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Characterization of the microbial community in three types of fermentation starters used for Chinese liquor production

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This study examined and compared the microbial community in three typical fermentation starters (called as *Daqu*, *Xiaoqu*, and *Fuqu* in China) used for liquor production by analysing the 16S and 18S rRNA gene clone library. The results show that the microbial diversity in the three types of fermentation starters (*JiuQu*) differs significantly. The bacterial species in *Daqu* and *Fuqu* were mainly thermophilic or thermotolerant. In *Daqu*, the dominant bacterial species were *Thermoactinomyces sanguinis* (53.85%) and *Pantoea agglomerans* (19.23%), followed by uncultured bacteria (15.39%). The lactic acid bacterium *Weissella cibaria* (50%) and a member of Enterobacteriaceae, *Enterobacter ludwigii* (10%), were the dominant bacterial species in *Xiaoqu*. Low abundances of other bacteria, including *Deinococcus radiodurans*, *Corynebacterium variabile* and *Acinetobacter baumannii*, were reported for *Xiaoqu*. *Enterococcus faecium*, *Clostridium beijerinckii* and *Bacillus cereus* were observed in *Fuqu* and accounted for 46.67, 23.33 and 16.67% of the total bacteria identified, respectively. Fungal diversity was high in *Daqu* and consisted exclusively of thermophilic moulds, such as *Aspergillus glaucus* (62.5%), *Thermomyces lanuginosus* (12.5%) and *Thermoascus crustaceus* (12.5%). Only two fungal species were reported for *Fuqu* and *Xiaoqu* and both contained the mould *Rhizopus oryzae*. *Saccharomyces cerevisiae* and the non-*Saccharomyces* yeast (*Saccharomycopsis fibuligera*) were also identified in *Fuqu* and *Xiaoqu*, respectively. This finding suggests that microbial community structure in *JiuQu* starters is the key factor to determine the variety of flavours.

Keywords: fermentation starters; *JiuQu*; bacterial community; eukaryotic community; clone library

Introduction

Chinese liquor is well-known worldwide as distilled spirit with a total annual production estimated at approximately 5 million tons per year and 12.26 million litres in 2013 *(1,2)*. Chinese liquors are often produced from cereals by solid-state fermentation using fermentation starters, which are called *JiuQu* in China. *JiuQu* acts as a saccharifying and fermenting agent that consists of a mixture of raw materials, microbiota, enzymes and aroma precursors, which have significant impact on the flavour of the liquor products *(3)*. *JiuQu* can be classified into three typical categories based on its ingredients and technological application: *Daqu*, *Xiaoqu* and *Fuqu (3)*.

Daqu is the grain-type *JiuQu* comprising wheat, barley and/or peas *(4)*. Most *Daqu* are prepared by a series of grinding and mixing, shaping, incubation and maturation steps *(4,5)*. Nowadays, 70% of Chinese liquors are made with *Daqu* fermentation starters, including most of the famous liquors in China, such as *Maotai*, *Luzhou* and *Fen*, which are typical representatives of sauce-, strong- and light-flavoured Chinese liquors, respectively *(4)*. The two other fermentation starters, *Xiaoqu* and *Fuqu*, consist of sorghum and bran, respectively *(4)*. Both are prepared mainly by steaming the raw materials followed by inoculation with pure-culture mould and yeast strains and incubation under suitable temperature and moisture conditions *(6,7)*. Sichuan *Xiaoqu* liquor, accounting for a third of the yearly yield of Sichuan liquors, is increasingly gaining popularity globally owing to its light and popular flavour and application in healthcare wine production *(6)*. In addition, *Fuqu* has been successfully used for production of sauce- *(7)*, light- *(8)* and sesame-flavour *(9)* Chinese liquor. The sesame-flavour liquor is a new taste produced by using *Fuqu* as the main fermentation starter coupled with *Daqu*; it is a recent development based on the production technology of the three-flavour liquors mentioned above *(9)*. The sesame-flavour Chinese liquor has been attracting increasing attention owing to its unique sesame taste. However, most studies have focused mainly on the production, technology and microbiota of *Daqu* because of its high yield.

Inoculation of bacteria, moulds and yeasts derived from raw materials and natural habitats plays a key role in *JiuQu* development. The microbial community of *Daqu* has been characterized in previous reports using culture-independent approaches, such as the widely used polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) *(2,10–12)* and small subunit ribosomal RNA (SSU rRNA) gene clone libraries *(13)*. Many factors influence the diversity and abundance of the microbial community in *Daqu*, including raw material constituents, production temperature,

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moisture and microhabitats. Knowledge of the microbiota in *Daqu* is still far from complete. In addition, limited information is available regarding the microbial composition and function in *Xiaoqu* and especially in *Fuqu*. Few studies have attempted to compare the microbial community of the three different *JiuQu*. In the present study, we investigated and compared the microbial community in three typical Chinese liquor fermentation starters (*Daqu*, *Xiaoqu* and *Fuqu*) using 16S and 18S rRNA gene clone libraries. The information obtained regarding the fermentation starters may aid in understanding the microbial composition of *JiuQu* and improving quality control for liquor products.

Materials and methods

Sample collection

Three *JiuQu* for liquor production (*Daqu*, *Xiaoqu* and *Fuqu*) were collected from different fermentation starter manufactures located in Qionglai City, Luojiang County and Lu County in the Sichuan province of China, respectively. All samples were stored at 4°C prior to microbial community analysis.

DNA extraction

After grinding, 2 g of each sample were resuspended in 30 mL phosphate buffered saline (PBS) (pH 7.4) and vortexed for 5 min at ambient temperature. The suspension was filtered through a single layer of gauze and the filtrate was transferred to a 50 mL centrifuge tube for centrifugation (10,000 rpm, 4°C) for 10 min. The resulting pellet was washed twice with 30 mL PBS buffer and resuspended in 2 mL PBS buffer for DNA extraction. Genomic DNA was extracted using the CTAB method as described by Griffiths et al. *(14)*. The extracted DNA was stored at −20°C prior to clone library construction.

Construction of SSU rRNA gene clone libraries

Prokaryotic (bacterial) and eukaryotic (fungal) SSU rDNA were amplified by PCR using specific primer sets (Eu27F, 5′-AGAGTTTGATCCTGGCTCAG-3′; and 518R, 5′-GTATTACCGCGGCTGCTGG-3′ for bacterial 16S rDNA; nu-ssu-0817-5′F, 5′-TTAGCATGGAATAATRRAATAGGA-3′; and nu-ssu-1536-3′R, 5′-ATTGCAATGCYCTATCCCCA-3′ for fungal 18S rDNA). The reactions were performed with a GeneAmp PCR System 2400 (Perkin Elmer, USA) under the following PCR conditions: 95°C for 9 min followed by 20 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The amplified 16S rDNA and 18S rDNA fragments were then cloned into the pT7 Blue T-vector plasmid (TaKaRa, Japan) using a DNA Ligation Kit (TaKaRa, Japan) according to the manufacturer's protocol. The recombined plasmids were then transformed into *Escherichia coli* DH5a cells (TaKaRa, Japan) and plated on Luria–Bertani agar plates containing 100 μg/mL ampicillin, 40 μg/mL X-gal (TaKaRa, Japan) and 24 μg/mL Isopropyl β-D-1-thiogalactopyranoside (TaKaRa, Japan). White clones were randomly selected for plasmid extraction. The presence of the SSU rDNA gene was verified by PCR.

Sequencing and phylogenetic analysis of SSU rRNA

Positive clones from each library were randomly selected and sequenced using a CEQ8000 Genetic Analysis System (Beckman Coulter, USA). Subsequent to performing the Chimera Check using the Bellerophon web services (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) as described by Huber et al. *(15)*, sequences showing >97% sequence similarity were grouped under one operational taxonomic unit (OTU). The OTU sequences were then aligned using the Clustal_X program *(16)* and phylogenetic trees were constructed based on the neighbour-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software *(17)*.

Statistical analysis of clone libraries

Statistical analysis of each library was conducted with the program Mothur *(18)*. Chao 1 and ACE estimator for the species richness estimation and the Shannon and Simpson indices for the diversity estimation were calculated with 95% confidence intervals, and sequence similarity cut-off was set at 3%. Good's coverage index of a given clone library describes the extent to which the sampled sequences in a library represent the total population. The Boneh index was calculated when sampling size was set at 50.

Nucleotide sequence accession numbers

The GenBank accession numbers for the bacterial and fungal OTU sequences obtained in this study are KP659364 to KP659383, and KP659384 to KP659393, respectively.

Results

Bacterial community diversity in the three fermentation starters

For the fermentation starters of *Daqu*, *Xiaoqu* and *Fuqu*, bacterial 16S rRNA libraries (DQ-P, XQ-P and FQ-P, respectively) were constructed. All coverage being >80% for the three libraries indicated that the obtained sequences in the library represented the entire population well (Table 1). Library DQ-P had much greater bacterial richness and diversity than the other libraries, as indicated by the

Table 1. Richness and diversity of SSU rRNA gene sequences in clone libraries

Library	No. of clones	No. of OTUs	Chao-1	ACE	Shannon	Simpson	Coverage (%)	Boneh
DQ-P	26	8	13	33.81	1.47	0.31	81	1.85
XQ-P	30	7	10	13.24	1.09	0.51	87	1.5
FQ-P	28	5	6	7.04	1.24	0.32	92.8	0.66
DQ-E	24	6	9	9.62	1.21	0.4	87.5	1.14
XQ-E	31	2	2	2	0.49	0.67	100	0
FQ-E	26	2	2	2	0.58	0.59	100	0

Shannon index, Chao1 and Ace values. The distribution of bacterial clones from the three libraries is shown in Table 2. Nearly all clones shared high sequence similarity (≥97%) with pure-culture bacteria sequences available from the GenBank database. These bacterial clones could be divided into eight OTUs for *Daqu*, seven OTUs for *Xiaoqu* and five OTUs for *Fuqu*. There were very few common bacterial genera among the three starters, other than *Weissella* and *Enterobacter* present in *Daqu* and *Xiaoqu* and *Enterococcus* found in *Xiaoqu* and *Fuqu*. However, the abundance of the common genera varied greatly in the different starters. These results demonstrate that the three fermentation starters each possess their own unique bacterial community.

Based on the phylogenetic analysis in Fig. 1, the bacterial clones in the *Daqu* sample were affiliated to three orders: Bacillales, Enterobacteriales and Lactobacillales. The majority of the clones (53.85%) were identified as *Thermoactinomyces sanguinis*, which belongs to the order *Bacillales* and is present only in *Daqu*. Enterobacteria including *Enterobacter cloacae* and *Pantoea agglomerans* were the second most abundant bacteria (23.08%) in *Daqu*. A low abundance of lactic acid bacteria (LAB) was reported; two LAB genera (*Leuconostoc mesenteroides* and *Weissella cibaria*) accounted for 7.7% of all bacteria identified. In addition, a number of uncultured bacterial clones were detected at high levels (15.39%) in *Daqu*. Three OTUs (DQ-P-5, DQ-P-6, and DQ-P-8) shared the highest sequence similarity with uncultured clone LJQX-14 (KF984325), clone 7s11 (DQ068858) and clone 1-1B-19 (JF417910), respectively. Clone LJQX-14 was obtained from certain ultrahigh-temperature *Daqu (19)*, while clone 1-1B-19 was found in a dry thermophilic methanogenic digester *(20)*. OTU DQ-P-8 in particular, had low sequence similarity with pure-culture bacteria (83%; highest with *Natronoanaerobium salstagnum*). The phylogenetic analysis shown in Fig. 1 indicates that this OTU should be classified under the phylum Firmicutes. These results suggest that some unknown bacteria in *Daqu* are involved in the production of Chinese liquor.

The bacteria present in *Xiaoqu* were affiliated to several orders, including Lactobacillales, Enterobacteriales, Pseudomonadales, Actinomycetales and Deinococcales (Fig. 1). Table 2 shows that the LAB are the most abundant bacteria in *Xiaoqu*. Three LAB species were identified: *W. cibaria* (70%), *Streptococcus lutetiensis* (3.33%) and *Enterococcus casseliflavus* (3.33%). Enterobacteria (*Enterobacter ludwigii*) comprised 10% of the clones. In addition, *Deinococcus radiodurans*, *Corynebacterium variabile* and *Acinetobacter baumannii* were less abundant and were identified solely in *Xiaoqu*.

The bacteria in *Fuqu* were associated with the orders Bacillales, Lactobacillales and Clostridiales (Fig. 1). *Enterococcus faecium* (LAB), *Clostridium beijerinckii* and *Bacillus cereus* were the main bacteria detected and accounted for 46.67, 23.33 and 16.67% of all identified bacteria, respectively. The genera *Clostridium* and *Bacillus* were only present in *Fuqu*. In addition, a low abundance of another LAB, *Pediococcus pentosaceus*, was detected in *Fuqu*.

Eukaryotic community diversity in the three fermentation starters

Eukaryotic 18S rRNA libraries (DQ-E, XQ-E and FQ-E, respectively) were constructed for each of the fermentation starters. Table 1 shows that library DQ-E had much greater eukaryotic richness and diversity. The 100% coverage for the libraries XQ-E and FQ-E was attributed to fewer OTU identifications. The distributions of eukaryotic clones from the three libraries are shown in Table 3. All clones shared 99 or 100% sequence similarity to pure-culture eukaryotic microorganisms. The results of the OTU analysis demonstrated that there were fewer fungi species than bacterial species involved in Chinese liquor production. The identified eukaryotic clones were associated with four orders: Sacch aromycetales, Eurotiales, Wallemiales and Mucorales (Fig. 2).

Greater fungal diversity was observed in *Daqu*. All identified fungal species were moulds present only in the *Daqu* sample. The

Table 2. Distribution of bacterial clones obtained from the three fermentation starters

Sample	Library OTU (clones)	Ratio (%)	Closest relative (accession no.)	Identity (%)
Daqu	DQ-P-1 (14)	53.85	*Thermoactinomyces sanguinis* (AJ251778)	99
	DQ-P-2 (5)	19.23	*Pantoea agglomerans* (FJ971873)	99
	DQ-P-3 (1)	3.85	*Weissella cibaria* (AB593341)	97
	DQ-P-4 (1)	3.85	*Leuconostoc mesenteroides* (CP003101)	99
	DQ-P-5 (2)	7.69	Uncultured clone LJQX-14 (KF984325)	97
	DQ-P-6 (1)	3.85	Uncultured clone 7s11 (DQ068858)	99
	DQ-P-7 (1)	3.85	*Enterobacter cloacae* (KJ126907)	99
	DQ-P-8 (1)	3.85	Uncultured clone 1-1B-19 (JF417910)	98
Xiaoqu	XQ-P-1 (1)	3.33	*Deinococcus radiodurans* (AE000513)	99
	XQ-P-2 (21)	70	*Weissella cibaria* (AB593341)	99
	XQ-P-3 (1)	3.33	*Streptococcus lutetiensis* (NR_121743)	99
	XQ-P-4 (1)	3.33	*Enterococcus casseliflavus* (NR_102793)	99
	XQ-P-5 (2)	6.67	*Corynebacterium variabile* (NR_102874)	99
	XQ-P-6 (3)	10	*Enterobacter ludwigii* (KM077046)	99
	XQ-P-7 (1)	3.33	*Acinetobacter baumannii* (LC014137)	99
Fuqu	FQ-P-1 (5)	16.67	*Bacillus cereus* (CP008712)	99
	FQ-P-2 (7)	23.33	*Clostridium beijerinckii* (CP006777)	99
	FQ-P-3 (14)	46.67	*Enterococcus faecium* (GU968165)	99
	FQ-P-4 (1)	3.33	*Bacillus subtilis* (JF346887)	99
	FQ-P-5 (1)	3.33	*Pediococcus pentosaceus* (AB598980)	100

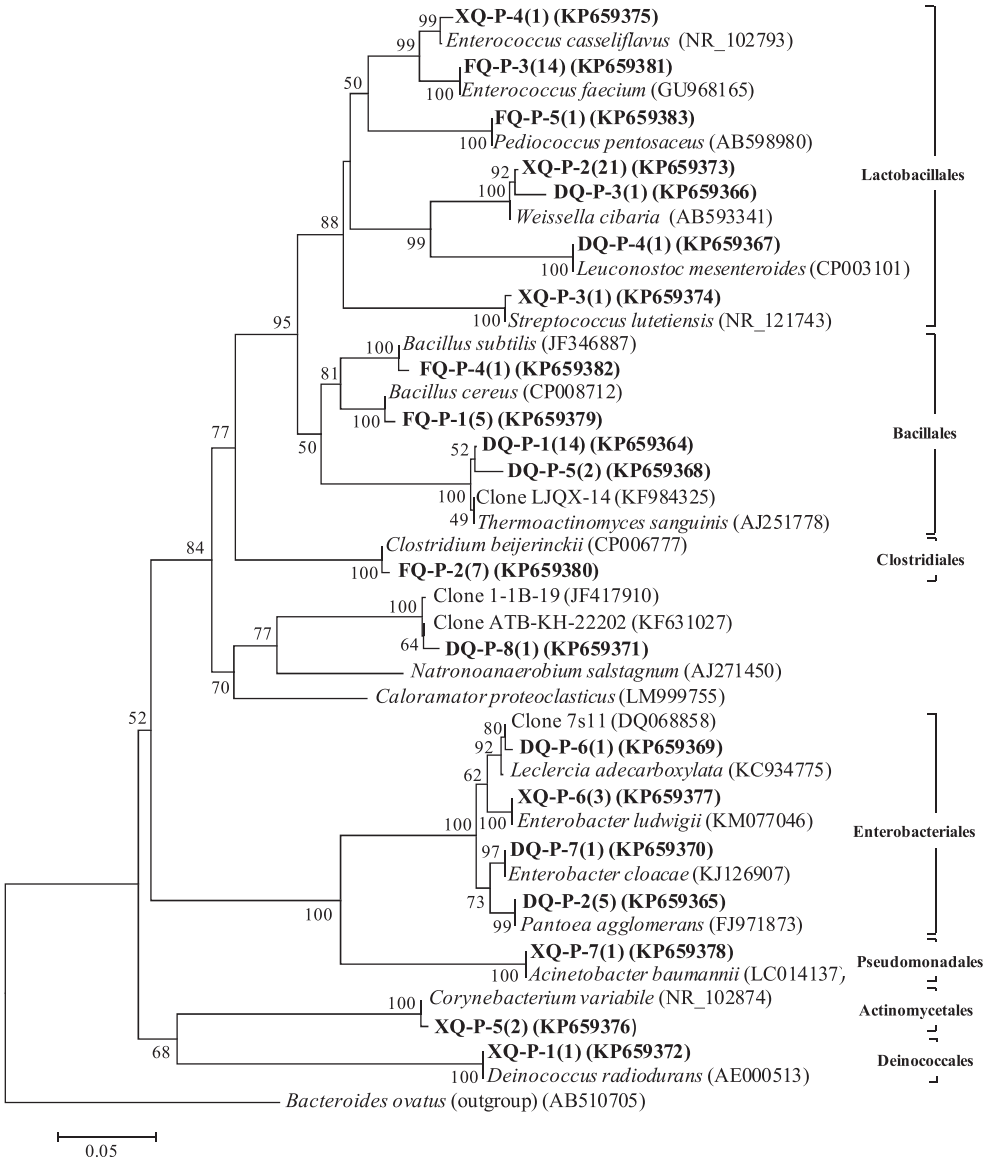

Figure 1. Phylogenetic tree demonstrating the genetic relationships among the bacterial clones from three fermentation starters used for Chinese liquor production. The tree was constructed by the neighbour-joining method with Kimura's two-parameter model using partial sequences of the 16S rRNA gene. The bootstrap analyses (1000 replications) were performed to test the confidence of nodes. The bar represents five substitutions per 100 nucleotide positions. Tree is rooted using *Bacteroides ovatus* as outgroup. DQ, XQ, FQ refer to the clones derived from *Daqu*, *Xiaoqu* and *Fuqu* samples, respectively. Numbers of clones with identical sequences in this study and accession numbers with all sequences are indicated in parentheses.

Table 3. Distribution of eukaryotic clones obtained from the three fermentation starters

Simple	Library OTU (clones)	Ratio (%)	Closest relative (accession no.)	Identity (%)
Daqu	DQ-E-1 (15)	62.5	*Aspergillus glaucus* (AY083218)	100
	DQ-E-2 (1)	4.17	*Rhizomucor pusillus* (HQ845298)	99
	DQ-E-3 (1)	4.17	*Lichtheimia corymbifera* (JQ004931)	99
	DQ-E-4 (3)	12.5	*Thermomyces lanuginosus* (AY706335)	99
	DQ-E-5 (3)	12.5	*Thermoascus crustaceus* (AY526486)	99
	DQ-E-6 (1)	4.17	*Wallemia sebi* (AF548108)	99
Xiaoqu	XQ-E-1 (25)	80.65	*Rhizopus oryzae* (AB250174)	99
	XQ-E-2 (6)	19.35	*Saccharomycopsis fibuligera* (KJ123706)	99
Fuqu	FQ-E-1 (7)	26.92	*Rhizopus oryzae* (AB250174)	99
	FQ-E-2 (19)	73.08	*Saccharomyces cerevisiae* (GQ458028)	99

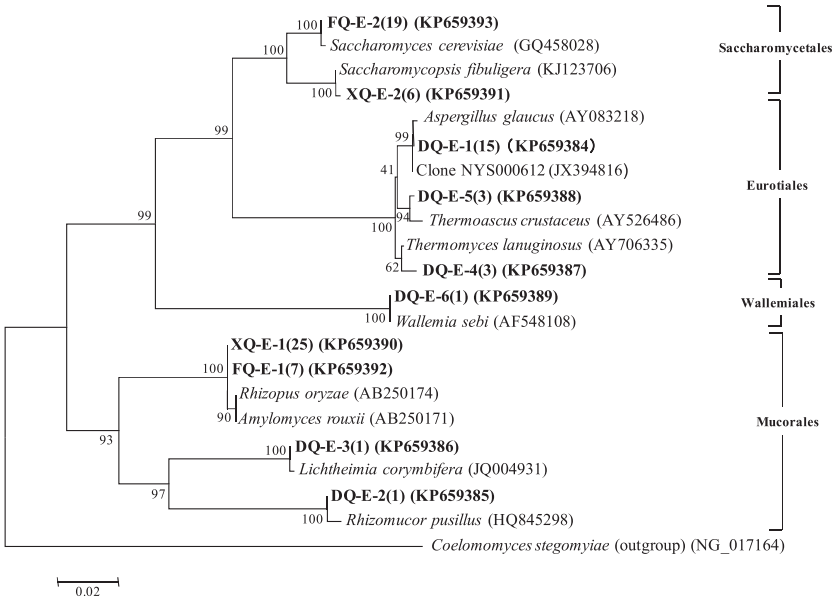

Figure 2. Phylogenetic tree demonstrating the genetic relationships among the eukaryotic clones from three fermentation starters used for Chinese liquor production. The tree was constructed using partial sequences of 18S rRNA gene. The bar represents two substitutions per 100 nucleotide positions. The tree is rooted by using *Coelomomyces stegomyiae* as outgroup. The other descriptions are the same as for Fig. 1.

majority of the clones were identified as Eurotiales fungi, including *Aspergillus glaucus* (62.5%), *Thermomyces lanuginosus* (12.5%), and *Thermoascus crustaceus* (12.5%). In addition, one Wallemiales clone and two Mucorales clones were affiliated with *Wallemia sebi*, *Lichtheimia corymbifera* and *Rhizomucor pusillus*, respectively. Similar fungal diversity was found in *Xiaoqu* and *Fuqu*, both of which contained mould and yeast clones. However, the most abundant fungi in *Xiaoqu* and *Fuqu* were the mould *Rhizopus oryzae* (80.65%) and the yeast *Saccharomyces cerevisiae* (73.08%), respectively, although *R. oryzae* was detected in both samples. *Saccharomycopsis fibuligera*, a non-*Saccharomyces* yeast, was observed only in *Xiaoqu*.

Discussion

Although the microbes present in *Daqu* used for liquor production have been observed in a number of studies, our knowledge regarding microbial composition in fermentation starters (*JiuQu*) is still quite limited, especially for *Xiaoqu* and *Fuqu*. Therefore, this study investigated and compared the microbial community in three types of *JiuQu* (*Daqu*, *Xiaoqu* and *Fuqu*) based on 16S and 18S rRNA gene clone library analysis.

Clone library analysis showed that bacterial species were more abundant than fungal species in *JiuQu* and that the bacterial community structure was unique for all three fermentation starters. Typical thermophilic or thermotolerant Bacillales, such as *Thermoactinomyces* and *Bacillus*, were observed in *Daqu* and *Fuqu*, suggesting that these two *JiuQu* are produced at higher temperatures than *Xiaoqu*. Several studies have reported that temperature greatly affects the microbial community structure of *Daqu (11,13)*. Therefore, temperature may be one of main factors contributing to the different bacterial communities in the three fermentation starters.

T. sanguinis was the main bacterial species in *Daqu*. It exhibits *Actinomycete*-like characteristics but is more closely related to *Bacillus* spp. based on phylogenetic relationship analysis *(21)*. *T. sanguinis* was identified as the dominant bacteria only in high- and ultrahigh-temperature *Daqu (13,19,22,23)* and may aid in forming flavour compounds by secreting alkaline phosphatase, esterase, lipase and phosphate hydrolase *(24)*. Enterobacteria, in particular *Pantoea agglomerans*, were the second most abundant species in *Daqu*. *Pantoea* spp. have been reported to exist in low, medium and high-temperature *Daqu* at low abundance and their function in liquor production are still unknown *(13,22)*. Maifreni et al. *(25)* identified the amino acid decarboxylase activity of a number of Enterobacteriaceae species in Montasio cheese, including *P. agglomerans*. In addition, a high ratio of uncultured clones (15.39%) was detected in *Daqu*. The uncultured Firmicutes clone (DQ-P-8) shared only 83% sequence similarity with pure-culture bacteria (*N. salstagnum*), suggesting that it may be a new species. These results indicate that uncultured bacteria play important and unknown roles in liquor production and the necessity for analysing the microbial community in *JiuQu* using culture-independent methods.

Many studies have demonstrated the abundance and importance of LAB in *JiuQu*; LAB can transform glucose or starch into lactic acid and provide substrates for yeasts to form ethyl lactate, one of the chief flavour components in Chinese liquor *(11)*. In the current study, various LAB genera were identified in the three types of *JiuQu*, including *Weissella*, *Enterococcus*, *Leuconostoc*, *Streptococcus* and *Pediococcus*. Although LAB is the most abundant bacteria in *Xiaoqu* and *Fuqu*, the bacterial species of the LAB were not identified. *W. cibaria* and *E. faecium* represented the largest LAB population in *Xiaoqu* and *Fuqu*, respectively. *W. cibaria* is a type of heterofermentative LAB widely detected during *Shochu* fermentation *(26)*. Liu et al. *(10)* inferred that *W. cibaria* may affect the textural properties and machinability of *Maotai*-liquor *Daqu* during shaping period of *Daqu* preparation. Several studies have indicated that the thermo-tolerant genus *Enterococcus* plays an important role in the flavour production of traditional fermented foods such as cheeses and sausages by proteolysis, lipolysis and citrate breakdown *(27)*. However, only Zheng et al. *(13)* have detected low numbers of *Enterococcus* clones in two types of *Daqu* samples. The roles of the abundant *E. faecium* in *Fuqu* require further investigation. Soria and Audisio *(28)* verified that the presence of *E.*

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faecium could inhibit the growth of *B. cereus* strains by antimicrobial metabolites. This might result in higher abundance of *E. faecium* than *B. cereus* in *Fuqu*. A number of reports have also indicated that *Bacillus* spp. could be inhibited by other species, such as *Lactobacillus* spp. *(29)*. Therefore, microbial interactions could also affect the microbial community in *JiuQu*. Another LAB found in *Fuqu*, *P. pentosaceus*, was previously detected in *Fen*-liquor *Daqu* used for flavour development through a culture-dependent method *(5)*. LAB was the dominant bacteria in many *Daqu* samples. However, only two LAB genera with low abundance (*L. mesenteroides* and *W. cibaria*) were identified in the *Daqu* sample in the present study. *L. mesenteroides* has been reported to possess high potential for producing tyramine or histamine in wine *(30)*. Although most studies revealed the presence of *Lactobacillus*. spp in other *Daqu*, no *Lactobacillus* clone was detected in the *Daqu* sample of this study. This result was similar with those reported by Zheng et al. *(13)* and Zhang et al. *(22)*, in which they found *Lactobacillus* spp. were only identified in medium- or low- temperature *Daqu* through clone library analysis. In addition, Lei *(31)* found a high abundance of LAB only at the beginning of certain types of *Daqu* production, which could be attributed to the increased temperature that resulted in the rapid growth of thermophilic bacteria compared with mesophilic LAB and might explain the low abundance of LAB in the final *Daqu* products.

In this study, *C. beijerinckii* was the second most abundant bacterium in the *Fuqu* sample; it has previously been reported to be capable of producing butanol from corn *(32)*. *Clostridia*, important contributors to that production of organic acids for liquor flavour formation, have been identified in the pit mud of *Luzhou*-flavour liquor *(33)*. *B. cereus* accounted for 16.67% of all bacteria identified in *Fuqu*. *Bacillus* species can produce various hydrolases, such as protease, cellulase and amylase, for the liquefaction and saccharification of carbohydrates *(34)*. In addition, they can produce nitrogenous pyrazine, which is one of the most important flavours in sauce- and sesame-flavor liquor *(4,9)*. However, *Bacillus* species are often detected in high-temperature *Daqu* owing to the formation of heat-resistant spores, which facilitate their survival under high-temperature (60–62°C) and low-moisture (13–14%) conditions *(2)*. In addition to the abundant LAB and *Enterobacter* sp. (10%), *C. variabile*, *A. baumannii* and *D. radiodurans* were present at low clone numbers only in *Xiaoqu*. *C. variabile* is often detected on cheese surfaces and contributes to the development of flavour and textural properties during cheese ripening *(35)*. A *Corynebacterium* clone was found in low-medium *Daqu* by Zheng et al. *(13)*. Actinobacteria have been detected in pit mud used for producing the flavour components of *Luzhou* liquor including esters, acids, aldehydes and ketones *(36)*. To the best of our knowledge, this is the first report of *D. radiodurans*, one of the most radiation-resistant microorganisms ever discovered, being detected in *JiuQu (37)*; its source and function in *Xiaoqu* is unknown.

A greater number of fungi species were detected in *Daqu*, all of which were thermophilic moulds. Three Eurotiales species (*A. glaucus*, *T. lanuginosus* and *T. crustaceus*) were found in *Daqu* with *A. glaucus* constituting the major fungi. Filamentous *Aspergillus* spp. have been reported as the common and dominant moulds in low-, medium- and high-temperature *Daqu*, contributing to the production of various hydrolytic enzymes for starch saccharification *(11,38,39)*. Starch cannot be utilized directly by most yeast and bacteria and needs be converted into sugars by filamentous fungi *(39)*. Xu et al. *(40)* revealed that the genera *Aspergillus* and *Rhizopus* are the two dominant fungi in different wheat varieties. Therefore, the abundant presence of this species in *Daqu* probably originates from wheat; most high-temperature *Daqu* is made from pure wheat with high concentrations of starch (40–60% of dry matter) *(4)*. It has been suggested that the raw materials used for *JiuQu* production could affect the fungal community. *Thermoascus* spp. are often found in high-temperature *Daqu*; however their functions in fermentation starters remain unknown *(10)*. *T. lanuginosus*, another thermophilic fungus able to survive at temperatures higher than 60°C, has been reported to be an efficient xylanase producer *(41)*. Other fungi such as *R. pusillus*, *L. corymbifera* and *W. sebi* were detected in *Daqu* with low clone numbers. *R. pusillus*, a thermophilic fungi, could affect liquor flavour by rapidly producing lactic acid, acid proteinase, glucanase and alcohol dehydrogenase *(42)*. *L. corymbifera* can grow at temperatures as high as 48–52°C and was detected during the last three stages of *Fen*-liquor *Daqu* fermentation *(43)*. *Wallemia* is a halophilic fungal genus *(44)*; however, its potential role in fermentation starters is not yet known.

It is widely established that the saccharification and alcoholic fermentation of Chinese liquor is accomplished by the combined activity of moulds and yeasts. However, no yeast species was found in the clone library of this *Daqu* sample. Xiong et al. *(2)* also found through DGGE analysis that the dominant fungi present in three types of high-temperature *Daqu* were moulds and not yeasts. These results suggested that the amounts of yeast may be less than that of mould in high-temperature *Daqu*, although there was some difference between the microbial communities obtained by different culture-independent methods. Generally, yeasts are more sensitive to heat and moisture than moulds. During the production of high-temperature *Daqu*, the temperature reaches $>$60°C with rapidly decreasing moisture, which might limit the growth and survival of yeast. Mohanty et al. *(48)* observed that higher temperature and lower moisture decreased *S. cerevisiae* activity. Therefore, this may well explain the absence of yeast species in the *Daqu* sample used in this study.

The fungal diversity in the *Xiaoqu* and *Fuqu* samples was similar and limited fungi species were observed (one mould and yeast). This may attributed to the similar production technology used for both starters – artificial inoculation of pure cultures (often mould and yeast strain) into the *JiuQu*, which is quite different from the spontaneous inoculation used during *Daqu* production *(6,7)*. *R. oryzae* was the most abundant mould detected in the *Xiaoqu* samples, consistent with previous results reported by Wang et al. *(46)*. *R. oryzae* is responsible for saccharification and proteolysis by secreting various types of extracellular enzymes, producing abundant volatile compounds from grains, such as ethanol, 2-methyl-1-butanol and 3 methyl-1-butanol *(47)*. The yeasts *S. fibuligera* and *S. cerevisiae* were observed in the *Xiaoqu* and *Fuqu* samples, respectively. It has been reported that, although a low level of *S. cerevisiae* accumulated during the production stage of *Daqu*, it could gradually become the dominant microbe and have a significant effect on subsequent ethanol production *(48)*. Therefore, the direct addition of *S. cerevisiae* in *Fuqu* could provide effective inoculation for liquor production. The absence of *S. cerevisiae* in the *Xiaoqu* sample may be due to their low level and therefore they could not be detected by the clone library analysis. Non-*Saccharomyces* yeasts (such as *S. fibuligera* and *Pichia anomala*) were detected in several starter samples. *S. fibuligera* has a strong saccharification ability and produces a large amount of α-amylase and glucoamylase for native starch conversion during the initial stages of liquor fermentation *(49)*. Although the effects of these non-*Saccharomyces* yeasts on liquor taste have attracted interest from liquor-making researchers, their function in liquor production remains poorly understood.

The microbial community in the three types of *JiuQu* differed significantly. Several researchers have found that the microbial community in *Daqu* is strongly influenced by temperature, moisture, raw materials, microhabitats and geographic location *(2,10,11,39)*. Zheng et al. *(13)* demonstrated that fungal diversity in *Daqu* is highly affected by production temperature and raw materials, while bacterial diversity is influenced by production temperature and geographic location. Similar results were observed in this study. In addition, microbial interaction and production technology may constitute other factors influencing microbial community in this study. The representative bacteria and fungi in each fermentation starter may be considered as biomarkers for distinguishing the three types of *JiuQu*.

In conclusion, to the best of our knowledge, this is the first report to compare the microbial community in three types of *JiuQu* (*Daqu*, *Xiaoqu* and *Fuqu*). The microbial communities in the three types of *JiuQu* differed significantly from each other. Mainly thermophilic or thermotolerant bacteria were identified in *Daqu* and *Fuqu*. *T. sanguinis*, *W. cibaria* and *E. faecium* were the most abundant bacteria in *Daqu*, *Xiaoqu* and *Fuqu*, respectively. Only thermophilic moulds were detected in *Daqu* and *A. glaucus* was the dominant fungi. Similar fungal diversity was found in *Xiaoqu* and *Fuqu*; both contained moulds and yeasts. The majority of fungi detected were *R. oryzae* and *S. cerevisiae* in *Fuqu* and *R. oryzae* and *S. fibuligera* in *Xiaoqu*. These differences may be ascribed to the different production temperatures, raw materials, moisture, microbial interactions and production technologies used during *JiuQu* production. These results may be useful for the improvement of liquor production and fermentation processes. Further research is required to establish the function of the microbiota in diverse types of *JiuQu*.

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