, 36, 37].

By both culture-dependent and culture-independent methods, a large variety of microbes have been detected in *Daqu*. These are considered to be the functional microbiota

in *Daqu* responsible for the formation of a range of lytic enzymes, substrates for alcoholic fermentation, and flavor compounds [24, 28, 30, 36]. *Daqu* substrates vary in their specific microbial compositions. This variation may arise from intrinsic and extrinsic factors, such as the raw material, moisture, temperature, and environment, that influence the microbial community of *Daqu* [30]. Because the majority of microbes in *Daqu* can not be cultured under laboratory conditions, the investigation of *Daqu* microflora by culture-independent methods has attracted increasing interest from researchers. PCR-denaturing gradient gel electrophoresis (DGGE) is a useful tool for investigating the composition of microbial communities, and has been used successfully to study the taxonomy of the microbial communities in fermentation products [4, 20, 32, 33, 37]. However, to date, only a few studies about the microflora in *Daqu* have been performed using culture-independent methods.

In past centuries, *Daqu* samples were manually prepared. This required a large amount of work force and increased the costs. Thus, in recent years, to lower the costs and to improve the production process, increasing amounts of *Daqu* were mass-produced by machine. Currently, the Baiyunbian Company uses three types of *Daqu* prepared by machine, humans, or a combination of machine and human (mixed prepared: mixing material by machine and stepping up the starter by humans). To determine the differences between the three types of *Daqu*, a systematic investigation was performed to determine the composition of the general bacterial, *Bacillus*, fungal, and yeast communities in *Daqu* by using reconditioning PCR-DGGE. The results may help to facilitate liquor production and process improvement.

Materials and Methods

Sample Collection

The three types of high-temperature *Daqu* (mechanically prepared, manually prepared, and mixed prepared) were obtained from Baiyunbian Liquor Industry Co. Ltd. in Songzi, Hubei, China. Five representative samples were collected for each *Daqu* by the quartering method. The samples were transferred to sterile bags, sealed, and stored at 4°C for analysis within 48 h.

DNA Extraction

Ten grams of each powdered *Daqu* sample was homogenized in 90 ml of 0.85% (w/v) sterile physiological saline, and subsequently filtered through four layers of sterile cheesecloth. The filtrates were centrifuged at 14,000 ×*g*, 4°C for 10 min, and then the pellets were subjected to DNA extraction using the commercial PowerSoil DNA Isolation Kit (Mo-Bio, Carlsbad, USA). For elution, the matrix-DNA complex was resuspended in 100 µl of elution buffer and stored at −20°C before use. The DNA concentration and quantity were tested on a Nanodrop ND-100 spectrophotometer (Thermo, Wilmington, USA). The extracted DNA was then used as a template for PCR.

PCR Amplification

Primers used in this study are shown in Table 1. The 50 µl reaction mixture consisted of 5 µl of 10× PCR buffer (Takara, Dalian, China), 4 µl of dNTPs (2.5 mM of each; Takara), 1 µl of each primer (10 µM), 2.5 U of *Taq* polymerase (Takara), and 50 ng of template DNA. A Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, USA) was used for amplification according to Table S1.

Table 1. Primers used in the study.

Target region	Name	Sequence (5′-3′)	References
V3 region of 16S rRNA, bacteria	357F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGC-CTACGGGAGGCAGCAG	[19]
	518R	ATTACCGCGGCTGCTGG	
16S rRNA of *Bacillus*	pBF	CGATGCGTAGCCGACCTGAG	[2]
	pHR	AAGGAGGTGATCCAGCCGCA	
V9 region of 16S rRNA, *Bacillus*	Ec1055F	ATGGCTGTCGTCAGCT	[10]
	Ec1392R-GC	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGC-CCGACGGGCGGTGTGTAC	
18S rRNA of fungi	NS1	GTAGTCATATGCTTGTCTC	[18]
	GC-fung	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCAT-TCCCCGTTACCCGTTG	
D1 region of 26S rRNA, yeast	NL1-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCCATAT-CAATAAGCGGAGGAAAAG	[11]
	LS2	ATTCCCAAACAACTCGACTC	

After the first PCR, a reconditioning PCR was performed as previously described [26].

To amplify *Bacillus* selectively, a three-step nested PCR approach was applied as previously described [4]. For the first PCR (referred to as "selective PCR"), primer sets pBF and pHR were used. The forward primer pBF was designed specifically for *Bacillus* and related genera, and the reverse primer pHR was a universal primer. The first PCR was performed using the 50 µl reaction system described above by following the PCR procedures listed in Table S2. Subsequently, the first PCR product was diluted (10-fold) and used as a template for a nested PCR targeting the V9 region of the 16S rRNA gene using primers Ec1055F and Ec1392-GC, designed specifically for *Bacillus* species. Finally, a reconditioning PCR was performed as described above to generate products for DGGE analysis.

DGGE Analysis

Before DGGE analysis, the five PCR products from each *Daqu* were mixed together as one sample, and then the three mixed PCR samples were cleaned using a PCR Purification Kit (Omega, Norcross, USA). Using a Model 475 Gradient Delivery System (Bio-Rad), gels containing 8% (w/v) polyacrylamide (acrylamide/bisacrylamide, 37.5/1) were formed in 1.0× Tris-acetate-EDTA (TAE) buffer (pH 8.0) with a 40% to 60% urea-formamide denaturing gradient (100% corresponding to 7 M urea and 40% deionized formamide). The gels formed were allowed to polymerize for 4 h before use. DGGE analysis was performed using the DCode Universal Mutation Detection System (Bio-Rad) by electrophoresing 20 µl of PCR products (25 ng/µl, plus 10 µl loading buffer) at 10 V for 10 min and then at 110 V for 10 h in 1.0× TAE buffer at 60°C. The DNA bands in gels were visualized by silver staining [27] and photographed with a Molecular Imager FX System (Bio-Rad).

To identify the species represented by DGGE bands, the bands were excised from the gels, washed twice with ultrapure water and then stored in 50 µl of ultrapure water at 4°C for 16–24 h to elute the DNA. Subsequently, 5 µl of DNA elution was used to re-amplify the DNA using the reaction mixture and conditions as described above, except that the primer without the GC clamp was used. The PCR products were cleaned using a PCR Purification Kit (Omega) and then sequenced (Invitrogen, Shanghai, China). Assembled sequences were aligned and compared with the 16S, 18S, or 26S rRNA sequences available in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) to identify their closest phylogenetic relatives. The identities of the relatives were determined on the basis of the highest score.

Analysis of DGGE Gel Images

The Shannon diversity index (H') was calculated using the software BIO-DAP (Fundy National Park, Canada), based on the quantity and relative intensity of each band, which were obtained by the software Quantity One (ver. 4.6.2; Discovery Series, Bio-Rad) [15].

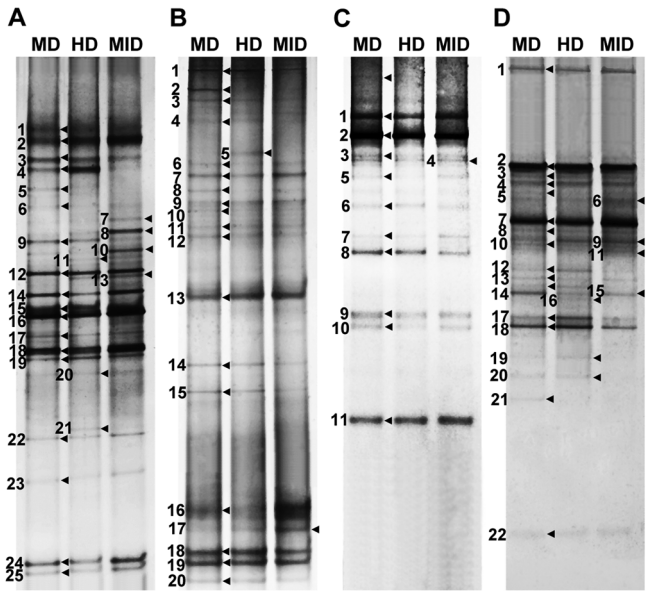

Fig. 1. DGGE profiles of bacterial (**A**), *Bacillus* (**B**), fungal (**C**), and yeast (**D**) communities of three types of *Daqu*.
The denaturant gradient ranges are from 40% to 60%. The major bands are numbered. MD, HD, and MID correspond to mechanically prepared, manually prepared, and mixed prepared *Daqu*, respectively.

Results and Discussion

***Bacillus* Species Are Dominant in the Bacterial Community**

The microbes found in *Daqu* play a key role in the production of alcohol and the formation of flavor. Fig. 1 shows the results of the DGGE analysis of the general bacterial, *Bacillus*, fungal, and yeast communities found in the three types of *Daqu*. Most of the microbes detected were the thermotolerant and thermophilic microbes (Tables 2–5), and the three types of *Daqu* showed only slight differences in microbial communities (Fig. 1).

For bacterial analysis, 25 bands in the DGGE profiles were eluted from gels and sequenced (Fig. 1A). The sequence identity of all the bands was ≥94% as compared with that in the GenBank database, and most of them were uncultured bacteria (Fig. 1A; Table 2). The most dominant bacterial species were *Bacillus* (11, 17, 20, and 22) and *Virgibacillus* (2, 12, 14, 15, and 18). Several previous studies also reported that *Bacillus* species were dominant in Fen-*Daqu* [24, 33, 37].

During the processing of the three types of *Daqu* tested here, the highest temperature at the center of the *Daqu* pile was about 60°C. *Bacillus* species have a better survivability than other bacteria under low moisture (13–14%) and high

Table 2. Summary of identification of bands in Fig. 1A (bacteria).

Band no.[a]	Closest relative and GenBank Accession No.	Identity[b]
1	Uncultured bacterium (KC414631.1)	98%
2	Uncultured *Trichococcus* sp. (EU919224.1)	95%
3	*Lactobacillus fermentum* (EU420175)	99%
4	Uncultured *Firmicutes* bacterium (JN409171.1)	98%
5	*Staphylococcus xylosus* (EU266748)	95%
6	Uncultured bacterium (JX133546.1)	94%
7	Uncultured bacterium (JQ337554.1)	95%
8	Uncultured bacterium (KC169782.1)	96%
9	Uncultured bacterium (JF546884.1)	94%
10	Uncultured *Trichococcus* sp. (EU919224.1)	95%
11	*Bacillus* sp. (AY433821.1)	97%
12	*Virgibacillus* sp. (HQ433442.1)	97%
13	*Lactobacillus helveticus* (EU483108)	95%
14	*Virgibacillus* sp. (GQ889491.1)	96%
15	Uncultured *Trichococcus* sp. (EU919224.1)	95%
16	Uncultured bacterium (KC414631.1)	98%
17	*Bacillus* sp. (FM865970.1)	97%
18	*Virgibacillus halodenitrificans* (AB741852.1)	99%
19	Uncultured bacterium (JX133320.1)	94%
20	*Bacillus licheniformis* (EU870512.1)	99%
21	*Lentibacillus salis* (NR_043170.1)	100%
22	Uncultured *Bacillus* sp. (EU834945.1)	94%
23	*Thermoactinomyces sanguinis* (AJ251778.1)	100%
24	Uncultured betaproteobacterium (DQ110058.1)	95%
25	*Thermoactinomyces sanguinis* (AJ251778.1)	96%

[a]Bands are numbered according to Fig. 1A.

[b]Identity represents the sequence identity (%) compared with that in the GenBank database.

Table 3. Summary of identification of bands in Fig. 1B (*Bacillus*).

Band no.[a]	Closest relative and GenBank Accession No.	Identity[b]
1	*Gracilibacillus* sp. (HQ738838.1)	98%
2	*Halobacillus profundi* (HE586572.1)	95%
3	*Virgibacillus salarius* (KC113161.1)	98%
4	*Oceanobacillus* sp. (EU817570.1)	99%
5	*Bacillus* sp. (GQ199746.1)	94%
6	*Gracilibacillus* sp. (HQ738838.1)	98%
7	*Bacillus thermoamylovorans* (GU125625.1)	95%
8	*Virgibacillus* sp. (JX258925.1)	100%
9	Uncultured *Bacillus* sp. (JX240772.1)	98%
10	*Bacillus thioparans* (JN208090.1)	98%
11	*Gracilibacillus* sp. (HQ738838.1)	98%
12	*Bacillus boroniphilus* (JN867119.1)	97%
13	*Bacillus pichinotyi* (JX203257.1)	99%
14	*Bacillus subtilis* (JN230522.1)	100%
15	*Bacillus subtilis* (EU302131.1)	100%
16	*Staphylococcus haemolyticus* (GU143876.1)	98%
17	*Bacillus licheniformis* (EU982479.1)	98%
18	*Bacillus licheniformis* (KC212001.1)	99%
19	Uncultured bacterium (KC414646.1)	97%
20	*Halobacillus profundi* (HE586573.1)	94%
21	*Bacillus* sp. (JN990411.1)	95%
22	*Bacillus humi* (FJ973532.1)	98%

[a]Bands are numbered according to Fig. 1B.

[b]Identity represents the sequence identity (%) compared with that in the GenBank database.

temperature (60–62°C) conditions because of their ability to produce spores. Thus, *Bacillus* species gradually become the dominant bacteria with an increasing ripening time [24, 28]. The current study showed a low variation of *Bacillus* in the three types of *Daqu* (Fig. 1B). More *Bacillales* species, including *Gracilibacillus*, *Halobacillus*, *Virgibacillus*, and *Staphylococcus*, as well as seven species of *Bacillus*, were detected (Table 3).

As the most dominant bacteria in Baiyunbian *Daqu*, *Bacillus* species may play an important role in the production of Chinese liquor. *Bacillus* species are important sources of proteases, amylases, lipases, cellulases, pectinases, glucanases, and other enzymes. These enzymes are important for the hydrolysis of the macromolecular components in cereal grains, leading to liquefaction and saccharification of starch. Furthermore, some metabolites from *Bacillus* species contain aromatic components such as diacetyl that facilitate subsequent reactions necessary for flavor production [28]. *Bacillus* species can also generate volatile compounds such as C_4 compounds, pyrazines, volatile acids, and aromatic and phenolic compounds in the liquor. These provide an improved sensory effect and various health benefits [5, 34]. However, the functionality of the *Bacillus* species in Baiyunbian *Daqu* requires further investigation.

Other bacterial species such as *Lactobacillus* (3) and *Trichococcus* (2, 15) were also dominant in *Daqu* (Fig. 1A; Table 2). Several *Lactobacillus* species, including *L. brevis*, *L. helveticus*, *L. panis*, and *L. fermentum*, were observed in

Daqu [30]. The *Lactobacillus* species regulate the diversity in a microbial consortium present in food and dairy manufacturing systems, and produce a variety of enzymes and organic acids to produce flavor compounds [13, 23]. *Trichococcus* species are frequently isolated from sewage sludge and the anaerobic bioreactor used to treat organic wastewater. However, little information is available about their role in fermented products [3].

The main differential bands in the three types of *Daqu* were related to the uncultured *Firmicutes* bacterium (4), uncultured bacteria (7, 8, and 9), uncultured *Trichococcus* (10), and *Lentibacillus salis* (21) (Fig. 1A; Table 2). Obtaining functional information about these bacteria is very difficult because most are uncultured. In the *Daqu* prepared by machine, a new band (21) related to *L. salis* was identified. *L. salis* is a moderately halophilic bacterium first reported in *Daqu*. *Lentibacillus* species belong to *Bacillaceae*, whose role in the liquor production may be similar to that of genus *Bacillus*.

Molds Are Dominant in the Fungal Community

The saccharification and alcoholic fermentation of Chinese liquor result partially from the combined actions of molds and yeasts that grow more or less in succession throughout the brewing process [30]. As shown in Fig. 1C and Table 4, the dominant fungi in the three types of *Daqu* were *Talaromyces emersonii* (1), *Penicillium oxalicum* (2), *Aspergillus* (3, 4, and 9), and *Monascus* (11). Previous studies have described the isolation and identification of fungi found in *Daqu* and in the fermentation of Chinese liquor [24, 28], and some functions of fungi in fermented foods were determined.

A study in 2012 proposed that, based on their genetic relationships, *T. emersonii*, *T. byssochlamydoides*, *Talaromyces eburneus*, and *Geosmithia argillacea* belong to *Rasamsonia* gen. nov. [6]. These are thermotolerant and thermophilic fungi, and little information is available about their roles in fermented products. However, *Penicillium* species are considered to be undesirable contaminants, as they can interfere with *Daqu* quality by inhibiting the growth of other beneficial microbes [36]. *Aspergillus* and *Monascus* species are very common in fermentation starters. The role of *Aspergillus* species in fermented products has been studied extensively. *Aspergillus* species can produce proteolytic and other lytic enzymes, which are linked to the transformation of insoluble grain compounds into water-soluble peptides, sugars, free amino acids, and other degradation products found in soy sauce, thus affecting liquor flavor [17, 30]. *Monascus* species can esterify mixed acids and ethanol to form esters such as ethyl caproate that contribute to the aroma of the Chinese liquor. This species can also produce citric acid, acetic acid, succinic acid, and substances with physiological activity that facilitate the formation of flavor and provide health benefits [14, 16].

Table 4. Summary of identification of bands in Fig. 1C (fungi).

Band no.[a]	Closest relative and GenBank Accession No.	Identity[b]
1	*Talaromyces emersonii* (D88321.2)	99%
2	*Penicillium oxalicum* (GU078431.1)	99%
3	*Aspergillus oryzae* (HM064501.1)	99%
4	*Aspergillus terreus* (FJ176935.1)	99%
5	*Thermoascus crustaceus* (JQ067913.1)	99%
6	*Rasamsonia argillacea* (AB032120.1)	99%
	Geosmithia argillacea (AB032111.2)	99%
	Talaromyces byssochlamydoides (AB023950.1)	99%
	Talaromyces eburneus (D88322.1)	99%
	Talaromyces emersonii (D88321.2)	99%
7	*Rhizomucor pusillus* (KC117252)	98%
8	*Saccharomycopsis fibuligera* (FJ176931.1)	99%
9	*Aspergillus niger* DAOM 221143 (JN938983.1)	100%
	Aspergillus tubingensis (KF018466.1)	100%
	Aspergillus awamori CF1 (JQ975055.1)	100%
10	*Absidia blakesleeana* (FJ176934.1)	99%
11	*Monascus purpureus* JCM 6934 (JN940466.1)	99%
	Monascus fuliginosus (HM188432.1)	99%
	Monascus ruber (HM188428.1)	99%
	Monascus pilosu (AB024047.1)	99%

[a]Bands are numbered according to Fig. 1C.

[b]Identity represents the sequence identity (%) compared with that in the GenBank database.

Other fungi such as *Thermoascus crustaceus* (5), *Rhizomucor pusillus* (7), and *Absidia blakesleeana* (10) were detected in the three starters (Table 4). *Thermoascus* is commonly found in high-temperature *Daqu*, but its functions in alcohol fermentation starters remain unknown [25, 32]. *R. pusillus*, a type of thermophilic fungi, is commonly present in cereals, oil seeds, meat products, and *Daqu*, and can affect liquor flavor by rapidly utilizing numerous carbon sources, and producing glycerol, lactic acid, glucanase, phosphatase, acid proteinase, and alcohol dehydrogenase [22, 35]. *A. blakesleeana* is the most common species in some kinds of *Daqu*. It can secrete hydrolyzing enzymes that decompose macromolecular materials and produce some metabolites that affect liquor flavor [30].

Table 5. Summary of identification of bands in Fig. 1D (yeast).

Band no.[a]	Closest relative and GenBank accession no.	Identity[b]
1	*Talaromyces striatus* (GU092971.1)	99%
2	*Saccharomyces cerevisiae* (HM236342.1)	98%
3	*Candida silvae* (FJ463644.1)	96%
4	*Saccharomyces cerevisiae* (HM236342.1)	98%
5	Fungal sp. (AB291680.1)	98%
6	*Saccharomycopsis fibuligera* (JX141337.1)	98%
7	*Saccharomycopsis fibuligera* (AM270990.1)	98%
8	*Saccharomyces cerevisiae* (EU441887.1)	100%
9	*Saccharomyces cerevisiae* (DQ285665.1)	98%
10	*Penicillium inflatum* (AF033393.1)	95%
11	*Aspergillus ochraceopetaliformis* (EF661429.1)	98%
12	*Pichia anomala* (KC222508.1)	98%
13	*Pichia anomala* (D29864.1)	96%
14	*Thermoascus crustaceus* (JF922031.1)	97%
15	*Debaryomyces hansenii* (KC485456.1)	96%
16	*Aspergillus intermedium* (HE974459.1)	98%
17	*Aspergillus intermedium* (HE974459.1)	98%
18	*Saccharomyces cerevisiae* (GQ121700.1)	99%
19	*Thermoascus aurantiacus* (KC585413.1)	98%
20	*Thermoascus aurantiacus* (KC585413.1)	99%

[a]Bands are numbered according to Fig. 1D.

[b]Identity represents the sequence identity (%) compared with that in the GenBank database.

In the current study, the results of the DGGE analysis showed that most of the fungi present were molds, and that yeasts were not the dominant microflora (Fig. 1C; Table 4). Temperature is an important factor that affects the growth and death of microbes. In general, the heat resistance of yeast is poorer than that of molds. During the preparation process of the three types of *Daqu*, the center of *Daqu* reaches 60°C, which is not compatible for the survival of most yeasts. The high temperature attained during the preparation process may explain the reason for the absence of yeast species in *Daqu*.

Although yeasts were not the dominant fungi in *Daqu*, they could gradually become the dominant microbes and have a significant effect on the subsequent fermentation processes used in liquor production. The dominant yeasts found in the three types of *Daqu* were *Saccharomyces cerevisiae* (8, 9, and 18), *Saccharomycopsis fibuligera* (7), *Pichia anomala* (12 and 13), and *Debaryomyces hansenii* (15) (Fig. 1D; Table 5). The yeasts identified in Baiyunbian *Daqu* are similar to those found in other alcoholic starters. Most of them are present naturally in the fermentation products [12, 28, 30, 37]. *S. cerevisiae* is usually dominant in alcoholic fermentations because of its competitive growth under strict anaerobic conditions and its tolerance to ethanol. Thus, *S. cerevisiae* is an efficient microbe for ethanol production [31, 37]. A previous study reported that *S. cerevisiae* was the major yeast species active during Fen-liquor fermentation [12].

Several non-*Saccharomyces* yeasts, including *S. fibuligera*, *P. anomala*, and *D. hansenii*, were found in Baiyunbian *Daqu*. *S. fibuligera* occurs commonly in fermentation starters and typically grows prior to the main alcoholic fermentation, thereby playing an important role during the initial stages [7]. *S. fibuligera* has a strong saccharification capability and produces various enzymes, particularly glucoamylase and α-amylase, that metabolize the native starch into maltose, dextrin, and glucose [1]. *P. anomala* is a film-forming yeast and can improve the taste, texture, yield, and safety of agricultural products by outcompeting undesirable fungi [8]. Supplementation with *P. anomala*, a potent producer of esters, during production can increase the ester content of liquor [8, 9, 30]. *D. hansenii* is a haloduric and lipid-accumulating oleaginous yeast that is found in the process of making Chinese liquor, and is beneficial for fermented foods [21, 30].

Overall, only small differences were detected in the composition of the fungal and yeast communities among the three types of *Daqu* (Figs. 1C and 1D). The main differences were observed in the mixed prepared *Daqu* (Fig. 1D) where the relative amount of *Aspergillus intermedium* (16 and 17) was increased, and the amounts of *S. cerevisiae* (2) and *Candida silvae* (3) were decreased (Table 5).

Similarity in Shannon Diversity Index

DGGE profiles were analyzed using the Shannon index (H′) to assess the relative intensity and the absence or presence of the bands (Fig. 1 and Table 6). The three types of *Daqu* had very similar diversity indexes of bacteria,

Table 6. Shannon index (H′) calculated from the DGGE banding patterns in Fig. 1.

Daqu samples	Bacteria	*Bacillus*	Fungi	Yeasts
Mechanically prepared	2.66	2.41	1.80	2.16
Manually prepared	2.67	2.44	1.82	2.35
Mixed prepared	2.64	2.42	1.77	1.84

Bacillus, and fungi. For yeast, the diversity indexes of manually prepared *Daqu* and mechanically prepared *Daqu* were similar, but higher than that of the mixed prepared *Daqu* (Table 6).

Several researchers have investigated the microbial communities and development of microbes in the *Daqu* preparation and ripening process as well as production in different regions using different culture temperatures [28, 30, 32]. Perhaps some other factors inherent in the different preparation methods would affect the microbial composition in *Daqu*. For example, mixing material by hand and stepping up the starter by foot may lead to microbial transfer from the human body to the starter. In addition, the force and heat produced by machine may affect the growth and death of microbes in *Daqu*. However, only slight differences in microbial communities were found among the three types of *Daqu*, and similar Shannon indexes (H′) were observed in manually prepared and mechanically prepared *Daqu*. During *Daqu* preparation, the culture was inoculated with 4% old *Daqu*. In this way, the *Daqu* has been handed down from one generation to the next. It is possible that the addition of old *Daqu*, temperature, and other factors played a decisive role in the microbial composition of *Daqu*.

In conclusion, this is probably the first study to identify the bacterial, *Bacillus*, fungal, and yeast communities in three types of *Daqu* by using reconditioning PCR-DGGE with four different groups of primers. The microbes detected were mainly thermotolerant and thermophilic microbes. The dominant bacterial and fungal species were *Bacillus* and molds. The preparation methods had minimal effects on the microbial composition, and based on these data, the mechanically prepared *Daqu* could replace manually prepared *Daqu* in Chinese liquor production. The findings of our study provide useful information for liquor production and fermentation process improvement.

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