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ABSTRACT

Daqu is a traditional fermentation starter that is used for Chinese liquor production. Although partly mechanized, its manufacturing process has remained traditional. We investigated the microbial diversity of Fen-Daqu, a starter for light-flavour liquor, using combined culture-dependent and culture-independent approaches (PCR–DGGE). A total of 190 microbial strains, comprising 109 bacteria and 81 yeasts and moulds, were isolated and identified on the basis of the sequences of their 16S rDNA (bacteria) and 26S rDNA and ITS regions (fungi). DGGE of DNA extracted from Daqu was used to complement the culture-dependent method in order to include non-culturable microbes. Both approaches revealed that Bacillus licheniformis was an abundant bacterial species, and Saccharomycopsis fibuligera, Wickerhamomyces anomalus, and Pichia kudriavzevii were the most common yeasts encountered in Fen-Daqu. Six genera of moulds (Absidia, Aspergillus, Mucor, Rhizopus, Rhizomucor and Penicillium) were found. The potential function of these microorganisms in starters for alcoholic fermentation is discussed. In general the culture-based findings overlapped with those obtained by DGGE by a large extent. However, Weissella cibaria, Weissella confusa, Staphylococcus saprophyticus, Enterobacter aerogenes, Lactobacillus sanfranciscensis, Lactobacillus lactis, and Bacillus megaterium were only revealed by DGGE.

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1. Introduction

Chinese liquor is one of the world's oldest distilled alcoholic beverages, and plays an important role in Chinese culture and people's daily life. The total annual production of Chinese liquor was estimated at approximately 5 million metric tons per year (Anonymous, 2008). The popularity of Chinese liquor is in part due to the diversity of flavours. Chinese liquor flavours can be distinguished as strong-flavour [Nongxiang in Chinese] (Zhang et al., 2011a), light-flavour [Qingxiang in Chinese] and sauce-flavour [Jiangxiang in Chinese] (Shen, 2001; Wu et al., 2009; Zhang et al., 2011b). The liquor is made from cereals, mainly sorghum, by fermentation, distillation and maturation. Mixed culture starters (Fan et al., 2007), which are locally called "Daqu", are used as inoculum for the solid-state fermentation, as shown in Fig. 1. The cooked cereals are inoculated by mixing with approximately 9–10%

powdered Daqu and this mixture is fermented in sealed jars for 21–28 days at 10–16 °C. Daqu is an important saccharifying and fermenting agent for the production of Chinese liquor and traditional vinegar, and has a significant impact on the flavour of the final product (Shen, 2001; Zheng et al., 2011).

Daqu itself is also made by fermentation, which takes about one month. This is followed by a storage period of 6 months for maturation. The manufacture of Daqu by traditional uncontrolled solid-state fermentation techniques results in products with inconsistent quality; this causes large losses of unsuccessful products which go to waste as animal feed (Shen et al., 2005). Therefore standardization of the Daqu production process is an important objective for Chinese liquor producers. So-far, this has resulted in the application of modernized machinery, but little attention has been given so-far to the functional composition, i.e. the microbiota of Daqu, in view of controlling its fermentation.

Fen-Daqu is a light-flavour Daqu from Shanxi province, which is made from barley and pea, and contains around 50% of starch (Zheng et al., 2011). "Fen-type" liquor has a flavour that is described as pure and sweet, with a refreshing aftertaste (Xiong, 2005).
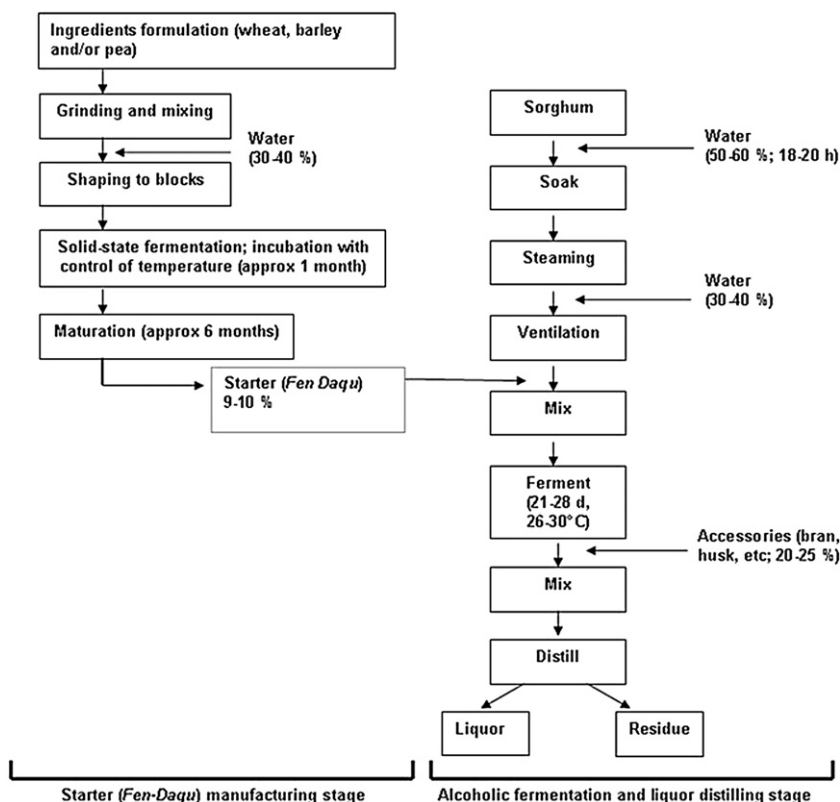
Fig. 1. Process diagram for the production of *Fen-Daqu* and its role in the production process of Chinese liquor.

During the production of *Daqu* by solid-state fermentation, the temperature in the *Daqu* blocks may increase up to 60 °C as a result of metabolic heat. In *Fen-Daqu* production, care is taken to restrict maximum temperatures to <50 °C by ventilation. Most microorganisms are expected to grow and survive these temperatures, and, therefore, *Fen-Daqu* is expected to contain a diverse microbial community.

Traditional microbiological methods such as enumeration using different general and selective media, can provide (i) quantitative data on the occurrence of different groups of microorganisms and (ii) isolated pure cultures for experimental fermentations. However, this classical culture-dependent approach only reveals the culturable microbes. Therefore, molecular microbiological methods such as analysis of direct DNA extracts by PCR-T/DGGE, pyrosequencing, sequencing of clone libraries, etc. are useful additions to realize rapid and high throughput microbial communities analysis (Aquilanti et al., 2007; Camu et al., 2007; Iacumin et al., 2009; Papalexandratou et al., 2011; Thanh et al., 2008). In this study we report an investigation of the microbial community in *Fen-Daqu* using combined culture-dependent and -independent approaches.

2. Materials and methods

2.1. Sample collection and storage of Daqu

Fen-Daqu was obtained from Shanxi Xinghuacun Fenjiu Group Company, the largest producer of light-flavour liquor. The dimensions of *Daqu* blocks are approximately 27 × 16 × 6 cm, weighing around 1.7 kg each. *Fen-Daqu* is fermented and matured in stacked layers of blocks. Three matured *Daqu* blocks (having been stored for 6 months of maturation) were randomly selected from upper, middle and lower layers in order to obtain an adequate

representation. Before performing microbiological analysis, each *Daqu* block was separated into two parts, namely its outer grayish-coloured surface layer of 1 cm thick, and the remaining brownish-coloured inner part. Both inner and outer parts were weighed before being ground to powder in an alcohol-disinfected grinder (Krups 75, model F203); the powder was kept in plastic jars at 4 °C during the experimental work.

2.2. Microbiological analysis

Samples of *Daqu* powder (10 g each) were transferred to stomacher bags and homogenized with 90 ml sterile PPS (Peptone Physiological Salt) solution containing (g/L) peptone (Oxoid LP0034) 1, and NaCl 8.5, in a Stomacher Lab-blender 400, twice 1 min at high speed. Appropriate serial dilutions were prepared using the same diluent. Unless otherwise indicated, 1 ml portions of the diluted suspension were mixed with molten (45 °C) medium and poured in duplicate counting plates of the media described below.

Total aerobic and anaerobic bacteria were enumerated on Plate Count Agar (PCA; Oxoid CM035) and Reinforced Clostridial Agar (RCA; Oxoid CM0151), respectively. Plates were incubated at 30 and 55 °C for 1–2 days.

For the enumeration of bacterial spores, the homogenized sample suspension was heated at 80 °C for 5 min. Serial dilutions were prepared from this heated suspension and mixed with PCA to which an additional top layer of 1.5% bacteriological agar (Oxoid, LP0011) was added to prevent spreading of colonies. Plates were incubated at both 30 and 55 °C for 2 days.

Lactic acid bacteria (LAB) were enumerated on MRSA (Oxoid CM0361) with 0.1% (w/v) natamycin (Delvocid, DSM, Delft, The Netherlands) to prevent yeast growth. Plates were incubated at 30 °C for 72 h. To confirm the presence of presumed LAB, Gram

staining and catalase reactions were carried out. The results were corrected, based on counting of Gram-positive and catalase negative organisms.

Enterobacteriaceae were counted with Violet Red Bile Glucose Agar (VRBGA; Oxoid CM0485). The medium was boiled twice before use. Plates were incubated at 37 °C for 24 h. To confirm the presence of Enterobacteriaceae, oxidase − and glucose fermentation tests on presumptive colonies were carried out. The results were corrected based on the counting of oxidase negative, but glucose fermenting colonies.

Yeasts and moulds were enumerated on three different media, namely Malt Extract Agar (MEA; Oxoid CM0059), Dichloran−Glycerol Agar (DG18; Oxoid CM0097) and Rose Bengal Chloramphenicol Agar (RBCA; Oxoid CM0549), to which 100 mg/L chloramphenicol (Oxoid, SR0078E) was added. The diluted suspension (0.1 ml) was added to make spread plates which were incubated at 25 °C for 2–4 days.

All enumerations of bacteria were carried out under both aerobic and anaerobic incubation conditions. Anaerobic growth conditions were achieved by flushing gas jars with a mixture of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen. All counts were made in duplicate for each of the duplicated samples and the results were reported as the means $\pm$ SD ($n = 4$). From plates with 20–300 colonies, the square root of the total number of colonies were randomly selected as isolates.

2.3. DNA extraction and PCR amplification

Genomic DNA of bacteria was extracted by a Bacteria Genomic DNA Purification Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The 16S rDNA gene was amplified using forward primer 5′-AACGCGAAGAACCTTAC-3′ and reverse primer 5′-CGGTGTGTACAAGACCC-3′ (Niemann et al., 1997). Amplifications were performed in the thermocycler GeneAmp PCR system 9700 (Applied Biosystems, USA) with an annealing temperature of 56 °C and using reagents and Taq DNA polymerase from Fermentas. The PCR products were analyzed by electrophoresis and stored at −20 °C for further sequencing analysis.

Genomic DNA of fungi was extracted by a Yeast Genomic DNA Purification Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The 26S rDNA and ITS regions were amplified by different primer sets. For yeast strains, forward primer NL1 (5′-TGCTGGAGCCATGGATC-3′) and reverse primer RLR3R (5′-GGTCCGTGTTTCAAGAC-3′) were used for amplification of the D1/D2 domain of the 26S rDNA gene; forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for amplification of the ITS 1 and ITS2 regions (Okoli et al., 2007). For moulds, forward primer V9G (5′-TTACGTCCCTGCCCTTTGTA-3′) and reverse primer LS266 (5′-GCATTCCCAAACAACTCGACTC-3′) were used for amplification of ITS1-5.8S-ITS2 (Zhang et al., 2011c). Amplifications were performed with an annealing temperature of 52 °C. The PCR products were analyzed as described before.

2.4. Sequencing and strain identification

The PCR products were purified using GFX columns (Amersham Pharmacia Biotech Inc., Roosendaal, The Netherlands). The bacterial and fungal rDNA was sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 3700 sequencer (Applied Biosystems, Foster City, CA). The sequences were compared with those present in the GeneBank/ NCBI database using the BLAST 2.0 program (Altschul et al., 1990). Sequences were assembled and edited with Seqman II software (DNAStar Inc., Madison, Wis.) and aligned with Megalign (DNAStar Inc., Madison, Wis).

2.5. DNA extraction and PCR−DGGE analysis

DNA from Fen-Daqu samples was extracted according to Wang et al. (2008b) and diluted to 1−50 ng/μL. Two sets of universal primers were tested for the amplification of fragments of the 16S rRNA and 26S rRNA genes, respectively. The V6−V8 region of the 16S rRNA gene was amplified using the primers EUB968GC-for and EUBL1401-rev (500 bp) (Nubel et al., 1996). The D1 region of the 26S rRNA gene was amplified using the primers NL1GC-for and LS2-rev (250 bp) (Flórez and Mayo, 2006). All PCR reactions were carried out on a AG 223B1 Thermoblock (Eppendorf, USA). Amplifications were performed as in Section 2.3. The sizes and quantities of the PCR products were determined using 1.5% agarose gel electrophoresis. The amplification products obtained as described above were subjected to DGGE analysis using the Dcode Universal Mutation Detection system (BioRad Laboratories, Richmond, CA, USA) on 20 cm × 16 cm × 1 mm gels. Electrophoresis was performed at 60 °C in 0.5 × TAE buffer (20 mM Tris-acetate, 2 mM EDTA; pH 8.0) using 8% polyacrylamide gels containing 30−60% urea−formamide linear denaturing gradient (100% corresponded to 7 M urea and 40% v/v formamide) increasing in the direction of electrophoresis for 16 h at 85 V. Following electrophoresis, the gels were stained by $AgNO_3$ solution as follows. The gel was fixed and shaken gently in Cairn's fixation solution (200 ml 96% ethanol; 10 ml acetic acid; 40 ml demi-water) for 3 min. The gel was transferred to a freshly made 2 g/l $AgNO_3$ staining solution and shaken gently for 10 min, followed by a brief rinse in water. The stained gel was developed in a freshly made developing solution (10 mg $NaBH_4$; 250 ml 1.5% NaOH solution; 750 μl formaldehyde) until the desired exposure was achieved. The gel was scanned with "Quantity One" software using a calibrated imaging densitometer GS-710 (BioRad, CA, USA). The identity of the microorganisms was revealed by sequencing selected bands from the DGGE profiles. Small fragments of selected DGGE bands were punched out from the gels, put in sterile water and boiled for 30 min to release DNA. The gel solutions were cooled and stored overnight. The obtained supernatant was used directly for re-amplification of the PCR products with primers described above without GC clamp.

2.6. Statistics

Statistical analysis was carried out using IBM-SPSS V19.0 (IBM® SPSS® Statistics; NY, U.S.A). A one-way ANOVA with Duncan's test was used to determine the significance of different microbial enumeration data.

3. Results

3.1. Enumeration of representative bacteria and fungi on selective media

The population levels of bacteria and fungi that were present in the inner and outer portions of the Fen-Daqu starter are summarized in Table 1. The total viable numbers of mesophilic and thermophilic bacteria in the inner and outer parts were not significantly different; whereas the numbers of bacterial spores were significantly higher in the inner part. The numbers of mesophilic bacteria, including total viable cells, LAB and Enterobacteriaceae, were similar when grown under aerobic or anaerobic conditions. However, total numbers of thermophilic bacterial spores were about 1 Log CFU/g higher when grown aerobically, especially for the inner portion. The levels of LAB and Enterobacteriaceae were similar in both inner and outer portions of Daqu. The total counts of yeasts and moulds in inner Daqu were about 1 Log CFU/g lower than in outer Daqu. No statistically significant differences were

Table 1
Microbiota of inner and outer parts of *Fen-Daqu* presented as Log CFU g^{-1}.

Log CFU g^{-1}	Aerobic counts		Anaerobic counts	
Microbial groups	Inner	Outer	Inner	Outer
Mesophilic bacteria	7.5 ± 0.4^{def}	7.5 ± 0.6^{def}	7.4 ± 0.2^{cdef}	7.7 ± 0.2^{ef}
Mesophilic bacterial spores	7.1 ± 0.6^{bcdef}	6.6 ± 0.2^{bcd}	7.7 ± 0.3^{ef}	6.8 ± 0.4^{bcdef}
Thermophilic bacteria	7.4 ± 0.1^{cdef}	6.9 ± 0.4^{bcdef}	7.5 ± 0.2^{cdef}	6.7 ± 0.5^{bcde}
Thermophilic bacterial spores	7.8 ± 0.3^{f}	6.7 ± 0.3^{bcde}	7.2 ± 0.2^{bcdef}	6.4 ± 0.5^{bc}
Lactic acid bacteria	4.0 ± 0.4^{a}	4.9 ± 0.3^{a}	4.0 ± 0.3^{a}	4.8 ± 0.2^{a}
Enterobacteriaceae	4.0 ± 0.6^{a}	4.8 ± 0.8^{a}	4.0 ± 1.2^{a}	4.8 ± 0.7^{a}
Fungi on MEA 25 °C	6.2 ± 0.7^{b}	7.1 ± 0.6^{bcdef}		
Fungi on MEA 37 °C	6.7 ± 1.2^{bcde}	6.9 ± 1.6^{bcdef}		
Fungi on DG18 25 °C	6.3 ± 0.5^{bcd}	7.1 ± 0.3^{bcdef}		
Fungi on DG18 37 °C	6.6 ± 0.7^{bcd}	7.1 ± 1.2^{bcdef}		
Fungi on RBCA 25 °C	6.7 ± 0.5^{bcde}	7.7 ± 0.6^{ef}		
Fungi on RBCA 37 °C	6.7 ± 0.5^{bcde}	7.6 ± 0.4^{def}		

Values represent means ± SD (n = 4). Means with different superscripts are significantly different (One-Way ANOVA; P < 0.05).

found for fungal counts when grown at 25 °C or 37 °C. We noticed that isolation using RBCA resulted in higher number of countable colonies if compared to the other two media used, viz. MEA and DG18. Bacteria were generally numerically dominant microorganisms in *Fen-Daqu*, followed by yeasts and moulds. The numbers of fungal colony forming units were more than 2 Log CFU/g higher than those of LAB and Enterobacteriaceae.

3.2. Bacterial diversity based on identification of 16S rDNA

A total of 109 bacterial strains were randomly selected and identified by sequencing the 16S rDNA. Thirteen species were encountered in *Fen-Daqu*. The results are presented in Table 2. *Bacillus* spp. were predominant in *Fen-Daqu*. Particularly, *Bacillus licheniformis* and *Bacillus subtilis* together represented about 57% of the bacteria isolated from inner and outer portions of *Fen-Daqu*. In addition, *Brevibacterium* sp., LAB, i.e. *Enterococcus faecalis*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*, represented 4, 17, 6, and 6% of the total number of isolates, respectively. Other bacteria such as *Salmonella enterica* and *Escherichia coli* were also encountered in outer *Daqu*.

3.3. Fungal diversity based on identification of D1/D2 domain of 26S rDNA and ITS region

A total of 81 fungal strains were randomly selected and identified by rDNA sequencing as shown in Table 3. *Absidia corymbifera*,

Table 2
Bacterial diversity in *Fen-Daqu*.

Species identification	Related GenBank sequence	% of bacterial isolates (n = 109)	Isolated from[a]	Identity
Bacillus cereus	EU111736	1	O	380/380(100%)
Bacillus licheniformis	AF399743	39	I & O	393/393(100%)
Bacillus pumilus	EU874880	1	I	387/392(98.7%)
Bacillus subtilis	FJ225312	18	I & O	392/392(100%)
Brevibacterium sp.	EU596384	4	O	396/396(100%)
Escherichia coli	EU026432	1	O	364/365(99.7%)
Enterococcus faecalis	AB507170	17	I & O	389/391(99.5%)
Lactobacillus plantarum	FJ749885	6	O	362/363(99.7%)
Leuconostoc citreum	FJ040203	1	O	364/366(99.5%)
Micrococcus luteus	FJ380953	1	O	381/382(99.7%)
Pediococcus pentosaceus	FM179609	6	I & O	352/352(100%)
Pseudomonas aeruginosa	GQ180118	2	O	369/370(99.7%)
Salmonella enterica	FJ997268	3	O	343/343(100%)

n = the total number of isolates.
[a] I/O: isolated from Inner or Outer part of *Fen-Daqu*.

Aspergillus flavus, *Rhizopus stolonifer* and *Saccharomycopsis fibuligera* occurred throughout the inner and outer portions of *Fen-Daqu*. Others were found only in the outer portion, namely *Mucor circinelloides*, *Penicillium commune*, *Rhizomucor variabilis* var. *regularior*, *Pichia kudriavzevii* (formally known as *Issatchenkia orientalis*), *Wickerhamomyces anomalus* (formally known as *Pichia anomalus*) and *Saccharomyces cerevisiae*. One species, *Rhizomucor pusillus* (1%), was isolated from the inner portion. The predominant isolates consisted of *S. fibuligera* (56%), *A. corymbifera* (11%), *W. anomalus* (8%), *P. kudriavzevii* (6%) and *R. stolonifer* (6%).

3.4. Analysis of bacterial and fungal populations using denaturing gradient gel electrophoresis (DGGE)

DGGE analysis of the amplified 16S rDNA fragments obtained from the samples of the inner and outer portions of *Fen-Daqu* provided the fingerprint shown in Fig. 2. Up to twelve bands, representing 10 different species were detected in the polyacrylamide gel. *Bacillus* spp., *Lactobacillus* spp. and *Weissella* spp. were the dominant bacterial species. A higher bacterial diversity was found in the outer layers of *Daqu* when compared to the inner part, as evidenced by the presence of *Staphylococcus saprophyticus*, *Lactobacillus sanfranciscensis* and *Bacillus megaterium* in the outer layers. DGGE fingerprints of the amplified fungal 26S rDNA fragments with primers NL1GC and LS2 are shown in Fig. 3. The identified mycobiota in the *Fen-Daqu* was less complex than the bacterial biota. Only four fungal species were detected, namely *W. anomalus*, *S. fibuligera*, *P. kudriavzevii* and *Debaryomyces hansenii*. The fungal biota of inner and outer *Daqu* layers were largely similar.

4. Discussion

The bacteria of *Fen-Daqu* represent higher numbers than the yeasts and moulds. It should be realised however that since yeast cells are larger than bacterial ones, this doesn't imply that bacteria have a larger metabolic impact on the eco-physiology or biochemistry of *Daqu*. Studies on other types of *Daqu*, i.e. light-flavour and strong-flavour *Daqu* types (Leimena, 2008; Qiao et al., 2004; Zhang, 1999) also showed the presence of high numbers of bacteria. This high level (7–8 Log CFU/g) of especially thermophilic bacteria and spores in *Daqu* makes this starter different from other Asian traditional alcoholic fermentation starters such as Men (Dung et al., 2007; Thanh et al., 2008) and Ragi (Hesseltine et al., 1988) of which the bacterial loads of 2.6–6.2 Log CFU/g and 4.3–5.8 Log CFU/g, respectively, represented mainly lactic acid bacteria.

Combining the results of culture-dependent and -independent analyses, we found that the outer layers of *Fen-Daqu* have a broader microbial biodiversity and higher numbers of mesophilic microorganisms than the inner part, which contained less species and higher numbers of thermophilic microorganisms, mostly *Bacillus* spp. This observation is consistent with those of previous studies (Shi et al., 2009; Wang et al., 2008a, b).

This "microbiota stratification" within the blocks of *Daqu* may be explained by the changes of temperature that take place during the processing of *Fen-Daqu* (Zheng et al., 2011), as well as the characteristics of the solid-state fermentation of *Daqu*, which results in mass - and heat gradients causing relatively high temperatures in the centre (inner portion) of the blocks (data not shown).

Fen-Daqu is a representative of light-flavour *Daqu*. During its manufacture by solid-state fermentation, the maximum temperature in the centre of the *Daqu* blocks during fermentation does not exceed 50 °C (Kang, 1991). Most bacteria tolerate these temperatures and, therefore, a wide range of bacterial species was observed in this type of *Daqu*, particularly at the somewhat cooler outside.

Table 3
Fungal diversity in *Fen-Daqu*.

Group of fungi	Species identification	Related Genbank sequence	%fungal population ($n = 81$)	Isolated from[a]	Identity
Filamentous fungi	*Absidia corymbifera*	AB305110	11	I/O	757/758(99.9%)
	Aspergillus flavus	FJ878681	4	I/O	651/655(99.4%)
	Mucor circinelloides	DQ118990	1	O	638/638(100%)
	Penicillium commune	AF236103	3	O	669/676(99.0%)
	Rhizomucor pusillus	AB369914	1	I	605/606(99.8%)
	Rhizomucor variabilis var. regularior	DQ119007	2	O	528/530(99.6%)
	Rhizopus stolonifer	DQ273817	6	I/O	242/244(99.2%)
Yeasts	*Pichia kudriavzevii*	AY939808	6	O	547/547(100%)
	Saccharomyces cerevisiae	EU798694	2	O	652/654(99.7%)
	Saccharomycopsis fibuligera	FJ475057	56	I/O	728/728(100%)
	Wickerhamomyces anomalus	EF449518	8	O	558/558(100%)

$n =$: the total number of isolates.
[a] I/O: isolated from Inner or Outer part of *Fen-Daqu*.

B. subtilis and *B. licheniformis* were the dominant members from the inner portion. Bacterial DGGE patterns also revealed the presence of *Weissella cibaria* and *Weissella confusa*. Interestingly, the total viable counts in the inner part of *Daqu* were sometimes lower than those of bacterial spores present, which may have been caused by heat-activation of spores during the preparatory heat treatment at
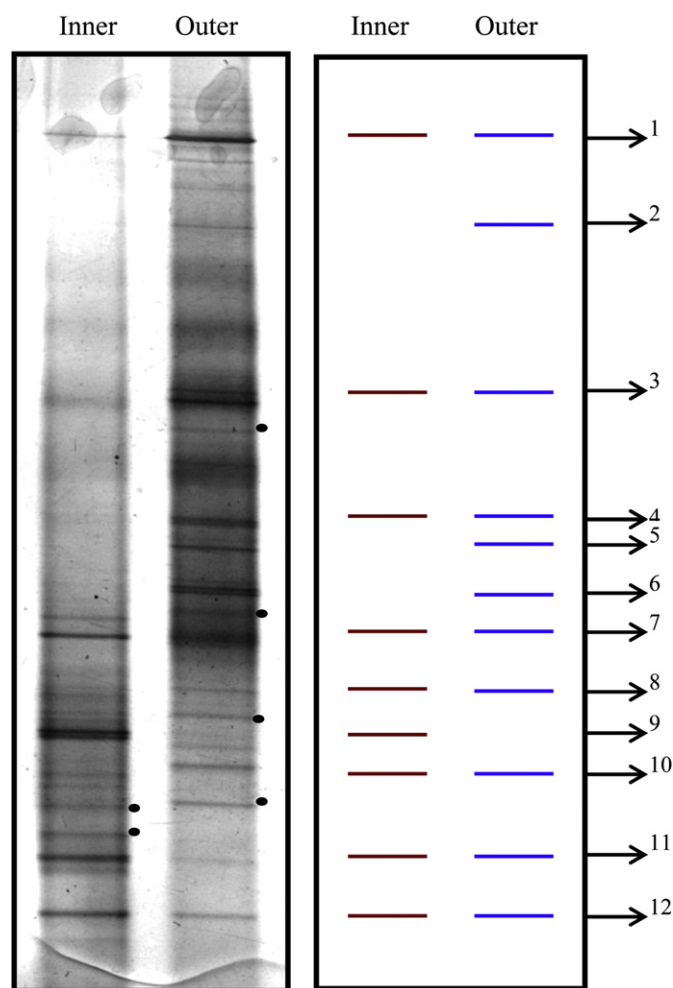

Fig. 2. DGGE profiles (30–60% denaturant) representing bacterial 16S rRNA gene fragments of *Fen-Daqu* samples. 1, *Weissella cibaria*; 2, *Staphylococcus saprophyticus*; 3, *Enterobacter aerogenes*; 4, *Lactobacillus sanfranciscensis*; 5, *Lactobacillus lactis*; 6, *Bacillus megaterium*; 7, *Lactobacillus plantarum*; 8, *Weissella confusa*; 9, *Uncultured bacterium*; 10, *Bacillus licheniformis*; 11, *Weissella cibaria*; 12, *Weissella confusa*. The dotted bands had also been excised but could not be identified since no amplicons were obtained.

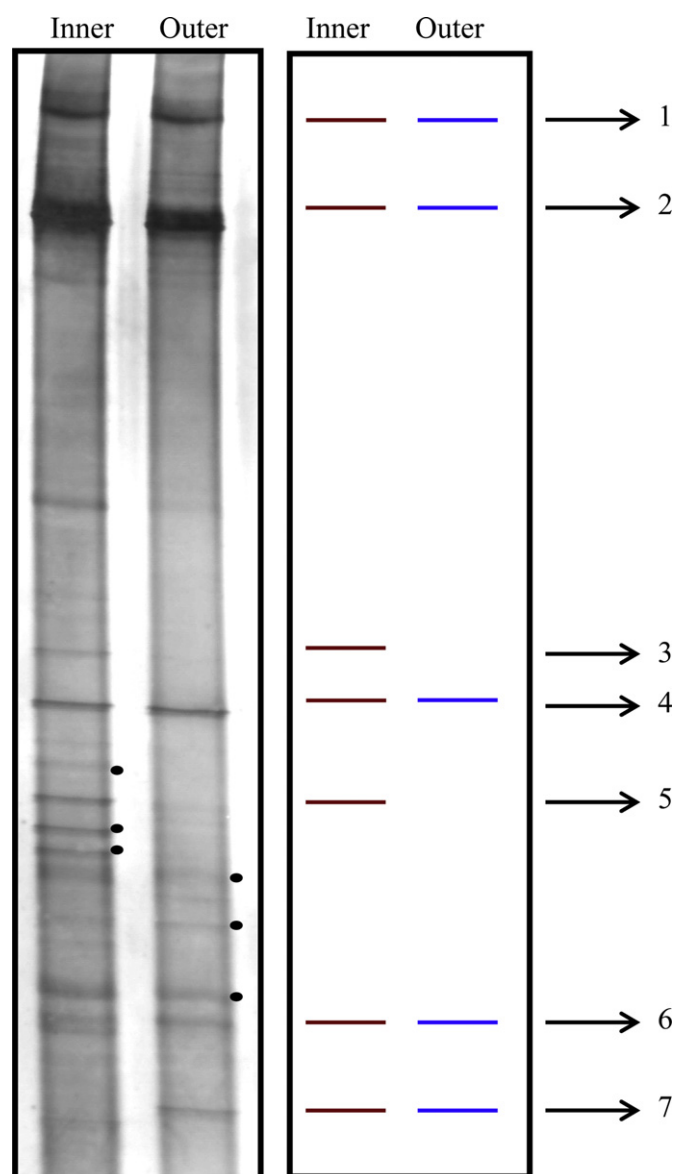**Fig. 3.** DGGE profiles (30–65% denaturant) representing 26S rRNA gene fragments of *Fen-Daqu* samples. 1, *Pichia kudriavzevii*; 2, *Saccharomycopsis fibuligera*; 3, *Wickerhamomyces anomalus*; 4, *Debaryomyces hansenii*; 5, *Pichia kudriavzevii*; 6, *Wickerhamomyces anomalus*; 7, *Pichia kudriavzevii*. The dotted bands had also been excised but could not be identified since no amplicons were obtained.

80 °C for 5 min before plating (Morn et al., 1990). We note that spore-forming bacteria are the predominant microbiota of Fen-Daqu, especially in the inner part.

The analyses of microbial communities in Fen-Daqu were carried out by both culture-dependent and -independent approaches. The Bacillus spp. encountered in Fen-Daqu were B. licheniformis, B. subtilis, Bacillus pumilus, B. megaterium and Bacillus cereus. Of these, B. licheniformis was most prevalent with 39% strains identified and the species was detected by both approaches. B. subtilis was another dominant species, with 18% identified strains, but it could not be detected by DGGE. In comparison, the method used by Shi et al. (2009) encountered two Bacillus spp. in Fen-Daqu namely B. licheniformis and Bacillus decisifrondis.

The functionality of the bacterial biota needs further investigation. B. subtilis, B. licheniformis and B. pumilus may facilitate the conversion of starch into fermentable carbohydrates due to their amylolytic activity, thus generating a suitable substrate for the second stage of liquor production, i.e. the alcoholic fermentation (Mukherjee et al., 2009; Wang et al., 2008a). In addition, Bacillus spp. produce nitrogenous flavour compounds such as diverse pyrazines (Li et al., 2011; Zhang et al., 2011c; Zheng et al., 2011) and we expect that these to contribute to Daqu flavour.

Several lactic acid bacteria viz., Ent. faecalis, Pc. pentosaceus, Lb. plantarum, Lb, lactis, Leuc. citreum, W. cibaria and W. confusa were identified that commonly occur in fermenting matter (Nout, 2009). Their contribution to the flavour development of Fen-Daqu or the final fermented liquor, for example, due to the formation of organic acids and other flavour compounds (Huang et al., 1993; Li and Ren, 2005; Wee et al., 2008) requires further attention.

Other bacteria including E. coli, Ps. aeruginosa, Micrococcus luteus, and S. enterica occurred in low numbers. Although we did not study their effects on quality or safety of Daqu we suggest that, because of their low numbers, they are not essential for the fermentation process, and that they originate from external contamination sources, such as air, soil, hands, or insects. Their presence, however, is not expected to pose a safety problem, as after fermentation, the product will be distilled to obtain the final liquor. During the steam distillation the cells of bacteria and fungi will be killed. Moreover, the alcohol concentration of the crude distillate is approximately 70% v/v, which is adequate to practically sterilize the liquor.

In this study we used three mycological media (MEA, DG18 and RBCA) (Boekhout and Robert, 2003) to increase the detection of diverse fungal biota. In Daqu analysis, RBCA was superior for yeasts and moulds enumeration, mainly because distinct individual colonies were obtained and spreading of mould colonies was inhibited due to the presence of rose bengal (Baggerman, 1981). DG18 proved most appropriate to distinguish between yeasts and moulds, although yeasts were occasionally overgrown by spreading fungi, such as Mucor spp.

In contrast with the distinct bacterial population, the yeast microbiota identified from Fen-Daqu is more similar to that described from other Asian traditional alcoholic starters (Hesseltine et al., 1988; Jeyaram et al., 2008; Saelim et al., 2008; Sujaya et al., 2004; Tsuyoshi et al., 2005). These studies revealed that S. fibuligera occurred commonly in these starters, and this species plays an important role during the initial stages of alcoholic fermentation. S. fibuligera typically grows prior to the main alcoholic fermentation and produces various enzymes, particularly glucoamylase and α-amylase, which contribute to glucose accumulation (Brimer et al., 1998; Horváthová et al., 2004; Knox et al., 2004; Lemmel et al., 1980; Steverson et al., 1984). It was also found that glucoamylase produced by S. fibuligera can digest native starch (Chi et al., 2009), which improves the degradation of starch from the raw materials (i.e. barley and pea) of Daqu. S. cerevisiae usually dominates in alcoholic fermentations (Li et al., 2011; Nout, 2009; Urso et al., 2008) as it has the ability to grow under strictly anaerobic conditions. However, in the DGGE profiles, S. cerevisiae was not detected and using isolation only one strain of S. cerevisiae was obtained. This demonstrates that S. cerevisiae is not a dominant yeast species in Daqu. Recently, a study of Li et al. (2011) investigated the species that involved in-situ Fen-liquor fermentation. This result indicated that the major active yeast species during this fermentation process was S. cerevisiae. The dominance of S. cerevisiae in alcoholic fermentation might be due to its competitive growth in the presence of fermentable sugars and its ethanol tolerance. We expect that it could grow quickly and become dominant during the liquor fermentation stage, such as has been observed in various wine fermentations (Dung et al., 2006, 2007; Jeyaram et al., 2008; Nyanga et al., 2007; Sujaya et al., 2004). Or it might be the host yeast species recycled in the fermentation jars. Mohanty et al. (2009) investigated the effect of different environmental factors on the S. cerevisiae fermentation. They observed that the combination of higher temperatures and lower moisture contents decreased the fermentation efficiency and ethanol production. During the Daqu production process, the fermentation temperature reached as high as above 55 °C, with moisture contents decreasing to around 10% at the end of maturation. Such conditions could be expected to limit the growth and survival of S. cerevisiae. This may thus well explain the low numbers of S. cerevisiae in matured Daqu. Other yeasts, such as P. kudriavzevii and W. anomalus, were observed in Daqu as well by both detection approaches. These yeast species are common in cereal fermentations (e.g. men, hamei, wheat qu and zaopei) and in combination with LAB, have been associated with the production of flavour and ethanol (Dung et al., 2006; Jeyaram et al., 2008; Nout, 2009; Thanh et al., 2008; Xie et al., 2007; Zhang et al., 2007).

Six genera of moulds, viz., Absidia, Aspergillus, Mucor, Penicillium, Rhizomucor and Rhizopus were obtained by direct plating. The predominant moulds in Fen-Daqu were A. corymbifera and R. stolonifer (Mucoraceae), which are known to be strong amylase producers (Blandino et al., 2003; Hesseltine et al., 1988; Thanh et al., 2008). M. circinelloides, Rhizomucor variabilis var. regularior, R. pusillus and P. commune were also isolated. In other fermentations, these moulds were responsible for volatile production during fermentation, such as ethanol, 2-methyl-1-butanol and 3-methyl-1-butanol (Sunesson et al., 1996, 1995; Wang et al., 2008a). In our analysis of Fen-Daqu, A. flavus was found as well. This species is known to produce aflatoxins (Degola et al., 2007) and, therefore, it may be prudent to check for the presence of mycotoxins in Daqu and derived products. Sometimes, the DGGE method using total DNA extracted from complex food products is unable to detect some fungal species, whereas these could be detected by culturing and identification by DNA sequencing. Cocolin et al. (2001) and Prakitchaiwattana et al. (2004) reported that individual species in a mixed population could be detected by DGGE when the concentrations were higher than 10^4 CFU/g, which may be the threshold level to detect species. Fen-Daqu is a kind of solid cake covered with mycelia of white moulds that mainly belong to the so-called zygomycetes. It seems that DNA of this group of fungi is more difficult to extract, even when pure cultures are used (Hrncirova et al., 2010). We assume that the absence of bands for this group of fungi in our DGGE gel is mainly caused either by (i) inadequate DNA extraction of filamentous fungi from the complex food matrix of Daqu, or (ii) by the presence of high concentrations of competing DNA, such as that from yeasts in Fen-Daqu.

Presently, the study of microbial diversity can be undertaken with culture-dependent and/or culture-independent analyses. Both approaches have their advantages and disadvantages. From our

study, we conclude that the culture-dependent analysis of *Daqu* samples resulted in a different and more complex microbiota than did DGGE analysis. A clear advantage of culturing is that a collection of pure cultures is obtained that can be used for further experimentation. A number of species (*B. licheniformis*, *Lb. plantarum*, *S. fibuligera*, *P. kudriavzevii* and *W. anomalus*) were detected using both approaches, that in total revealed 30 species, most of which were found by either the culture-dependent or culture-independent technique. This shows that although PCR–DGGE analysis provides a broad picture of the different groups of microorganisms present in *Daqu*, including unculturable ones, a combined approach is preferred for the detection of dominant and minor species in order to better understand the complete microbial ecosystem present in such natural mixed fermentation starters.

Fen-Daqu is a representative of low-temperature *Daqu*. Although the manufacturing techniques have been modernized during recent years, the inconsistency and instability of *Daqu* is still a problem that hampers the standardization of Chinese liquor making. Our study provides qualitative and quantitative information on the microbial diversity present in *Fen-Daqu*. The knowledge of microbial composition and functionality will enable further upgrading of the Chinese traditional *Daqu* making processes, e.g. by selection of functionally important strains, and optimization of microbial composition and quality control.

Acknowledgements

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