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ORIGINAL ARTICLE

Characterization of activity and microbial diversity of typical types of Daqu for traditional Chinese vinegar

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Abstract Daqu is a fermentation starter used in traditional Chinese vinegar production and determines the quality of the final vinegar products. In this study, the activity and microbial diversity of three typical types of Daqu were investigated using a nested PCR–denaturing gradient gel electrophoresis (DGGE) and plating technique. The bacterial DGGE profile and plate analysis indicated that the bacterial composition of Daqu was dominated by *Bacillus* sp., *Pantoea* sp., *Saccharopolyspora* sp., *Enterobacter cloaceae* and *Cronobacter sakazakii*, and lactic acid bacteria including *Weissella* sp., *Pediococcus pentosaceus*. DGGE analysis of fungal plates identified *Aspergillus niger*, *Absidia corymbifera*, *Rhizopus* sp. and *Penicillium* sp. as the predominant fungi involved in Daqu. However, DGGE results indicated that the bacterial microbiota of QC (Qing-Cha) and HX (Hong-Xin) were more complex than that of HR (Huai-Rang) Daqu. The total populations of microorganisms in QC were about 1 log CFU g^{-1} higher than in HR and HX Daqu. Moreover, the saccharifying power of QC was significantly higher than in HR and HX Daqu, and the liquefying power of QC was significantly higher than in HR, but slightly lower than in HX Daqu. Our results indicate that microbial diversity plays a crucial role in determining the activity of typical types of Daqu.

Keywords PCR-DGGE · Microbial diversity · Activity · Daqu · Vinegar · Fermentation starter

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Introduction

Vinegar is consumed worldwide as a food seasoning and plays an important role in people's daily lives. In China, vinegar is brewed traditionally from cereal with the addition of a vinegar fermentation starter, known locally as Daqu. Generally, Daqu is prepared by spontaneous solid-state fermentation in a traditional four-stage process (Zheng et al. 2011): (1) material mixing and shaping; (2) inoculation and enrichment of the microbe in its surrounding; (3) spontaneous solid-state fermentation with incubation at a controlled temperature (the most important factor in determining the quality of Daqu as the dominant microorganisms can be enriched by controlling the temperature); and (4) drying and ripening.

Traditional Chinese vinegar fermentation employs liquefying and saccharifying enzymes including α-/β-amylase and glucoamylase, which play a crucial role in productivity and directly affect the flavor formation of vinegar. Daqu starter is a primary liquefying and saccharifying agent for the fermentation of traditional vinegar. Thus, the types and activities of liquefying and saccharifying enzymes originating from microbes involved in Daqu (Xiu et al. 2012) play a significant role in determining the activity of liquefying and saccharifying power, which finally contribute to the quality of the fermented vinegar. Recent studies have described the microbial diversity of traditional fermentation starters, including Chinese traditional liquor (Li et al. 2014a) and rice wine fermentation starter (Lv et al. 2013), Korean meju (Kim et al. 2011), Japanese koji (Yoshikawa et al. 2010), and Vietnamese banh men (Thanh et al. 2008). These studies have given us a better understanding of the relationship between the microbial diversity in the starter and its special flavor and characteristics. Nowadays, the microbial diversity and dynamics that occur throughout vinegar fermentation have been well investigated (Haruta et al. 2006); however, there is little information on the activity and microbial diversity of typical types of traditional

Chinese vinegar Daqu, despite the fact that Daqu is especially prominent in the fermentation of vinegar.

Daqu starters play a key role in determining the flavor and characteristics of vinegar. Traditionally, the use of three typical types of Daqu, i.e., Qing-Cha (QC), Hong-Xin (HX) and Huai-Rang (HR), as starter cultures contributes to the unique production technology of Chinese light aroma style, sauce-flavor style and strong aroma style vinegar, respectively (Zheng et al. 2011). The three typical types of Daqu starters can be distinguished according to the maximum incubation temperature during Daqu preparation, which range between 53 and 55 °C, 55 and 58 °C and 58 and 60 °C, respectively. Temperature control during Daqu production plays a key role in influencing the richness and structure of the microbial community. According to previous results (Xiu et al. 2012; Lv et al. 2013; Zhang et al. 2014), different types of Daqu have specific microbial compositions, and the microbial diversity of typical types of Daqu impart unique flavors to the vinegar and affect the quality and characteristics of the final products. Consequently, it is important to analyze the microflora of Daqu starter in order to provide high quality products for consumers.

Molecular techniques for the detection, identification and characterization of microorganisms in foods are currently welcomed and considered indispensable tools for the description of food microflora (Porcellato et al. 2012). Culture-dependent methods, such as traditional plate techniques using different selective media and nucleotide sequence analysis of 16S/18S rRNA genes, can provide quantitative data on the main groups of microorganisms. However, culture-dependent methods reveal only culturable microbes. Therefore, in order to circumvent this limitation, culture-independent methods are widely employed in food microbiology. In our previous research, we found that low population levels and variations in abundance of fungal species in Daqu play a crucial role in tampering with the quality of the resulting vinegar (Li et al. 2014b). In this study, we performed nested PCR–denaturing gradient gel electrophoresis (PCR–DGGE) combined with a plate method to investigate the microbial diversity in typical types of vinegar Daqu. In addition, chemical activity analysis was performed to complement analysis of the relationship between activity and the microbial diversity of Daqu.

Materials and methods

Sample collection and preparation

The three typical types of traditional fermentation starters—QC, HX and HR Daqu samples—used in this study were obtained from a vinegar-production factory in Shanxi province, China. The moisture content of Daqu samples is about 12 %. In order to obtain adequate information and representation before carrying out microbiological analysis, six pieces of each type of Daqu sample were selected randomly from upper, middle and lower locations in the storage room, ground to powder in a sterile grinder and mixed as a sample. All samples were stored in a refrigerator at 4 °C and subjected to microbial analysis within 24 h, and at −20 °C until activity analysis.

Enumeration, isolation and identification of culturable microbes

For the enumeration and isolation of microorganisms using the plate method, 10 g Daqu samples were transferred to sterile conical flasks and homogenized with 90 mL 0.85 % NaCl solution for 30 min at 150 rpm. Appropriate serial 10-fold dilutions were prepared using the same diluent, then 100 μL portions of the diluted suspension were spread onto Luria Bertani agar [LB, 1 % tryptone (Oxoid, Basingstoke , UK), 0.5 % yeast extract (Oxoid), 1 % NaCl and 1 % agar], de Man, Rogosa and Sharpe agar (MRS; Difco, Detroit, MI) and Potato Dextrose agar (PDA; Difco), respectively. LB plates were incubated at 37 °C for 1–3 days, and MRS and PDA plates were incubated at 30 °C for 2–5 days. After incubation, colonies of variable bacteria and fungi were picked and microbial enumeration was performed on selective media based on their morphological features and phenotypic tests (Li et al. 2014b). After purification by streaking, the isolates were stored at −80 °C with 25 % glycerol for sequent analysis. Sampling was performed in triplicate.

For sequencing analysis of isolates, genomic DNA of bacteria and fungi was extracted from pure cultures of isolates using a Bacterial Genomic DNA Extracting Kit (PuBoXin, Beijing, China) and Soil DNA Kit (Omega, Norcross, GA) according to the manufacturer's instructions. The genomic DNA was then used as a template for PCR to amplify the bacterial 16S rRNA and fungal 18S rRNA using the primers 27 F (5′-AGAGTTTGATCCT GGCTCAG-3′)/1492R (5′-GGCTACCTTGTTACGACTT-3′) (Lane 1991) and NS1 (5′-GTAGTCATATGCTTGTCTC-3′)/FR1 (5′-AICCATTC AATCGGTAIT-3′) (Vainio and Hantula 2000), respectively. Amplified 16S and 18S rRNA fragments were analyzed electrophoretically on 1 % (w/v) agarose gels. After purification with a Gel Extraction Kit (Omega), the 16S rRNA and 18S rRNA fragments were ligated into pMD18-T vector (TaKaRa, Dalian, China) and then sequenced by Invitrogen (Shanghai, China). The sequences obtained were compared to the GenBank database with the BLAST program. A phylogenetic tree was constructed using software MEGA5 (http://www.megasoftware.net/) with 1,000 repetitions.

DNA extraction of total DNA and PCR amplification

For the DGGE analysis of bacterial diversity in Daqu, direct DNA extraction from Daqu powder was performed using a Soil DNA Kit (Omega) according to the manufacturer's

instructions. PCR amplification of 16S rRNA was conducted using the primers 27F/1492R (Lane 1991) in the first PCR step, followed by nested PCR (Kim et al. 2010) using DGGE primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 518R (5′- ATTACCGCGGCTGCTGG-3′) (Cocolin et al. 2001) to obtain profiles of the V3 region of 16S rRNA. For DGGE analysis of fungal diversity in Daqu, 18S rRNA was amplified using the primers NS1/FR1 (Vainio and Hantula 2000) in the first PCR step. Subsequently, the initial products of the first PCR were diluted and used as a template for nested PCR using DGGE primers NS3 (5′-GCAAGTCT GGTGCCAGCAGCC-3′)/YM951R (5′-TTGGCAAATGCT TTCGC-3′). A GC-clamp (5′-CCCGCCGCGCGCGGCG GGCGGGGCGGGGGCACGGGGGG-3′) (Xu et al. 2011) was attached to the 5′ end of primers 338F and NS3 to create a DNA fragment suitable for DGGE analysis. PCR amplification was performed in 50 μL reaction mixtures containing 25 μL Premix *ExTaq* (TaKaRa), 2 μL of each primer (20 μM) and 3 μL template DNA. A touch-down PCR program was carried out with an initial denaturation step of 98 °C for 5 min, followed by 20 cycles of denaturation at 98 °C for 45 s, annealing temperature starting at 65 °C for 45 s and decreasing by 0.5 °C /cycle, and 72 °C for 1 min for extension. This step was followed by 15 cycles of 98 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min before holding at 16 °C. PCR products were visualized by 1 % agarose gel electrophoresis before DGGE analysis.

PCR-DGGE analysis

The DGGE was performed with a Dcode System apparatus (Bio-Rad, Hercules, CA). The PCR products were applied onto 8 % (w/v) polyacrylamide gels in 0.5× TAE running buffer. For the DGGE analysis of bacterial communities in vinegar, a denaturing gradient ranging from 20 % to 60 % denaturant (100 % denaturant was defined as 7 M urea and 40 % formamide) was used. For the DGGE analysis of microbial diversity in Daqu, a denaturing gradient from 20 % to 40 % denaturant was applied. The gels were electrophoresed at 160 V at 60 °C for 4.5 h, followed by silver staining for 15 min. The gel image was scanned on a HP ScanJet G4050 scanner (Hewlett Packard, Palo Alto, CA). Selected bands were excised from the gel using a clean blade and eluted by incubation in 30 μL sterile distilled water at 4 °C overnight to allow diffusion the DNA and then stored at −20 °C for further analysis.

Sequencing, phylogenetic analysis and accession numbers

For sequencing, the DNA was re-amplified with the same primers without the GC clamp. After purification, the PCR products were inserted into pMD18-T vector (TaKaRa) and sequenced by Invitrogen. The sequences obtained were compared to the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using software MEGA 5 with 1,000 repetitions. The sequences reported in this study were deposited with GenBank. The sequences obtained from DGGE are under accession numbers KP119777–KP119807, KJ158323–KJ158325, KJ158328–KJ158332 and KJ158343–KJ158344, and those of the isolates are under accession numbers KP119808–KP119823.

Activity analysis of Daqu samples

For evaluating the activity and quality of Daqu samples, amino acid nitrogen content (g 100 g^{-1}), saccharifying power (U g^{-1}) and liquefying power (U g^{-1}) were determined according to the general methods of determination for industrial enzymes (Ministry of Light Industry of China 1993).

Results and discussion

Enumeration and identification of isolates

The results of total microbial counts on selective media are presented in Fig. 1. The total levels of microbes in HR (Huai-Rang) and HX (Hong-Xin) were not significantly different, whereas the total populations of bacteria, lactic acid bacteria (LAB), yeasts and molds in QC (Qing-Cha) were about 1 Log CFU g^{-1} higher than in HR and HX Daqu, and were about 5.0, 4.2 and 4.2 Log CFU g^{-1} on LB, MRS and PDA agar, respectively.

For sequencing analysis, a total of 96 isolates was picked randomly, including 44 isolates from LB plates, 32 isolates (gram-positive and catalase-negative) from MRS plates and 20 isolates from PDA plates, and subjected to preliminary

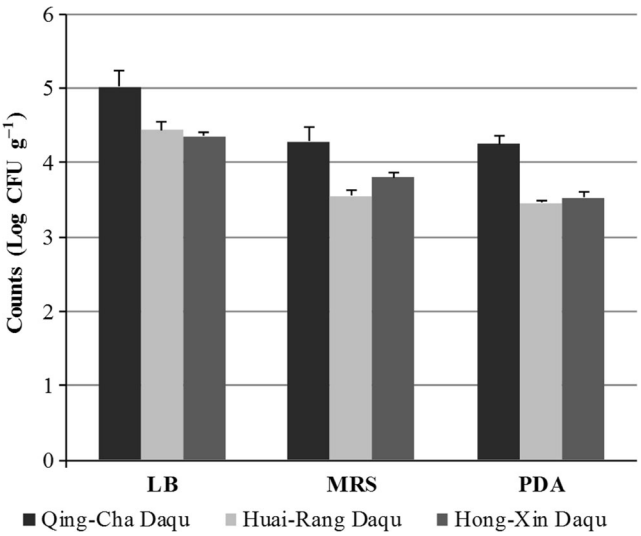

Fig. 1 Total microbial counts on selective media in typical types of Daqu starters. Microbial counts are given in Log_{10} CFU g^{-1}

identification based on morphological differentiation and phenotypic tests. A total of 16 isolates was subjected to sequencing analysis, according to the phylogenetic analysis: the 16 isolates were identified as *Bacillus licheniformis*, *B. amyloliquefaciens*, *Pediococcus pentosaceus*, *Cronobacter sakazakii*, *Enterobacter hormaechei*, *Absidia corymbifera*, and *Saccharomycopsis fibuligera* (Fig. 2). All of these species were detected in the three typical types of Daqu samples except *B. licheniformis*, which was not detected in QC Daqu (Fig. 2).

Bacterial diversity of typical types of Daqu

From bacterial DGGE profiles, slight differences in band distribution were observed between QC and HX; however, a distinctively higher bacterial diversity was found in QC and HX as compared to HR Daqu. Bands 1–4 were detected only in HR, and bands 18–32 were only detected in QC and HX Daqu (Fig. 3a).

A total of 32 strong bands were selected for amplification and sequencing. The sequences obtained were assigned into four orders: *Enterobacteriales*, formed by the genera *Enterobacter*, *Pantoea*, *Escherichia* and *Cronobacter*; *Actinomycetales*, integrated by the genera *Streptomyces* and *Saccharopolyspora*; *Lactobacillales*, composed by the genera *Weissella* and *Pediococcus*; and *Bacillales*, combined with the genera *Bacillus* and *Staphylococcus* (Fig. 4). According to the relative intensity of DGGE bands, *Bacillus* sp., *Weissella* sp., *Saccharopolyspora* sp., *Pantoea* sp., *Pediococcus pentosaceus*, *E. cloaceae* together with *C. sakazakii* were the main representative bacteria in the three typical types of Daqu.

Among the bacteria detected in Daqu, *B. licheniformis* was detected by PCR-DGGE but not detected by plate analysis in QC Daqu, which was consistent with the results of an earlier report (Bae et al. 2006) and discussed previously (Prakitchaiwattana et al. 2004), possibly due to different affinities of primer DNA to template DNA for different species and competing influences when template DNAs are present in different amounts. *Bacillus* sp. including *B. licheniformis* and *B. amyloliquefaciens*, could secrete a variety of hydrolytic enzymes, such as amylases and proteases (Zheng et al. 2012), which can play an important role in the liquefaction and saccharification process, generating suitable substrates for alcoholic fermentation and leading to flavor formation (Zheng et al. 2011). The main LAB species detected in Daqu starters were *Weissella* sp. and *Pediococcus pentosaceus*, which are the major LAB in fermenting matter (Nout 2009) and cheese (Tzanetakis and Litopoulou-Tzanetaki 1989). LAB play a strong role in improving the taste of vinegar, which can produce lactic acid from glucose or starch, and provide substrates for esterification of yeasts, affecting both the technological properties and microbial stability of the final products through the production of lactic acid (Yousif et al. 2010). Meanwhile, LAB might affect the growth of yeasts and molds, which might contribute significantly to the quality of the fermented vinegar. However, in addition to *Bacillus* and LAB species, other bacteria, such as *Enterobacter cloaceae*, *Escherichia coli*, *Cronobacter sakazakii*, *Pantoea* sp. and *Staphylococcus* sp., which are involved in the koji-making process of Douchi (Chen et al. 2011), were also detected and occupied a dominant position in the bacterial community in Daqu. *Saccharopolyspora* sp.

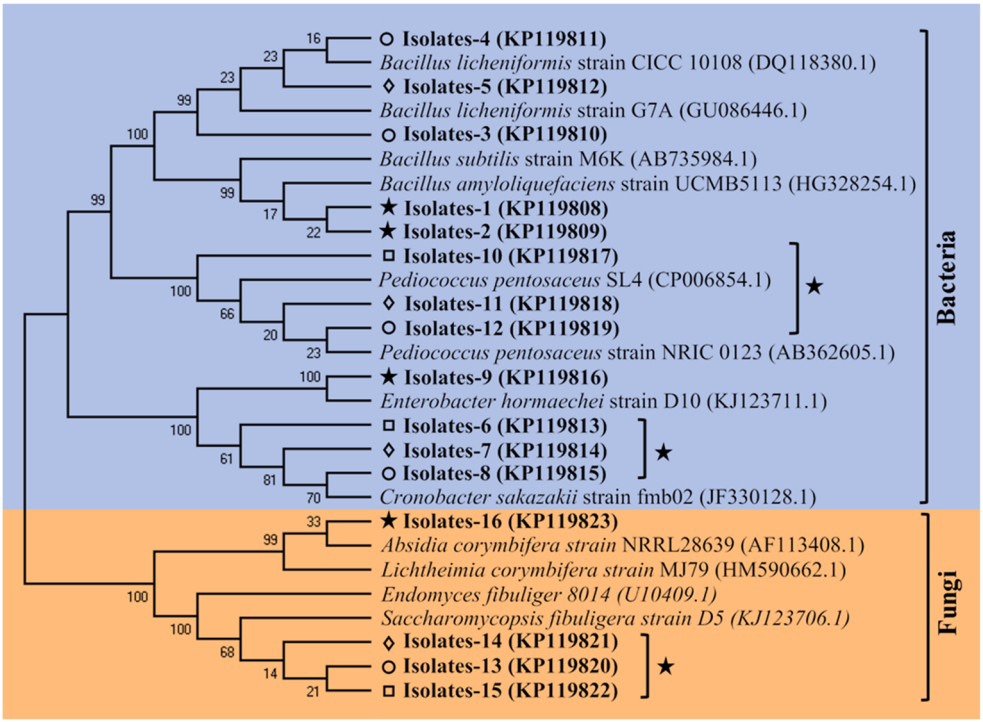

Fig. 2 Phylogenetic tree based on 16S and 18S rRNA fragments sequences of the isolates from Daqu and reference sequences from GenBank. The tree was constructed using neighbor-joining method with 1,000 repetitions. *Squares* Isolated from QC Daqu, *circles* isolated from HX Daqu, *diamonds* isolated from HR Daqu, *stars* detected in all of the three types of Daqu

including *Saccharopolyspora rectivirgula*—one of the major agents responsible for farmer's lung disease, a form of hypersensitivity pneumonitis (Duchaine et al. 1999)—was newly detected in vinegar Daqu starters. These bacteria are widely known for their pathogenicity and are considered as food-borne bacterial pathogens; however, some pathogens could also contribute to flavor formation, for instance, *Enterobacter* sp. can also produce amylase and lipase (Sinkuniene et al. 2008), and *Staphylococcus* sp. helps to produce volatile fatty acids (Wah et al. 2013). Thus, further investigation is required to evaluate the detailed role and function of these pathogens in Daqu.

Fungal diversity of typical types of Daqu

From the fungal DGGE profiles, nine strong bands were selected for amplification and sequencing. Bands 1, 2, 3, 5 and 6 were detected in all three typical types of Daqu; however, band 4 was detected only in HX and bands 7, 8 and 9 only in HR (Fig. 3b). The sequences obtained were grouped into five families. Bands 1 and 5 (similar to *Aspergillus niger*), band 8 (near to *A. oryzae*) together with band 9 (close to *Penicillium* sp.) were classified as *Aspergillaceae*. Band 4, as *Saccharomycopsis fibuligera*, was identified as *Saccharomycopsidaceae*. Band 7, close to *Pichia kudriavzevii*, was detected as *Pichiaceae*. Band 2, corresponding to *Absidia corymbifera*, was detected as *Lichtheimiaceae*. Bands 3 and 6, near to *Rhizopus microsporus* and *R. azygosporus*, were relatives of *Rhizopodaceae* (Fig. 5).

In the case of the fungal community, *Absidia corymbifera*, *Aspergillus niger* and *Rhizopus* sp. were the most frequent and predominant species detected in all three typical types of Daqu starters. Of these, *Absidia corymbifera* is considered as a strong amylase producer (Thanh et al. 2008) and significant contributor to vinegar fermentation. Meanwhile, *Aspergillus niger* and *Rhizopus* sp. also have the ability to secrete large amounts of various enzymes into their environments (Oda et al. 2006). Therefore, we expected that *Absidia corymbifera*, *Aspergillus niger* and *Rhizopus* sp. would be the main contributors to the saccharifying and liquefying power in Daqu. In constrast to the fungal microbiota identified in the three typical types of Daqu starters, other alcoholic starters (Hesseltine

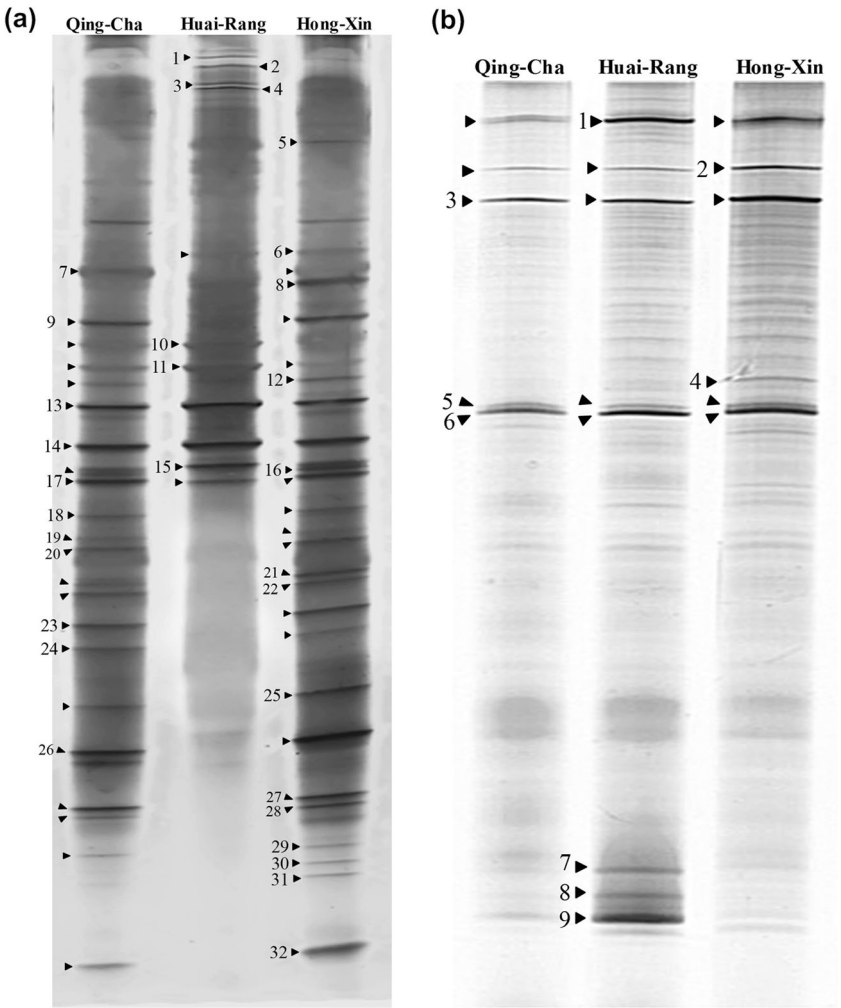

Fig. 3 Denaturing gradient gel electrophoresis (DGGE) profiles representing **a** bacterial 16S rRNA gene fragments, and **b** fungal 18S rRNA gene fragments of the three typical types of Daqu starters. A 20–60% denaturing gradient for bacteria and a 20–40% denaturing gradient for fungi were used. All bands indicated were excised but amplicons were obtained only for those with numbers

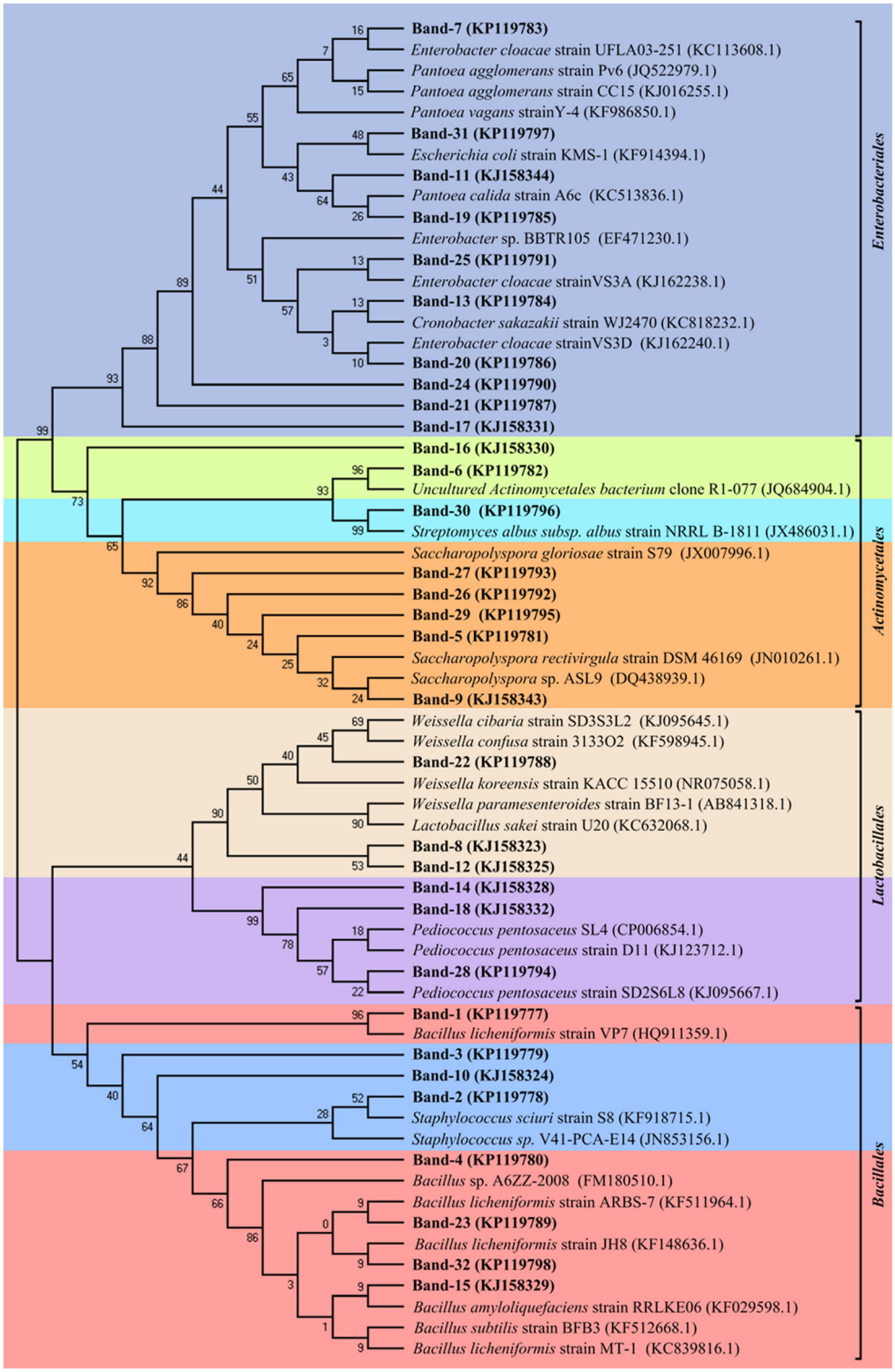

Fig. 4 Phylogenetic tree based on sequences of the bacteria from Daqu and reference sequences from GenBank. The tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA) method with 1,000 repetitions

et al. 1988; Sujaya et al. 2004; Jeyaram et al. 2008) often commonly include *Saccharomycopsis fibuligera*—a species that produces various enzymes including glucoamylase and α-amylase and plays an important role during the initial stages of alcoholic fermentation (Jeon et al. 2009). *Pichia kudriavzevii* is common in cereal fermentations and, in combination with LAB, has been associated with the production of flavor and ethanol (Nout 2009). However, *Saccharomycopsis fibuligera* was detected only in HX, whereas *Pichia kudriavzevii* and *Penicillium* sp. were detected only in HR. *Penicillium* sp. also possess proteolytic activity and contribute to the taste and aroma of fermented sausages (Sunesen and Stahnke 2003).

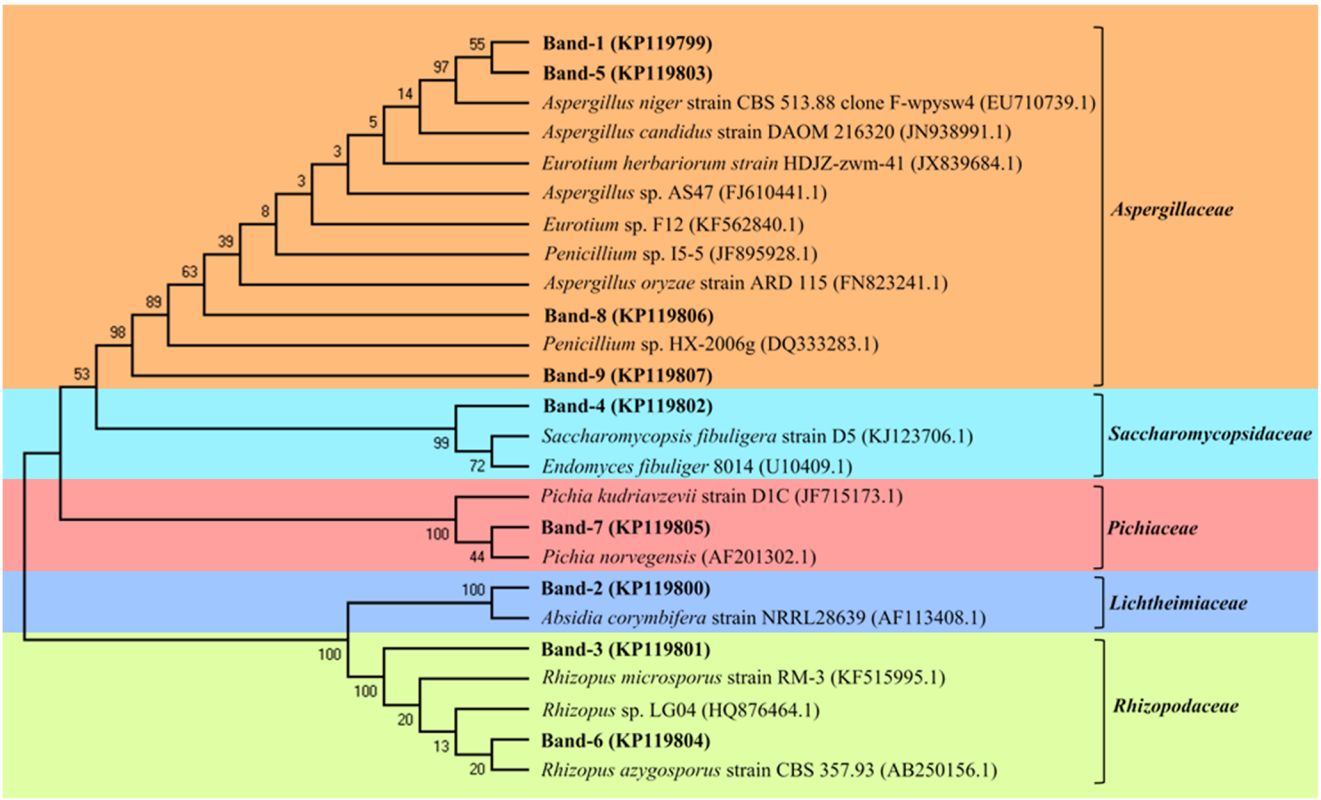

Fig. 5 Phylogenetic tree based on sequences of the fungi from Daqu and reference sequences from GenBank. The tree was constructed using the neighbor-joining (NJ) method with 1,000 repetitions

These results indicate that *Saccharomycopsis fibuligera*, *Pichia kudriavzevii* and *Penicillium* sp. may play a crucial role in flavor formation in HX and HR, respectively.

Temperature was an important technological parameter affecting the growth and death of microorganisms and finally determining the microbial structure and community in Daqu starters. The effect of temperature on bacteria was weaker than that on yeast and molds (Wang et al. 2011). In this study, we observed distinct differences in bacterial structure and diversity between QC or HX and HR Daqu. Meanwhile, the DGGE results revealed that the bacterial microbiota were more complex than the fungal microbiota. In addition, *Saccharomycopsis fibuligera* was detected only in HX, whereas *Pichia kudriavzevii* and *Penicillium* sp. were detected only in HR. According to the maximum temperatures reached during the incubation period, we presumed that incubation temperature

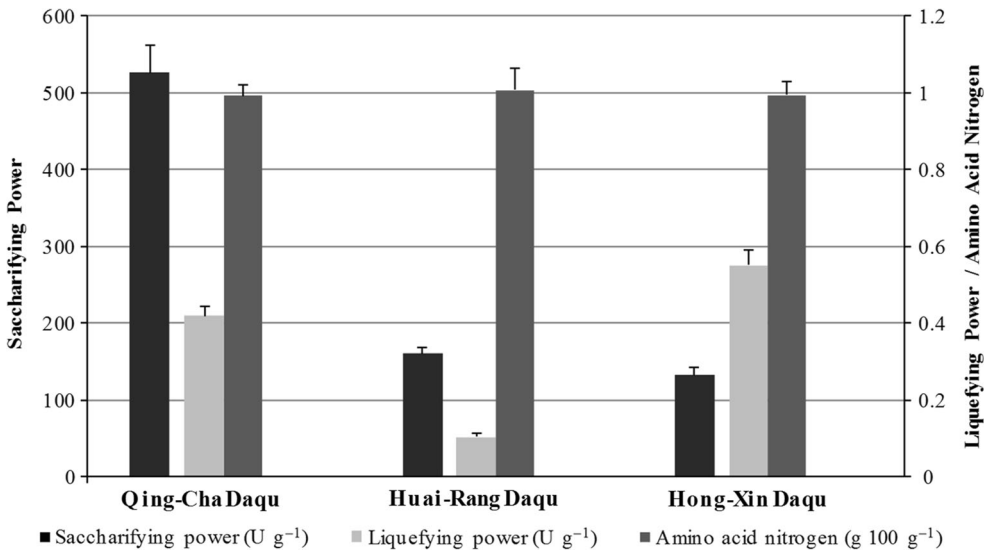

Fig. 6 The liquefying and saccharifying activity of typical types of Daqu starters

during Daqu production played a crucial role in the microbial diversity and structure in Daqu.

Activity of typical types of Daqu

To evaluate the effect of microbial community on the activity of three typical types of Daqu, the activities of saccharifying power and liquefying power were also determined; these exhibited significant differences in the three typical types of Daqu (Fig. 6). The saccharifying power of QC was about 524.8 U g^{-1}, i.e., significantly higher than in HR (159.6 U g^{-1}) and HX (131.7 U g^{-1}). The liquefying power of QC was about 0.42 U g^{-1}, which was slightly lower than in HX (0.55 U g^{-1}), whereas significantly higher than in HR (0.10 U g^{-1}). Based on the aforementioned results and discussion, there are several possible explanations for these discrepancies: (1) the total populations of microbes in QC were higher in QC than in HR and HX Daqu (Fig. 1); (2) a distinctively higher bacterial diversity and complexity existed in QC and HX as compared to HR Daqu (Fig. 2); (3) the difference in incubation temperature during Daqu production influenced the microbial community in Daqu and finally affected the activity of three typical types of Daqu. Thus, we concluded that microbial diversity and structure in Daqu plays a decisive role in determining the activity of Daqu, and a positive correlation between microbial diversity and activity of typical types of Daqu was expected. However, no significant difference in amino acid nitrogen content was found among the typical types of Daqu, which indicated that there were only slight flavor differences in the three Daqu starters. By contrast, the obvious difference in liquefying and saccharifying powers, which resulted from the predominant microbial communities, determined the decomposition ability of macro-molecular materials and yield of metabolites, and finally affected flavor formation in the final vinegar products.

In conclusion, this study provided more comprehensive information on the microbial diversity and activity in the three typical types of Daqu. Our study provides qualitative and quantitative information on the microbial diversity present in Daqu. This knowledge of microbial composition, functionality, and activity can give us novel insights into microbial structure and activity, and enable further upgrading of Chinese traditional Daqu-making processes. However, in order to determine the exact functional microorganisms involved in Daqu, more detailed studies should be performed further in the future.

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Conflict of interest The authors declare no conflict of interest.

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