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World Journal of Microbiology and Biotechnology

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https://doi.org/10.1007/s11274-014-1776-z

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World J Microbiol Biotechnol (2015) 31:199–208
DOI 10.1007/s11274-014-1776-z

ORIGINAL PAPER

Characterization of the microbial community in different types of *Daqu* samples as revealed by 16S rRNA and 26S rRNA gene clone libraries

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Received: 18 August 2014 / Accepted: 7 November 2014 / Published online: 14 November 2014
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Abstract *Daqu* is a fermentative saccharification agent that is used to initiate fermentation in the production of Chinese liquor and vinegar. Different types of *Daqu* can be distinguished based on the maximum fermentation temperature, location of production, and raw materials used. We aimed to characterize and distinguish the different types of *Daqu* using a culture-independent cloning method. The lowest microbial diversity was found in *Daqu* produced at high-temperature. Principal component analysis (PCA) was used to compare the bacterial composition of *Daqu* from different regions (i.e., northern *Daqu* and southern *Daqu*). *Staphylococcus gallinarum* and *Staphylococcus saprophyticus* were found in southern *Daqu*, and were absent in northern *Daqu*. The fungi *Saccharomycopsis fibuligera* and *Lichtheimia ramosa* dominated in low/medium-temperature *Daqu,* whereas *Thermomyces lanuginosus* occurred in high-temperature *Daqu*. Our study identified potential biomarkers for the different types of *Daqu,* which can be useful for quality control and technology development of liquor or vinegar production.

Keywords Starter · Bacterial community · Fungal community · PCA · Clone library · Biomarker

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Introduction

Chinese liquor and vinegar have a long history of production and consumption and are produced through unique brewing processes. They are typically produced from cereals, such as sorghum, by solid-state fermentation using a natural fermentation starter termed *Daqu*. *Daqu* comprises a microbial community and is rich in enzymes. *Daqu* is made by a natural fermentation process running for a few weeks such that a microbial succession occurs (Zheng et al. 2011, 2014). Being a major source of microorganisms and enzymes, *Daqu* is crucial for the quality, safety, and flavor of its derived products, such as liquor and vinegar. Different types of *Daqu* can be distinguished (Zheng et al. 2011), for instance, according to the maximum incubation temperature during the fermentation. *Daqu* can be grouped into three classes based on the production temperature: (1) high-temperature *Daqu* (60 and 70 °C), (2) medium-temperature *Daqu* (50–60 °C), and (3) low-temperature *Daqu* (40–50 °C). According to the raw materials used for production, *Daqu* can be classified as single-grain *Daqu* or multi-grain *Daqu*. *Daqu* can also be classified geographically into southern and northern *Daqu*. Generally, southern *Daqu* is classified as a single-grain product produced at medium to high temperature. The northern variant is commonly a multi-grain, low to medium temperature *Daqu* (Shen 2001). Several studies have shown the diversity of the microbial community in *Daqu* (Wang et al. 2011a). We

hypothesized that the microbial composition of *Daqu* correlates with environmental factors prevailing during the fermentation process. Thus, the microbial community in similarly classified *Daqu* is predicted to harbor common species or similar dominant groups of microorganisms.

The microbial community of *Daqu* has been analyzed in previous studies using culture-dependent methods, such as isolation and enumeration on selective media (Li et al. 2009; Ma et al. 2011; Zheng et al. 2012), as well as by culture-independent methods, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), and amplified fragment length polymorphism (Gao et al. 2010; Meng et al. 2010; Yan et al. 2012). In the present study, a semi-quantitative culture-independent cloning method was used for the analysis of microbial communities in *Daqu*. By comparing clone libraries, not only qualitative information on the composition of the microbial community is obtained, but also quantitative information of the relative abundance of the identified species. The main objective of this study was to obtain an overview of the composition of the microbial communities in different types of *Daqu*. This analysis is expected to deliver potential biomarkers for fast and reliable verification of the authenticity of *Daqu* types.

Materials and methods

Sampling

Eight types of brick-shaped *Daqu* were obtained from five commercial distilleries located in northern and southwestern China. *Daqu* was produced and matured according to the procedures of the different distilleries. An overview of the types of *Daqu* and their technological parameters is presented in Table 1. In order to obtain adequate repetition, three blocks of each type of *Daqu* were randomly selected from each of the upper, middle, and lower stacked layers, and ground together. About 100 g of these *Daqu* powders was used as an experimental *Daqu* powder sample. Samples were then collected in sterile Stomacher® bags (Seward Laboratory Systems Inc., London, UK), transported to the laboratory in a cool box, and stored at −20 °C until analysis.

DNA extraction and PCR amplification

DNA from seven powdered *Daqu* samples was extracted according to the method of Wang et al. (Wang et al. 2008) and diluted to a DNA concentration of 50 ng/μL. The 16S rRNA bacterial gene was amplified using universal primers "B-for" (5′-AGAGTTTGATCCTGGCTCAG-3′) and "B-rev" (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al. 1989). The D1/D2 domain of the 26S rRNA fungal gene was amplified using universal primer "NL1" (5′-TGCTGGAGCCATGGATC-3′) and reverse primer "RLR3R" (5′-GGTCCGTGTTTCAAGAC-3′) (Okoli et al. 2007). PCR was performed in a total reaction volume of 50 μl containing 26.6 μl ddH_2O, 5 μl PCR buffer, 3 μl $MgCl_2$ (25 mM), 10 μl dNTP (2 mM), 2 μl of each primer (10 μM), 1 μl DNA template (approximately 50 ng), and 0.4 μl Taq DNA polymerase (5 U/μl) (Fermentas, USA). PCR was performed using a GeneAmp PCR system 9700 (Applied Biosystems, USA) with the following PCR conditions: initial denaturation for 5 min at 94 °C; 35 cycles each consisting of 30 s at 94 °C, 20 s at 56 °C (bacteria) or 52 °C (fungi), and 1 min at 72 °C; and extension of incomplete products for 7 min at 72 °C, followed by cooling at 4 °C. The sizes and quantities of the PCR products were determined using 1.5 % (wt/vol) agarose gel electrophoresis. The PCR products were analyzed by electrophoresis and then stored at −20 °C for future experiments.

Table 1 *Daqu* samples investigated

Sample code	Type	Raw materials	Maximum temperature reached during the fermentation (°C)	Location
9-H–S–W*	High temperature	Wheat	62.7 ± 0.2	28.32°N(S)
5-H–S–W	High temperature	Wheat	65.2 ± 0.1	28.88°N(S)
8-M–N–BP	Medium temperature	Barley and pea	54.2 ± 0.1	40.02°N(N)
5-M–S–W	Medium temperature	Wheat	54.6 ± 0.1	28.88° N(S)
4-M–S–W	Medium temperature	Wheat	56.5 ± 0.2	28.55°N(S)
7-L–N–BP'	Low temperature	Barley and pea	50.1 ± 0.2	37.31°N(N)
7-L–N–BP	Low temperature	Barley and pea	45.2 ± 0.1	37.31°N(N)
8-L–N–BP	Low temperature	Barley and pea	50.7 ± 0.1	40.02°N(N)

* indicate the codes of *Daqu* samples: The number in codes means the name of liquor factory; the letter "H" "M" and "L" indicates the high temperature *Daqu*, Medium temperature *Daqu* and Low temperature *Daqu*, respectively. The letter "N" and "S" indicates that the *Daqu* is produced in northern China and southern China, respectively. The letter "W" and "BP" indicates that the *Daqu* is produced from wheat and "barley and peas", respectively. The only difference between samples 7-L–N–BP and 7-L–N–BP' is maximum temperature

DNA clone library construction

Clone libraries of 16S rDNA and 26S rDNA amplicons from *Daqu* samples were constructed. Amplicons derived from PCR products were purified with a QIAquick PCR purification Kit (Qiagen, Hilden, Germany), cloned using a pGEM-T Easy Cloning Kit (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 High Efficiency Competent Cells (Promega), following the manufacturer's instructions. Around 90 positive clones (white colonies) were randomly picked from the plates of each sample. These plasmid-harboring clones were transferred with a sterile toothpick into 50 μl of Tris–EDTA buffer, lysed, and amplified with "T7" and "Sp6 pGem-T"-specific primers to confirm the appropriate size of the insert (approximately 1,500 bp for bacteria and 700 bp for fungi). Clones containing the plasmid with an insert were sent for sequencing at the Beijing Genomics Institute (Beijing, China). Sequences were assembled and edited with Seqman II software (DNAStar Inc., Madison, WI, USA) and aligned with Megalign (DNAStar Inc.). Chimeric sequences in the clone library were identified and discarded using the software package Chromas v.2.31 (Technelysium Pty Ltd.). The nucleotide sequences obtained were identified in GenBank using BLAST (http://blast.ncbi.nlm.nih.gov/) to determine the closest known relatives of the partial ribosomal DNA sequences obtained.

Calculation of species diversity indices

To determine the diversity of species in *Daqu* samples (as revealed by cloning), Shannon's diversity index ($H' = -\sum p_i \ln(p_i)$, where p_i is the proportion of taxon i), was calculated.

Statistical analysis

The composition of microbiological communities in all *Daqu* samples was analyzed by principal component analysis (PCA) using the software package SIMCA-P 12.0 (Umetrics, Umeå, Sweden) to cluster the samples into different groups. Samples were plotted in two dimensions based on scores for the first two principal components to evaluate relationships among samples. The proportion of variance explained by each principal component was calculated.

Results

Composition of microbial communities in *Daqu*

The composition of the microbial communities in *Daqu*, representing three temperature types obtained from five factories, is shown in Table 2. About 69 bacterial species and 19 fungal species were detected by the cloning method. Only three species (*Bacillus licheniformis*, *Saccharomycoposis fibuligera* and one uncultured bacterium) were detected in all types of *Daqu*. Twenty bacterial species and 11 fungal species were found in high-temperature *Daqu*, but only five species, i.e. *B. licheniformis*, *Enterobacter* sp., *Pichia kudriavzevii*, *S. fibuligera*, and *Thermomyces lanuginosus* were found in all high-temperature *Daqu* samples. Forty-three bacterial species and 10 fungal species were found in medium-temperature *Daqu*, but only four species, i.e. *B. licheniformis*, *Bacillus* sp., *S. fibuligera*, and *Lichtheimia ramosa* were found in all medium-temperature *Daqu*. Twenty-nine bacterial species and four fungal species were found in low-temperature *Daqu*, with *B. licheniformis*, *S. fibuligera*, *Lichtheimia corymbifera*, and *Pichia kudriavzevii* as common species occurring in all low-temperature *Daqu*. The lowest bacterial diversity ($H' = 1.19$) was found in sample 9-H–S–W, and the highest bacterial diversity ($H' = 3.40$) was found in sample 4-M–S–W. For fungi, the lowest diversity ($H' = 0.49$) was in sample 8-M–N–BP, and the highest ($H' = 1.59$) in sample 5-H–S–W (Table 2).

Characteristics of different types of *Daqu*

Group-wise PCA comparisons of the bacterial and fungal composition of the different types of *Daqu* were constructed (Figs. 1, 2). The loading plots indicate the species that are responsible for the separation of the clusters (Figs. 1b, 2b).

Based on bacteria detected, five of the eight samples (8-L–N–BP, 4-M–S–W, 5-H–S–W, 5-M–S–W and 9-H–S–W) clustered together (cluster 1). Furthermore, samples 7-L–N–BP and 7-L–N–BP′ clustered together (cluster 2) and sample 8-M–N-BP was separated from all other samples (Fig. 1a). With one exception (i.e., sample 8-L–N–BP), all *Daqu* samples in the main cluster 1 are from southern China, while the *Daqu* samples 7-L–N–BP, 7-L–N–BP′ and 8-M–N–BP are from northern China. The loading plot (Fig. 1b) indicates the bacterial species that contributed to this discrimination. The microbial species that most significantly characterized different types by their increased relative abundance were in cluster 1 are: *Saccharypolyspora rosea*, *Streptomyces albus*, *Thermomonospora chromogena*, *Staphylococcus gallinarum*, *Staphylococcus* sp., *Staphylococcus saprophyticus*, *Bacillus* sp., *Ent. cowanii*, and *E. hermannii*. In cluster 2, *W. confusa* showed a marked increase in relative abundance and in cluster 3 the species *Thermoactinomyces sanguinis*, *Saccharopolyspora* sp., *Saccharopolyspora rectivirgula* were detected with an increased abundance in the microbial population.

The PCA of three temperature types of *Daqu* based on fungal composition (see Fig. 2), showed three groups

Table 2 Microbial composition of three types of *Daqu*

Microorganisms	High temperature *Daqu* (60–70 °C)		Medium temperature *Daqu* (50–60 °C)			Low temperature *Daqu* (40–50 °C)		
	9-H–S–W	5-H–S–W	8-M–N–BP	5-M–S–W	4-M–S–W	7-L–N–BP	7-L–N–BP′	8-L–N–BP
Bacteria								
Acinetobacter baumannii					[7]			
Acinetobacter sp.					[1]			
Actinopolyspora salina			[1]					
Altererythrobacter sp.					[1]			
Bacillus atrophaeus						[1]		
Bacillus licheniformis	[27]	[21]	[5]	[18]	[4]	[4]	[10]	[55]
Bacillus oleronius		[1]			[1]			
Bacillus pumilus						[1]	[3]	
Bacillus sp.		[29]		[21]		[1]	[2]	[6]
Bacillus subtilis					[3]		[8]	
Bacillus shackletonii								[1]
Bacillus sonorensis						[3]		
Brevundimonas sp.					[1]			
Corynebacterium sp.						[1]		
Desmospora activa			[1]					
Endophytic bacterium					[4]			
Enterobacter asburiae		[2]			[7]			
Enterobacter cowanii	[21]							
Enterobacter cloacae		[1]						
Enterobacter sp.	[4]	[5]			[11]		[5]	[1]
Enterococcus canintestini					[1]	[1]		
Enterococcus saccharolyticus						[1]		
Escherichia hermannii	[28]							
Kocuria sp.					[1]			
Klebsiella pneumonia					[8]			
Klebsiella sp.					[2]			
Klebsiella variicola					[5]			
Kurthia gibsonii						[1]		
Lactobacillus farciminis						[2]	[4]	
Lactobacillus fermentum					[1]			
Leclercia sp.					[1]			
Legionella taurinensis					[1]			
Leuconostoc citreum					[1]			
Leuconostoc pseudomesenteroides						[1]		
Microbispora bispora			[2]					
Myxococcus xanthus		[1]						
Oceanobacillus sp.			[3]					[11]
Pantoea agglomerans						[1]		
Pantoea sp.		[2]						
Pantoea vagans						[2]		
Patulibacter minatonensis					[1]		[4]	
Pediococcus acidilactici				[2]				
Nilaparvata lugens					[1]			
Saccharopolyspora hordei		[2]		[2]	[1]			
Saccharopolyspora rectivirgula			[13]					[4]
Saccharopolyspora rosea			[6]			[2]		
Saccharopolyspora sp.			[12]					[3]

Table 2 continued

Microorganisms	High temperature *Daqu* (60–70 °C)		Medium temperature *Daqu* (50–60 °C)			Low temperature *Daqu* (40–50 °C)		
	9-H–S–W	5-H–S–W	8-M–N–BP	5-M–S–W	4-M–S–W	7-L–N–BP	7-L–N–BP′	8-L–N–BP
Saccharopolyspora spinosa			[1]					
Sphingomonas aurantiaca						[1]	[2]	
Staphylococcus gallinarum	[2]	[2]		[9]	[1]			
Staphylococcus saprophyticus	[2]	[3]		[20]	[3]			
Staphylococcus sciuri						[4]	[3]	
Staphylococcus saprophyticus								
Staphylococcus sp.				[18]	[5]	[6]	[7]	
Stenotrophomonas maltophilia			[1]		[6]			
Streptomyces cacaoi								[3]
Streptomyces albus			[4]					[3]
Streptomyces sp.			[1]					
Tepidanaerobacter sp.		[2]						
Thermoactinomycetaceae bacterium		[2]						
Thermoactinomyces sanguinis		[1]	[36]		[1]			[6]
Thermoactinomyces vulgaris		[1]						
Thermobispora bispora			[2]					
Thermomonospora chromogena			[3]					
Weissella cibaria		[1]				[5]	[12]	
Weissella confusa		[2]			[10]	[50]	[38]	
Weissella paramesenteroides						[2]		
Uncultured bacterium	[3]	[9]	[1]	[1]	[3]	[2]	[1]	[3]
Fungi								
Absidia idahoenis					[20]			
Aspergillus flavus	[3]							
Aspergillus fumigatus	[2]			[2]				
Coelometopinae sp		[1]						
Cucujus clavipes		[5]						
Eurotium amstelodami				[2]				
Lichtheimia ramosa			[5]	[21]	[5]			
Lichtheimia corymbifera		[1]			[15]	[11]	[18]	[15]
Pichia kudriavzevii	[31]	[21]				[21]	[19]	[21]
Pselaphacus signatus		[1]						
Pselaphacus vitticollis		[5]						
Rhizomucor miehei					[2]			
Rhizomucor pusillus					[39]			
Saccharomycopsis fibuligera	[21]	[20]	[52]	[42]	[7]	[53]	[48]	[45]
Stephanoascus ciferrii					[1]			
Talaromyces luteus		[1]						
Thermomyces lanuginosus	[28]	[30]						
Trichomonascus ciferrii				[2]				
Wickerhamomyces anomalus						[12]		
Total	[172]	[167]	[153]	[156]	[184]	[187]	[184]	[177]
Bacterial diversity scores (Shannon's diversity, H_b')	1.19	2.03	2.13	1.62	3.40	1.92	1.88	1.61
Fungal diversity scores (Shannon's diversity, H_f')	1.28	0.97	0.49	1.59	1.49	1.16	1.08	0.98

The number in the brackets [] indicates the number of clones isolated

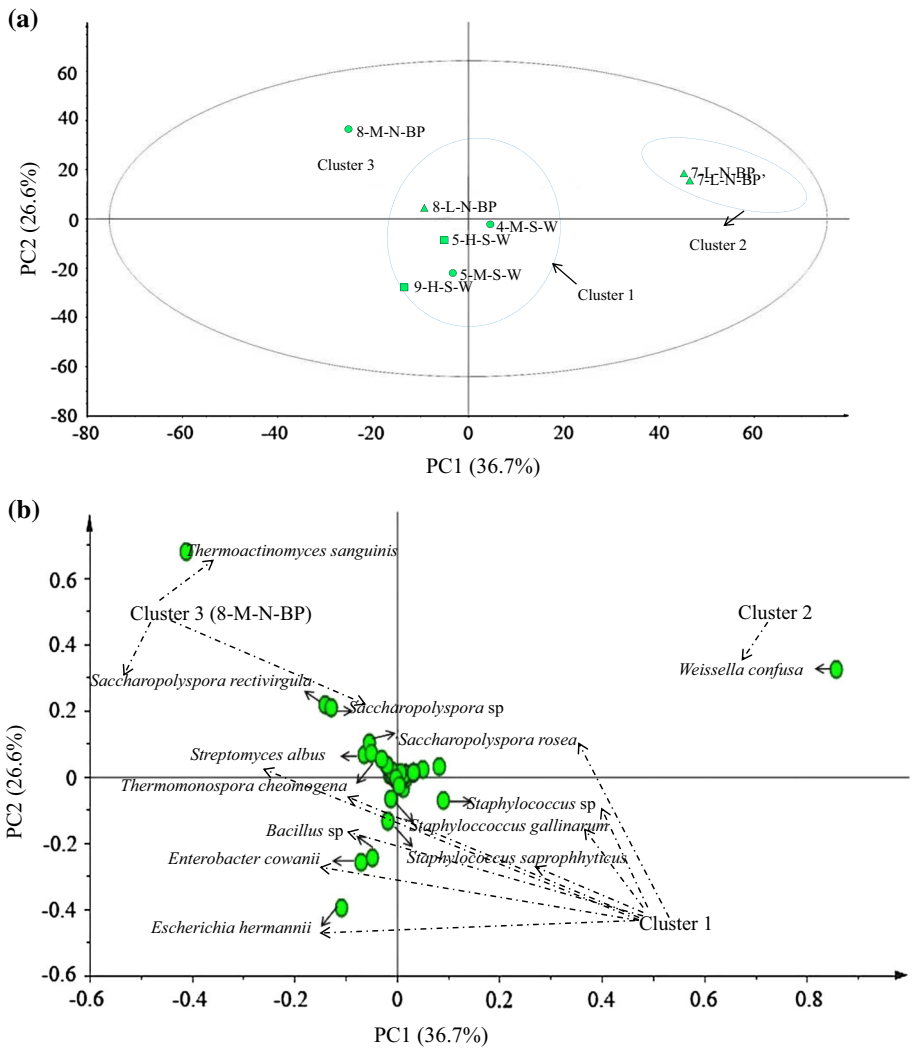

Fig. 1 PCA of *Daqu* extracts on bacterial composition **a** Score plots of three temperature types of *Daqu* **b** Loading plots of PC1 and PC2. *Traingle* low-temperature *Daqu*; *circle* medium-temperature *Daqu*; *square* high-temperature *Daqu*

(Fig. 2a). The species that most significantly characterized the different clusters by their fungal composition were in cluster 1 *Rhizomucor pusillus*, *Absidia idahoenis* and *L. corymbifera*; in cluster 2 *S. fibuligera* and *L. ramosa* and in cluster 3 *Thermomyces lanuginosus* and *A. flavus* (Fig. 2b).

Discussion

Knowledge of the microbiota of *Daqu* is still far from complete. Therefore, this study was initiated to understand the composition of the microbial community in three temperature types of this saccharafication agent. *Daqu* is made from different ingredients (barley, pea, or wheat), and is produced in different locations in China, each location applying different fermentation conditions. The most variable parameter is the maximum temperature of fermentation. Three arbitrary classes can be distinguished with regard to the latter parameter: (1) high-temperature-, (2) medium-temperature-, and (3) low-temperature processes for *Daqu* production. It is expected that the relative abundance of several of the identified microorganisms correlates with specific environmental conditions. For instance, the prevailing temperature is expected to have a major selective effect on the microbiota. The presence or relative abundance of other microorganisms could be associated with available substrates for fermentation, the location of production facility, and unique factory conditions.

The lowest bacterial diversity as measured by the Shannon index ($H' = 1.19$) was found in a high-temperature *Daqu* (9-H–S–W). Temperatures higher than 65 °C occurred during the production of high-temperature *Daqu*, and such temperatures only permit the survival and growth of thermophilic or thermotolerant bacteria and fungi, such as *Bacillus* spp. and *Thermomyces* spp., respectively (Moretti et al. 2012). This explanation is in line with our observations of the high abundance of *B. licheniformis*, and *Thermomyces lanuginosus* in high-temperature *Daqu* (samples 9-H–S–W and 5-H–S–

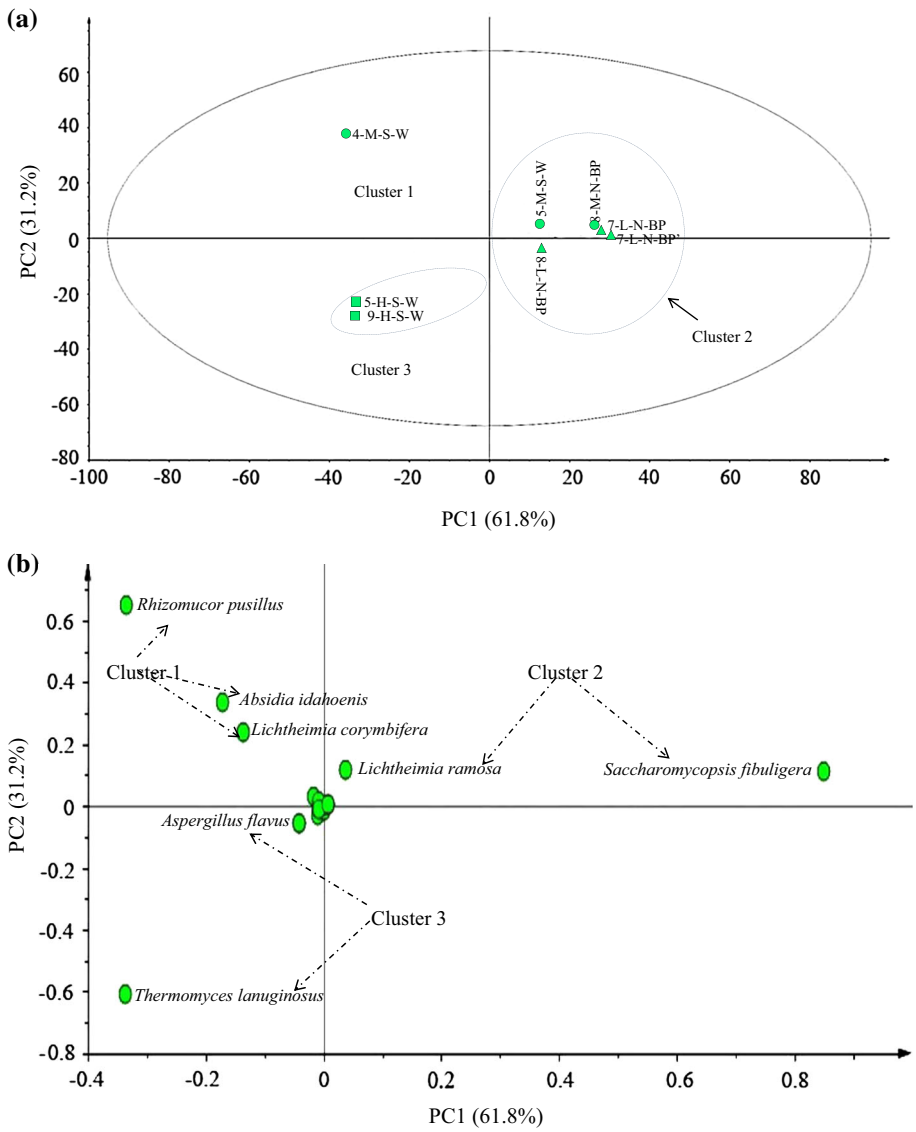

Fig. 2 PCA of *Daqu* extracts on fungal composition **a** Score plots of three temperature types of *Daqu* **b** Loading plots of PC1 and PC2. *Traingle* low-temperature *Daqu circle* medium-temperature *Daqu*; *square* high-temperature *Daqu*

W). Samples 5-H–S–W and 5-M–S–W were made from the same raw materials (wheat) and produced in the same factory; they only varied in their fermentation temperatures (about 10 °C differences). The comparison of the microbial diversity between samples 5-H–S-W and 5-M–S–W revealed a reduction in fungal diversity upon elevation of the fermentation temperature: sample 5-H–S–W had a lower number of species and a lower value of Shannon's diversity index compared to sample 5-M–S–W. Two samples obtained from factory 8 (i.e., 8-L–N–BP and 8-M–N–BP) were also produced in the same factory and they revealed the same trend: the higher the temperature, the lower the diversity in fungal composition (Table 2). However, the bacterial composition revealed a opposite trend. This indicates that the bacterial composition, in comparison to the fungal composition, is affected more by other factors such as moisture content and oxygen condition. The production technique used could be another factor affecting the bacterial community in *Daqu.* One specific technique called "back-slopping" was used in factory 9. The *Daqu* (4–8 %) that was produced 1 year ago (named "mother *Daqu*") was added to the raw materials, and the mixture was used to carry out the *Daqu* fermentation. On the one hand, the mild acidity of the "mother *Daqu*" could inhibit the growth of fungi (Li 2013), on the other hand however, the dominant microorganisms in the "mother *Daqu*" could dominate the *Daqu* fermentation, thereby suppressing the less prevalent microorganisms. This presumably explains why only seven bacterial species were detected in the sample 9-H–S–W.

Bacillus licheniformis, *S. fibuligera* and one uncultured bacterium were detected in all tested *Daqu* samples. This result is in agreement with the study of Wang et al. (2011b). *B. licheniformis* is a ubiquitous spore-forming bacterium associated with a variety of fermented food products (Lima et al. 2012; Ramos et al. 2010; Wang et al.

2011a.), and it is a well-known producer of proteases and amylases (Karataş et al. 2013). The high relative abundance of *B. licheniformis* in *Daqu* suggests that it plays an important role in flavor formation in products such as Chinese liquor and vinegar by hydrolysis of complex carbohydrates and proteins during fermentation. *B. licheniformis* was found to produce more than 70 metabolites, most of which are flavor compounds and flavor precursors important for the aroma of fermented products (Yan et al. 2013). Yan et al. (2013) reported high levels of acetic acid and lactic acid produced by *B. licheniformis*. These organic acids may give rise to a variety of aroma compounds by esterification with ethanol. This corresponds well with the fact that the key aroma compounds in light-flavor liquor, such as *Fen* liquor (factory 7), are mainly ethyl acetate and ethyl lactate. An abundance of *B. licheniformis*, *B. subtilis*, and non-specified *Bacillus* sp. was found in high-temperature *Daqu*, as has been observed elsewhere (Huang et al. 2006; Yan et al. 2007). Wu et al. (2009) analyzed the metabolite composition in different types of *Daqu*, and found higher concentrations of amino acids, such as isoleucine and leucine, in high-temperature *Daqu.* This correlates with the abundance of *Bacillus* spp. in high-temperature *Daqu*, since *Bacillus* spp. were shown to be important thermophilic protease producers (Zhang et al. 2007).

Saccharomycoposis fibuligera was encountered in different types of *Daqu* (Wang et al. 2011b). The role of *S. fibuligera* in *Daqu* production may be the secretion of amylases, acid proteases, and β-glucosidases, which have high potential application in the fermentation industry (Chi et al. 2009). *S. fibuligera* also has been reported to degrade and assimilate raw starch as a carbon source (Chi et al. 2009); thus, it may contribute to the formation of fermentable carbohydrates for subsequent alcoholic fermentation. In the current study, various genera of lactic acid bacteria (LAB) were identified in *Daqu* samples, including *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weisella*. In general, LAB were found in low abundance, except *W. confusa* which was found at high abundance in two *Daqu* samples (i.e., 7-L–N–BP and 7-L–N–BP'). Based on this, the high abundance of *W. confusa* can be used potentially to distinguish *Daqu* from factory 7 from *Daqu* samples originating from other production locations. Among the LAB species, *L. fermentum*, *L. citreum*, *P. acidilactici*, *W. confusa*, and *W. cibaria* were reported in earlier studies on other types of *Daqu* (Wang et al. 2011a; Zheng et al. 2012). Several studies mentioned the importance of LAB during the production of *Daqu*, but it was only found at high abundance during the beginning of the *Daqu* production process (Lei 2011). The study of Katina et al. (2002) indicated that some species of *Lactobacillus* inhibit the growth of *Bacillus* spp., especially *B. subtilis* and *B. licheniformis*. This might explain that a high abundance of LAB was present at early stages of *Daqu* production. However, the increase in temperature throughout the fermentation process results in the fast growth of thermophilic bacteria such as *Bacillus* spp. in *Daqu* and slower growth of mesophilic LAB (Lei 2011). This might explain the low abundance of LAB in the final *Daqu* products.

Thirteen species of actinomycetes were detected in *Daqu*, i.e., *Actinopolyspora salina*, *Saccharopolyspora hordei*, *Saccharopolyspora rectivirgula*, *Saccharopolyspora rosea*, *Saccharopolyspora spinosa*, *Saccharopolyspora* sp., *Streptomyces cacaoi*, *Streptomyces albus*, *Streptomyces* sp., *T. sanguinis*, *T. chromogena*, *Thermoactinomyces* bacterium and *Thermobispora bispora* (Table 2). Of these, *Saccharopolyspora rectivirgula*, *Saccharopolyspora* sp. and *Streptomyces albus* were detected particularly in 8-M–N–BP and 8-L–N–BP, which both originate from the same factory (i.e., factory 8), but were processed at different fermentation temperatures. Since *Saccharopolyspora rectivirgula*, *Saccharopolyspora* sp. and *Streptomyces albus* were not present in other *Daqu* samples, these three actinomycetes may represent the "house microbiota" of the factory 8. Wang et al. (2012a) monitored the presence of actinomycetes during the production of liquor and observed that >80 % of all the identified actinomycetes (especially *Streptomyces* spp.) originated from the air in the production room. This result was in line with our hypothesis that *Saccharopolyspora rectivirgula*, *Saccharopolyspora* sp., and *Streptomyces albus* belong to the "house microbiota". To date, no studies have been published on the role of actinomycetes in the production of *Daqu*, even though they commonly occur in *Daqu*. However, other studies have reported the ability of *Thermoactinomycetes* sp. and *Streptomyces* spp. to secrete alkaline phosphatase, esterase, lipid esterase, and phosphate hydrolase (Wang et al. 2012a; Yang et al. 2012), which might play important roles in the formation of the flavor compounds or flavor precursors during *Daqu* fermentation processes.

Analysis of different samples of southern *Daqu* revealed that *Staphylococcus* spp., especially *Sta. gallinarum* and *Sta. saprophyticus* could be considered as biomarkers of southern *Daqu* (Gao et al. 2010; Wang et al. 2012b), since these two bacteria were absent in the northern *Daqu* samples (8-M–N–BP, 8-L–N–BP, 7-L–N–BP and 7-L–N–BP'). This indicates that the bacterial community of *Daqu* is highly dependent on locations. In addition, the selection of raw materials and the environmental conditions (soil, air, etc.) could also influence the bacterial community in *Daqu* (Gao 2010; Xu et al. 2004). *Klebsiella* was found to be heavily associated with the soil used for planting wheat. Only one *Daqu* sample (4-M–S–W) contained *Klebsiella*

(including *K. pneumonia*, *K. variicola*, and *Klebsiella* sp.), and its presence probably indicates soil contamination. Another study reports that bacteria belonging to the genera *Bacillus* and *Micrococcus* were the only dominant bacterial species in wheat (Xu et al. 2004). The high number of *Bacillus* sp. in the *Daqu* samples, 5-H–S–W and 5-M–S–W, may be attributed to the dominance of *Bacillus* in the wheat samples originating from factory 5.

Temperature is an important environmental parameter that affects the growth and survival of microorganisms and, consequently, largely contributes to the microbial community structure of *Daqu* (Wang et al. 2011b). In general, yeasts and molds are more sensitive to heat than bacteria (Wang et al. 2011a). PCA confirmed grouping of the composition of the fungal communities of *Daqu* according to the fermentation temperature (Fig. 2). *Thermomyces lanuginosus*, a thermophilic fungus that survives at temperatures higher than 60 °C (Singh et al. 2003), is a candidate biomarker for high-temperature *Daqu*. *Tm. lanuginosus* has been reported to be an efficient xylanase producer, and the xylanase from this fungus is active over a wide pH range (Singh et al. 2003). This might imply that *Tm. lanuginosus* also plays a role in degrading xylan, as reported previously (Archana and Satyanarayana 1997). *S. fibuligera* occurred in all tested types of *Daqu* and represented about 50 % of the total fungal community in low/medium-temperature *Daqu*. This observation suggests an important role for this species in *Daqu*. *L. ramosa* is known to occur on wheat (Shang et al. 2012). Liu et al. (2010) compared the microbial diversity on wheat and its derived *Daqu*, and observed that *L. ramosa* and *Rhizomucor pusillus* occurred in both *Daqu* and wheat. In our study, *Rhizomucor pusillus* was present in relatively high numbers in the *Daqu* sample 4-M–S–W, and this species probably originated from the wheat used in factory 4 (Xu et al. 2004). A comparison of dominant microorganisms in different wheat varieties (Xu et al. 2004) revealed two dominating fungal genera (*Rhizopus* and *Aspergillus*) in wheat. Therefore, the *Aspergillus flavus* and *Aspergillus fumigatus* species detected in the southern *Daqu* samples (9-H–S–W and 5-M–S–W) could possibly be associated with the wheat used. All these findings indicate that the fungal communities in *Daqu* also depend on the raw materials formulation used during production.

Sample 9-H–S–W showed a relatively high abundance of Enterobacteriaceae (*Ent. cowanii* and *E. hermannii*) and this may indicate problems with hygienic processing in factory 9. Also, 9-H–S–W was the only sample with *Aspergillus flavus* (but at low abundance). Although we did not study the effect of *A. flavus* on the quality and safety of *Daqu*, the fact that this species is potentially able to produce aflatoxins, indicates that factory 9 requires a more strict quality control than other factories during the whole *Daqu* production process. Fortunately, this fungus was not observed in other types of *Daqu*, and therefore we do not regard this as a potential safety risk during *Daqu* production in general.

Until now, little attempt has been made to compare the microbial community structures of different types of *Daqu*. We have demonstrated that the fungal diversity in *Daqu* is highly influenced by fermentation temperature and raw materials, and that the bacterial diversity is influenced by fermentation temperature and geographic environment (i.e. climate, water, and air). The microbial communities of different types of *Daqu* samples differed significantly from each other. However, the relative abundances of species belonging to the genus *Bacillus* was higher than that of species of other bacterial genera. Among the *Bacillus* species, *B. licheniformis* was predominant and found in all *Daqu* samples, consistent with previous studies (Yiao et al. 2005; Zheng et al. 2011). On the other hand, each type of *Daqu* contained a high proportion of sample-specific bacteria. These bacteria and fungi are regarded as candidate biomarkers to distinguish different types of *Daqu*.

Differences in abundance of specific microorganisms present in *Daqu* samples as a function of regional origin potentially facilitate the selection of starters for creation of unique, region-specific flavors. Further research is required to establish the impact of *Daqu* composition on other quality aspects such as health effect of its derived product. This work may help liquor and vinegar industries to understand the microbial ecology of *Daqu*, and this enables further optimization of using different types of *Daqu* for liquor and vinegar production.

Acknowledgments This project was supported by the Specialized Research Fund for the Doctoral Program of Higher Education (No.20130008110013) and grant KNAW-China Joint Research Project 07CDP015 of the Royal Netherlands Academy of Arts and Sciences.

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