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Exploring the impacts of raw materials and environments on the microbiota in Chinese Daqu starter

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ABSTRACT

Many traditional fermented foods and beverages in both eastern and western countries are produced with the addition of starter cultures. Daqu is the undefined starter used for Chinese liquor, which contributed many fermentation functional fungal and bacterial communities to liquor fermentation process. However, the sources of these microbial communities and how these microorganisms formed in Daqu are still unclear. In this study, high-throughput sequencing combined with microbial source tracking analysis were applied to analyze the sources of the microbial communities in Daqu. We found fungal communities in Daqu were mainly originated from Daqu making environments (mainly tools and indoor ground), including *Saccharomycopsis fibuligera*, *Pichia kudriavzevii*, *Rhizopus oryzae*, *Sterigmatomyces elviae*, *Aspergillus flavus/oryzae*, *Hyphopichia burtonii* and *Lichtheimia corymbifera*. Meanwhile, most of bacterial communities in Daqu were from raw materials, including Gammaproteobacteria, Alphaproteobacteria and Bacilli. During Daqu-making process, the abundance of Gammaproteobacteria and Alphaproteobacteria that were not beneficial to liquor fermentation declined, but the abundance of the fermentation functional yeasts and Bacilli increased. Moreover, network analysis showed the bacteria in Daqu might be the drivers for the microbial succession during the Daqu-making process. This study shows that the Daqu production technology is a good way to screen fermentation functional microorganisms from complex environmental microbial communities.

1. Introduction

Fermented foods and beverages are widely consumed both in eastern (Chinese liquor and kimchi) and western (bread and cheese) countries (Alfonzo et al., 2017; Jung et al., 2012; Wolfe et al., 2014; Zheng et al., 2014). Many fermented foods and beverages are produced with the addition of defined or undefined starter cultures. Defined starter cultures have often replaced traditional undefined cultures due to their superior performance and reliability (Parente et al., 2016). However, undefined starters are still used in the manufacture of many traditional fermented foods and beverages, such as cheeses (Parente, 2006), kombucha (Marsh et al., 2014) and Chinese liquor (Zheng et al., 2014).

Daqu, an undefined starter culture, is one kind of jiuqu (a sort of equivalence of Koji) (Zhu and Tramper, 2013). Daqu can provide diverse microorganisms, which can secrete diverse enzymes, such as amylases, proteinases, cellulases, and phytases. Similar starters are used for many traditional oriental fermented foods and beverages, for example, liquor, rice wine, sake, vinegar and soy sauce (Jin et al., 2017). Typically, Daqu is an important saccharifying and fermenting agent for traditional vinegar (Li et al., 2016b) and liquor (Zheng et al., 2014) in China. Our previous studies have showed that Daqu contributes 61.06–80.00% of fungi during Chinese light-flavor liquor fermentation, mainly *Pichia* and *Saccharomycopsis* (Wang et al., 2018). *Pichia* is the major fermentation functional fungus during liquor fermentation, which can utilized sucrose and glucose to produce various aromatic compounds, including ethanol, ethyl acetate and 4-hydroxy-2-butanon (Li et al., 2016a; Li et al., 2011). *Saccharomycopsis* is known to produce extracellular proteolytic and saccharolytic enzymes with high activities (Huang et al., 2017; Li et al., 2017). However, where the microbial communities in Daqu originated from and how these microorganisms enriched in Daqu are unclear.

The production of Daqu is a traditional spontaneous solid-state fermentation (SSF) process in an open-work environment (Huang et al.,

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2017). During fermentation, the non-autoclaved raw materials (wheat) will be exposed to many environments (such as air, ground and equipment surface) on its journey from raw materials to mature Daqu (Li, et al., 2016). Thus, both the raw materials and the processing environments act as important sources for Daqu microbiota. Therefore, study on the microbiota in raw materials and environments is necessary for understanding the microbial ecosystem of Daqu, and is critical to control and rationalize the Daqu fermentation process.

Several research groups have reported the microbial community compositions in low-temperature Daqu (Li et al., 2013; Zheng et al., 2014), medium-temperature Daqu (Zhang et al., 2014) and high-temperature Daqu (Su et al., 2015; Xiong et al., 2014). Previous study showed that bacterial communities in low-temperature Daqu are dominated by Firmicutes and Actinobacteria, and nearly all of the fungal communities are *Pichia kudriavzevii*, a member of *Saccharomycetaceae* (Li et al., 2013). Metatranscriptomics has been used to explore the fermentation functional microbial communities in medium-temperature Daqu, and indicated fungi are the most active community members (Huang et al., 2017). Moreover, Xiong et al. showed that the microbes in the high-temperature Daqu were mainly thermotolerant and thermophilic microbes (Xiong et al., 2014). However, to our knowledge, only Liu et al. (Liu et al., 2010) tried to analyze the microbial community of wheat (raw materials) based on culture method, and indicated the strains isolated from wheat were the same with those isolated from Daqu. Nevertheless, the origins of the microbial communities and how these microorganisms formed in Daqu has seldom been investigated.

In this study, to better understand the origins of microbiota and how these microorganisms formed in Daqu, high throughput sequencing was applied to analyze the microbial community structure in Daqu, raw materials and environments during Daqu fermentation. Microbial source tracking was further applied to analyze the origins of microbiota in Daqu. Network analysis was used to analyze the interactions among the microorganisms in Daqu. This study provides new insights into where the microbial communities in Daqu originated from and how the microbial community structure in Daqu formed.

2. Materials and methods

2.1. Experimental design and sample collection

In the fermentation room of a traditional liquor production distillery in Hebei province, China (37°, 71′N; 115°, 69′E), we carried out the spontaneous solid-state fermentation (SSF) of the medium temperature Daqu starters. Initially, raw materials (wheat) were ground to powder, stirred with the addition of 30–32% water, and then the mixture was shaped into bricks (20.6 cm × 12.6 cm × 5.5 cm, new Daqu). After that, the new Daqu bricks were layer-by-layer piled on the fermentation rooms ground. The stacked layers of Daqu bricks were incubated for about 30 days, and were manually turned every 2 days for adequate aeration and temperature. Finally, the Daqu bricks were stored for six months to be mature Daqu, and then the mature Daqu bricks were ready to be used in liquor fermentation process (Figs. S1 and S2).

Triplicate new or mature Daqu samples were collected from the upper, middle, and lower locations in fermentation room, respectively. Raw material samples (5 kg) were collected randomly from the storage. The environmental samples were taken from the air, tools, indoor ground in fermentation room and the outdoor ground before the Daqu-making process. Air samples were collected via a liquid impact aerosol collector coupled with a vacuum pump (Kangjie Instrument Institute, Liaoning, China). We placed the aerosol collector as close as possible to the center of the fermentation chamber, which approximately 2 m above the ground. Prior to sampling, the aerosol collector and airway tube were ultrasonic cleaned with ethanol, and filled with 20 ml of 0.1 M phosphate-buffered saline (PBS) solution (pH = 7.0). A continuous 10 l/min flow of air from fermentation room was collected between 08:00–12:00 am for three days at the beginning of Daqu-making process. The PBS solutions were filtered through a 0.22 μm cellulose nitrate filter (Solarbio, Beijing, China) to capture the aerosol samples. The other environmental samples [tools (grinder and mold), indoor ground and outdoor ground samples] were collected with sterile degreasing cotton pre-moistened with sterile 0.1 M PBS. Degreasing cotton were rubbed across the tools and ground surface (1.00 m^2). Finally, all 21 samples (triplicate mature Daqu, new Daqu, raw materials, tools, air, indoor ground and outdoor ground samples) were transferred into lab on ice and stored at −80 °C until DNA extraction.

2.2. Total DNA extraction, amplification and sequencing

We isolated the total DNA via the EZNA™ (Easy Nucleic Acid Isolation) Soil DNA Kit (Omega bio-tek; Norcross, GA) according to the manufacturer protocol. For bacteria, the universal primer sets F338 and barcode-R806 were used to amplify the V3-V4 hypervariable region of the 16S rRNA gene (Soergel et al., 2012). For fungi, primers ITS3 and ITS4 were used to amplify the internal transcribed spacer (ITS) region (Hertz et al., 2016). PCR products were purified and carefully assessed via Thermo Scientific NanoDrop 8000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Then the barcoded PCR products were sequenced via a Miseq Benchtop Sequencer for 250 bp pair-end sequencing (2 × 250 bp; Illumina, San Diego, CA).

We processed all the raw sequences generated via Qiime (v1.8.0) (Caporaso et al., 2010). In brief, the raw sequences were quality trimmed, the sequences with quality scores < 30 were trimmed, and only the sequences over 200 bp in length were chosen for further analysis. After that, the sequences that had non-assigned tags, had N base, or did not perfectly match the PCR primer were removed (Caporaso et al., 2010). We removed the chimera sequences via the Uchime algorithm (Edgar et al., 2011). Then a distance matrix was calculated from the aligned sequences, and OTUs were clustered with a 97% identity threshold by Qiime's uclust pipeline (Edgar, 2010). Before further analysis, singleton OTUs were removed. Then the representative sequence from each clustered OTU was aligned to the Greengenes database (v13.8) (DeSantis et al., 2006) or the UNITE fungal ITS database (v6.0) (Koljalg et al., 2005). For further accurate verification of species information, the OTUs were manually checked through a BLAST search against the NCBI database (http://www.ncbi.nlm.nih.gov) for bacteria, and CBS database (http://www.westerdijkinstitute.nl) for fungi.

2.3. Statistical analysis and microbial source tracking

Alpha diversity was calculated by analyzing the observed species and Shannon index via Qiime after rarefying all samples to the same sequencing depth (17,359 reads for bacteria, and 37,564 reads for fungi). Statistical significance of the differences between alpha diversity were investigated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test via IBM® SPSS® Amos™ 22 (Arbuckle, 2013). Principal-coordinate analysis (PCoA) was displayed to examine community dissimilarity of different samples based on weighted UniFrac distances. Hierarchical clustering analysis (HCA) was implemented with the Vegan package in R v.3.3.1 (http://www.r-project.org) to illustrate the differences of microbial community compositions among samples. To analyze the relationships among microbial communities in Daqu, we calculated all possible Spearman's rank correlations between the dominant species in Daqu (with average abundance > 1%) via IBM® SPSS® Amos™ 22. Only the significant correlation ($p < 0.05$, with false discovery rate correction) was considered valid. Network was created by Gephi (Web Atlas, Paris, France) to visualize the correlations (Bastian et al., 2009).

We calculated the Pearson correlation coefficient among the Daqu, raw materials and environmental samples to analyze the relationships among them. SourceTracker (version 0.9.8) was used to predict the sources of microbial communities in Daqu (Knights et al., 2011). New

Daqu samples were set as sink. Raw materials and environmental samples (tools, air, indoor ground and outdoor ground samples) were set as sources.

3. Results

3.1. Microbial alpha diversity of Daqu, raw materials and environmental samples

We utilized high-throughput sequencing to characterize the microbial community structures in mature Daqu, new Daqu, raw materials and environments (tools, air, indoor ground and outdoor ground). A total of 487,867 high quality reads from V3-V4 region of 16S rRNA gene sequences, and 849,282 high quality reads from internal transcribed spacer (ITS) region were obtained from all 21 samples. For bacteria, there was an average of 23,231 reads per sample, with a range from 17,359 to 28,120 reads. For fungi, there was an average of 40,442 reads per sample, with a range from 37,564 to 42,759 reads. The rarefaction curves of both bacterial and fungal communities approached the saturation plateau, which indicated that the microbial communities were well represented at the sequencing depth (Fig. S3). Alpha diversity of Daqu, raw materials and environments were determined by Shannon index and observed species (the relative number of species) (Fig. 1). For Daqu samples, we found that both the bacterial (Fig. 1A and B) and fungal (Fig. 1C and D) diversity of mature Daqu were significantly ($p < 0.05$) lower than those of new Daqu. For raw materials and environmental samples, the bacterial diversity of air was significantly higher than that of raw materials and other environment samples, whereas the fungal diversity of raw materials was highest. Meanwhile, both the bacterial and fungal diversity of tools were lowest.

3.2. Bacterial community compositions of Daqu, raw materials and environmental samples

For bacteria, we subsampled this database at 17,359 reads per sample. It was considering that only OTUs appeared to be equal or greater than twice in a sample type a valid OTU, which yielded 822 OTUs from 21 samples. Pairwise comparison of bacterial OTUs revealed the greatest overlap between new Daqu and raw materials samples. However, the mature Daqu shared the greatest OTUs with indoor ground samples (Fig. 2A).

At class level, we obtained 42 classes in all samples. Among them, five classes (Bacilli, Gammaproteobacteria, Alphaproteobacteria, Actinobacteria and Sphingobacteriia) were dominant (with > 5% relative abundance) in Daqu, raw materials or environmental samples, and comprised 83.48% (air) to 99.83% (mature Daqu) of the total sequences. The mature Daqu was dominated by Bacilli (94.13 ± 1.06%). However, the bacterial community structure of new Daqu was similar with that of raw materials, among them Gammaproteobacteria accounted for a large percentage (53.13 ± 1.29%), followed by Bacilli (13.09 ± 0.09%), Alphaproteobacteria (12.47 ± 0.18%), Actinobacteria (9.73 ± 1.45%) and Sphingobacteriia (5.26 ± 0.76%). For environmental samples, Bacilli was more abundant in indoor ground (30.25 ± 2.19%) and tools (19.14 ± 6.52%), and less abundant in outdoor ground (1.62 ± 0.10%) and air (0.86 ± 0.07%). Gammaproteobacteria and Actinobacteria accounted for a large proportion in all of the environmental samples. Alphaproteobacteria and Sphingobacteriia were only dominant in air, indoor ground and outdoor ground samples (Table S1).

At genus level, we obtained 245 bacterial genera in all samples. Among them, 173 genera were identified in new Daqu samples, only 52

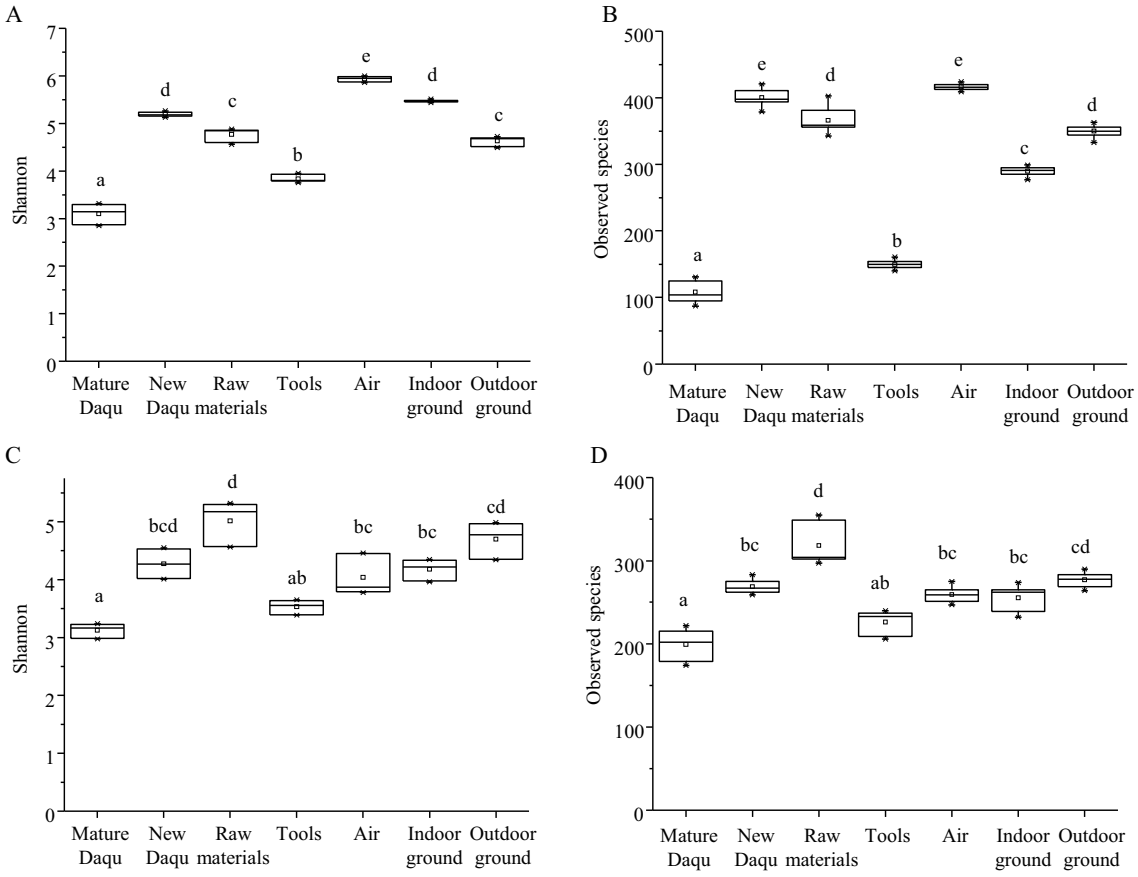

Fig. 1. Box plots showing Shannon index values and the number of observed species for bacterial (A, B) and fungal communities (C, D) among Daqu, raw materials and environmental samples. Sample groups with different letters mean significant differences ($p < 0.05$) as determined by one-way ANOVA Tukey's post hoc test.

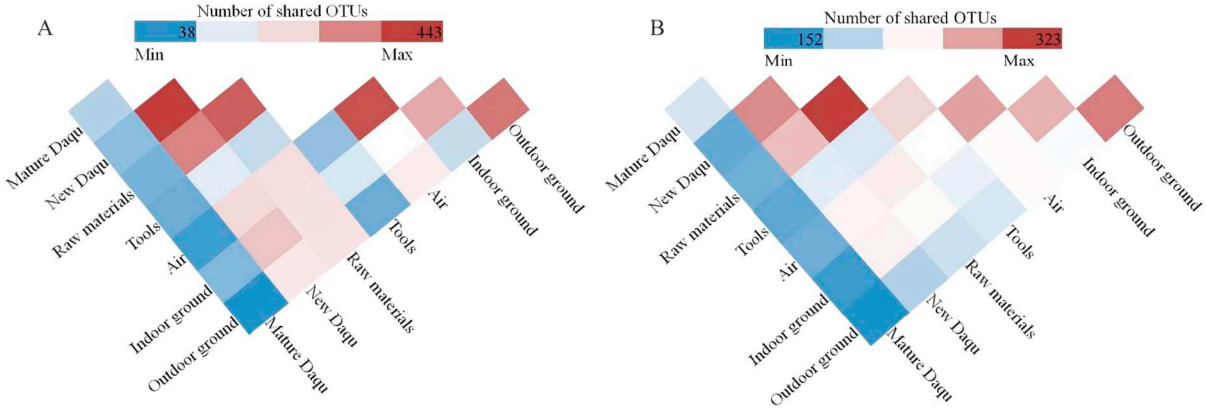

Fig. 2. Widespread sharing of microbial taxa among Daqu, raw materials and environmental samples. (A) Shared bacterial taxa. Pooled samples were rarified to an even depth of 17,359 reads. (B) Shared fungal taxa. Pooled samples were rarified to an even depth of 37,564 reads.

genera could be identified in mature Daqu. Meanwhile, 66 genera were only identified in raw materials or environmental samples, but could not be identified in Daqu samples (Dataset S1).

Fig. 3A showed the bacterial species with over 1.00% average relative abundance in new Daqu or mature Daqu. New Daqu was dominated by *Pantoea agglomerans* (34.19 ± 1.39%), followed by *Sphingomonas desiccabilis* (4.60 ± 0.16%), *Staphylococcus xylosus* (3.67 ± 0.28%), *Enterobacter aerogenes* (3.26 ± 0.22%), *Pseudomonas koreensis* (3.22 ± 0.20%), *Weissella cibaria* (3.14 ± 0.26%) and nine other bacteria. Interestingly, we found the relative abundances of *Pantoea agglomerans*, *Sphingomonas desiccabilis*, *Enterobacter aerogenes* and *Pseudomonas koreensis* decreased in the mature Daqu, whereas the relative abundances of *Staphylococcus xylosus*, *Weissella cibaria* and the other low abundance bacteria increased (Fig. 3A). The mature Daqu was dominated by *Staphylococcus xylosus* (41.55 ± 5.30%), *Bacillus amyloliquefaciens* (18.19 ± 0.96%), *Weissella cibaria* (8.51 ± 1.01%) and *Pediococcus acidilactici* (6.44 ± 0.12%), followed by *Lactobacillus fermentum* (4.26 ± 0.92%), *Lactobacillus pontis* (4.14 ± 0.42%), *Staphylococcus condimenti* (2.53 ± 0.24%), *Kroppenstedtia sanguinis* (2.45 ± 0.43%), *Saccharopolyspora phatthalungensis* (1.73 ± 0.20%), *Leuconostoc holzapfelii* (1.59 ± 0.39%) and *Lactobacillus plantarum* (1.66 ± 0.23%). For raw materials and environmental samples, we found the bacterial community structure of raw materials was similar with that of new Daqu. However, all the bacterial community structure of raw materials and environmental samples were different from that of mature Daqu (Fig. 3A).

PCoA of bacterial community was carried out based on weighted UniFrac distances (Fig. 3B). Overall, the two axes explained 73.91% of the total variance in bacterial community differentiation. Results showed the bacterial community structure of mature Daqu was clearly differentiated from all other samples. Meanwhile, the bacterial community structure of new Daqu was close with that of raw materials. This was also verified by HCA, which showed a similar clustering pattern with PCoA (Fig. 3C).

3.3. Fungal community compositions of Daqu, raw materials and environmental samples

For fungi, we subsampled this database at 37,564 reads per sample, and yielded 472 OTUs (97% identity) from all the samples. More than a quarter of OTUs were detected in all samples, and these 126 OTUs composed between 90.45 and 99.50% of sequences in each sample. Meanwhile, 110 OTUs were detected exclusively in a single sample type, which composed < 1% of sequences in each sample. Pairwise comparison of fungal OTUs sharing between Daqu and environmental samples revealed a similar pattern with that of bacterial community (Fig. 2B).

At class level, we obtained 15 fungal classes in all samples. Eight classes (Saccharomycetes, Zygomycetes, Agaricostilbomycetes, Eurotiomycetes, Microbotryomycetes, Dothideomycetes, Tremellomycetes and Sordariomycetes) comprised 56.09% to 98.01% of the total sequences (Table S2). Mature Daqu was dominated by Saccharomycetes (82.29 ± 1.72%) and Zygomycetes (8.56 ± 0.84%). New Daqu was dominated by Saccharomycetes (28.67 ± 4.86%), Zygomycetes (22.31 ± 4.00%), Agaricostilbomycetes (11.08 ± 2.61%) and Eurotiomycetes (10.89 ± 1.77%), which were also dominant in tools and indoor ground samples. Moreover, Microbotryomycetes was most abundant in raw materials (10.52 ± 4.28%), Dothideomycetes was most abundant in air (46.74 ± 10.69%) and outdoor ground (38.89 ± 5.48%), whereas Tremellomycetes (20.96 ± 2.91%) and Sordariomycetes (10.45 ± 0.53%) were only abundant in outdoor ground samples.

At genus level, 119 fungal genera were obtained in all samples (Dataset S2). Among them, 74 genera were identified in new Daqu samples, 60 genera were identified in mature Daqu, and 40 genera were only identified in raw materials and environmental samples.

Fig. 4A showed the fungal species with over 1.00% average relative abundance in at least one sample type. New Daqu was dominated by *Saccharomycopsis fibuligera* (20.52 ± 5.04%), *Rhizopus oryzae* (18.65 ± 3.66%), *Sterigmatomyces elviae* (10.26 ± 2.34%) and *Aspergillus flavus/oryzae* (6.11 ± 0.88%). However, the relative abundance of *Rhizopus oryzae*, *Sterigmatomyces elviae* and *Aspergillus flavus/oryzae* decreased in mature Daqu. The mature Daqu was dominated by *Hyphopichia burtonii* (37.74 ± 0.87%), *Saccharomycopsis fibuligera* (24.31 ± 3.59%), *Pichia kudriavzevii* (12.49 ± 1.91%) and *Lichtheimia corymbifera* (5.24 ± 1.15%). Furthermore, both PCoA and HCA based on fungal diversity showed the fungal community structure of mature Daqu was different from all other samples. Interestingly, the fungal community structure of new Daqu was close with that of tools and indoor ground, but was different from that of raw materials (Fig. 4B and C).

3.4. Microbial source tracking analysis highlights the sources of Daqu microbiota

We tested the correlations among the Daqu, raw materials and environments via Pearson correlation analysis (Fig. 5). Results showed raw materials were more likely to be sources of bacterial communities in new Daqu than environments (Fig. 5A), but tools and indoor ground were more likely to be sources of fungal communities in new Daqu (Fig. 5B).

SourceTracker was applied to track the sources of microbes found in Daqu (Knights et al., 2011). For bacterial communities, results revealed that raw materials (82.67%) were the main contributor of bacterial

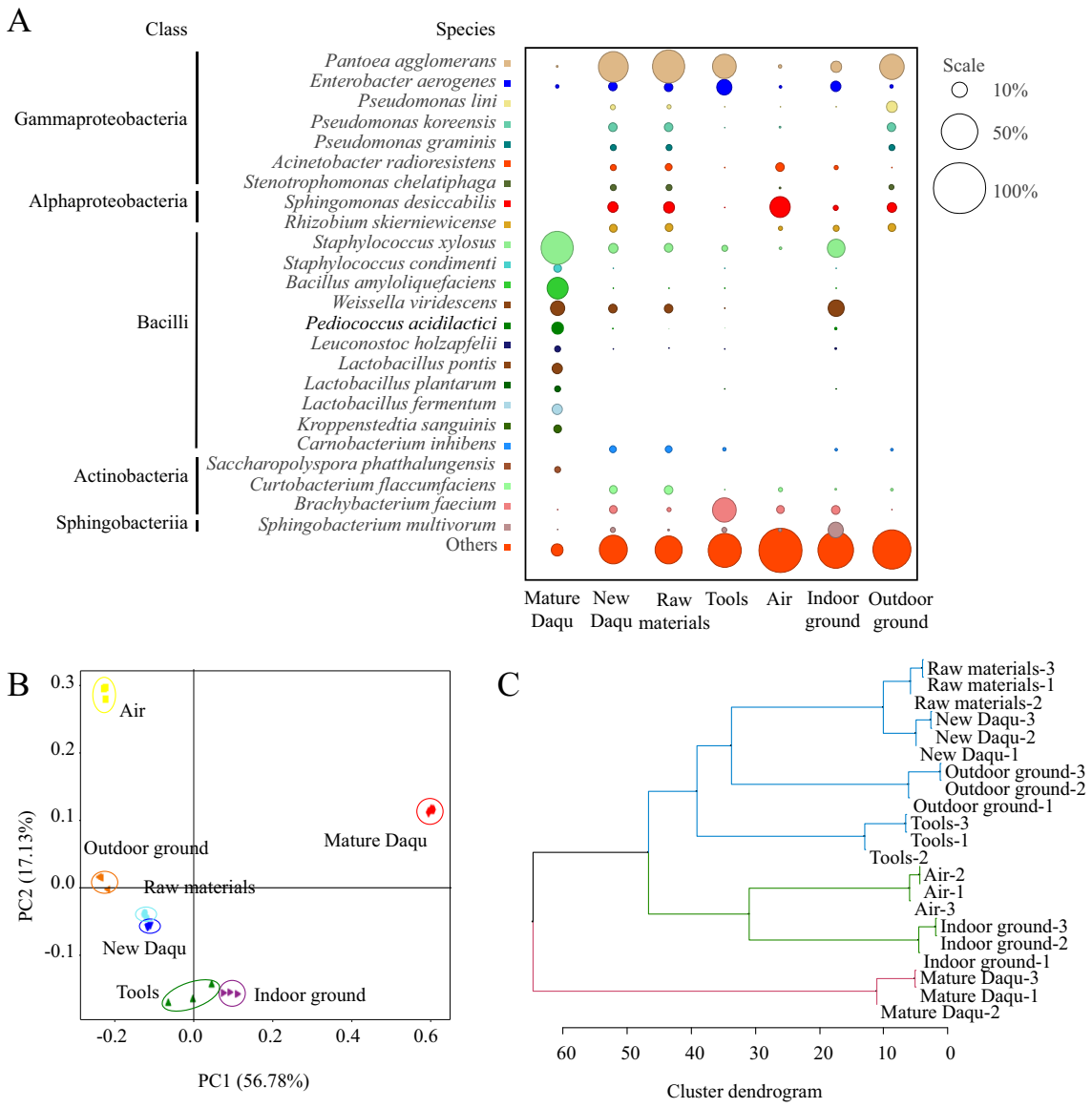

Fig. 3. Bacterial community compositions of Daqu, raw materials and environmental samples. (A) Abundance of the dominant bacterial communities in Daqu at species level. The radius of each circle represents the average relative abundance (n = 3) on square-root scale. (B) Principal coordinate analysis (PCoA) of the bacterial communities based on weighted UniFrac distances. Data points are colored and shaped by sample type. (C) Hierarchical clustering analysis (HCA) of bacterial communities. Triplicate samples are shown as "#-1" to "#-3"; e.g., "mature Daqu-1" represents the first replicate sample of mature Daqu.

communities in new Daqu (Fig. 6A). In details, raw materials contributed most of *Pantoea agglomerans* (95.52% of total *Pantoea agglomerans* in new Daqu), *Enterobacter aerogenes* (76.15%), *Pseudomonas koreensis* (98.91%), *Sphingomonas desiccabilis* (88.87%) and other low abundance species in new Daqu (Fig. 6B). Moreover, raw materials was also the main source for the species enriched in mature Daqu, such as *Staphylococcus xylosus* (65.74%), *Weissella cibaria* (64.01%), *Leuconostoc holzapfelii* (85.02%), *Lactobacillus pontis* (100.00%) and *Lactobacillus plantarum* (100.00%). However, indoor ground and tools were the main sources of *Bacillus amyloliquefaciens* (96.18%) and *Pediococcus acidilactici* (100.00%) that were also enriched in mature Daqu (Fig. 6B).

For fungal communities, tools (55.18%) was the main contributor for new Daqu, followed by raw materials (17.39%) and indoor ground (15.97%, Fig. 6C). In details, tools contributed most of *Saccharomycopsis fibuligera* (79.08%), *Rhizopus oryzae* (71.59%), *Sterigmatomyces elviae* (76.64%) and *Aspergillus flavus/oryzae* (84.36%) in new Daqu. Meanwhile, the environmental samples (mainly tools and indoor ground) were also the main sources of the fungi enriched in mature Daqu. Among them, *Hyphopichia burtonii* was mainly from indoor ground (41.04%) and tools (24.40%). *Pichia kudriavzevii* was mainly from tools (37.63%) and indoor ground (8.48%). *Lichtheimia corymbifera* was mainly from indoor ground (18.86%) and unknown environments (79.18%). However, raw materials mainly contributed *Candida athensensis*, *Botrytis cinerea*, *Sporobolomyces roseus* and other low abundance fungi to both new and mature Daqu (Fig. 6C).

3.5. Correlation networks of microbial communities in Daqu

To illuminate the formation mode of the microbial community structure in Daqu, we explored the co-occurrence and co-exclusion patterns of microbial communities in Daqu based on Spearman's rank correlations (Fig. 7). Totally, 132 pairs of significant and robust relationships were identified from 20 species. Among them, 80 pairs of positive correlations (edges) were identified from 19 species (nodes) with an average degree (AD): 8.0 (Fig. 7A), and 52 pairs of negative correlations were identified from 20 species with an AD: 5.2 (Fig. 7B).

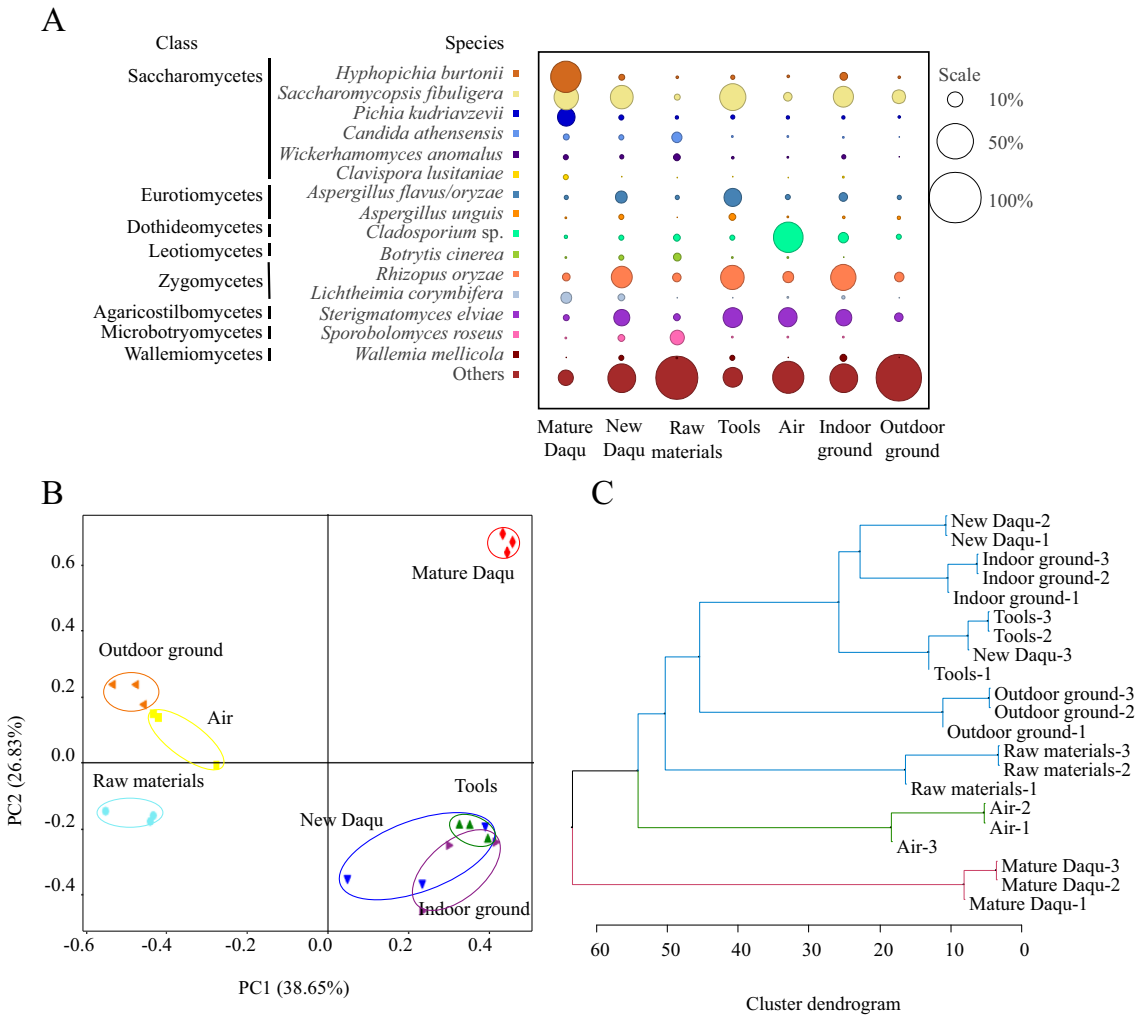

Fig. 4. Fungal community compositions of Daqu, raw materials and environmental samples. (A) Abundance of the dominant fungal communities in Daqu at species level. The radius of each circle represents the average relative abundance ($n = 3$) on square-root scale. (B) Principal coordinate analysis (PCoA) of the fungal communities based on weighted UniFrac distances. Data points are colored and shaped by sample type. (C) Hierarchical clustering analysis (HCA) of fungal communities.

Topological properties were calculated to describe the patterns of relationships between species in the co-occurrence and co-exclusion network of microbial communities in Daqu. For the co-occurrence network (Fig. 7A), the average path length (APL) between nodes was 1.29 edges with a network diameter (ND, the longest distance between nodes) of three edges, with a high clustering coefficient (CC) of 0.86. Fig. 7A also showed there were higher co-occurrence incidence among the species from yeasts and lactic acid bacteria in Daqu than those among filamentous fungi. For the co-exclusion network (Fig. 7B), the APL between nodes was 2.10 edges with a ND of four edges and CC of 0.00. We found members from filamentous fungi was negatively related with the occurrence of *Pichia kudriavzevii*, *Wickerhamomyces anomalus*, *Weissella cibaria*, *Leuconostoc holzapfelii*, *Lactobacillus pontis*, *Lactobacillus plantarum* and *Bacillus amyloliquefaciens* (Fig. 7B).

4. Discussion

Traditional Chinese Daqu is an important saccharifying and fermenting agent for traditional vinegar (Li et al., 2016b) and liquor (Zheng et al., 2014) in China. Previous studies have showed that Daqu contributes many fermentation functional fungal and bacterial communities to liquor fermentation process (Wang et al., 2018). The production of Daqu is processed in an open environment. The community of Daqu form under controlled conditions. Many studies have showed that the core community of Daqu are replicate and are easily sampled at various stages (Li et al., 2017; Li et al., 2016b; Zheng et al., 2014). With regard to some else microbes, the environment change also can induce the instability of Daqu quality. Moreover, the Daqu-making process covered variety environmental microbes, however, how these diverse microorganisms form the stable quality Daqu is unclear. In this study, we combined high throughput sequencing with microbial source tracking to analyze the origin of the microbial communities in Daqu and how did these microbial communities in Daqu formed.

A total of 179 bacterial genera and 79 fungal genera were identified in mature and new Daqu (Dataset S1 and S2). Among them, 110 bacterial genera (*Staphylococcus*, *Bacillus*, *Lactobacillus*, *Weissella*, *Pediococcus*, *Pantoea*, *Pseudomonas*, *Sphingomonas*, etc.) and 48 fungal genera (*Hyphopichia*, *Saccharomycopsis*, *Pichia*, *Candida*, *Lichtheimia*, *Rhizopus*, *Aspergillus*, etc.) had been reported in previous studies of Chinese Daqu starters (Li et al., 2015; Su et al., 2015; Xiong et al., 2014; Zhang et al., 2014; Zheng et al., 2012; Zheng et al., 2014). Interestingly, 69 bacterial genera (*Paracocccus*, *Chitinophaga*, *Salinicoccus*, *Lysinimonas*, *Epilithonimonas*, *Arcticibacter*, *Chroococcidiopsis*, *Saccharibacillus*, etc.) and 31 fungal genera (*Sterigmatomyces*, *Guehomyces*, *Exophiala*, *Blastobotrys*, *Xylaria*, etc.) were identified for the first time in Daqu (Dataset S1 and S2). Besides high throughput sequencing used in this study could identify more microbes in Daqu, this detection may be attributed to the different environments for Daqu-making process.

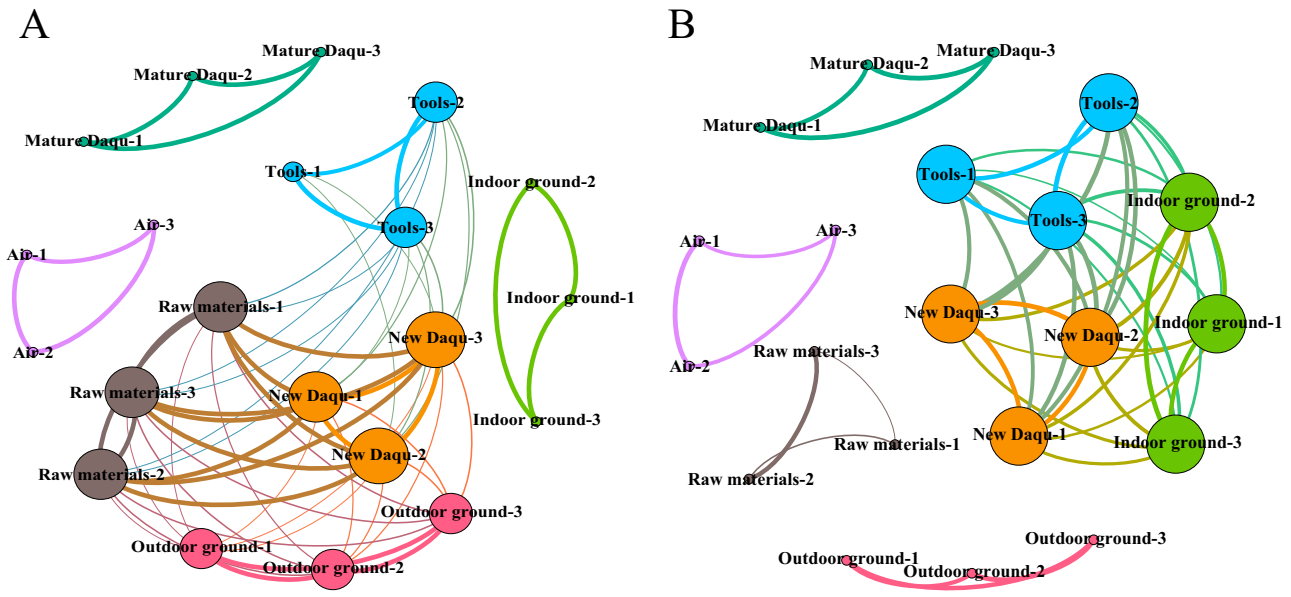

Fig. 5. Network analysis depicting the interactions among Daqu, raw materials and environments. Population data was considered at the OTU level and statistically significant Pearson correlations were defined among Daqu, raw materials and environments by OTU level taxonomic associations (A, based on bacterial community; B, based on fungal community). A connection stands for a significant ($p < 0.05$) and positive (Pearson correlation > 0.6) correlation. The thickness of edge is proportional to the value of Pearson correlation coefficients.

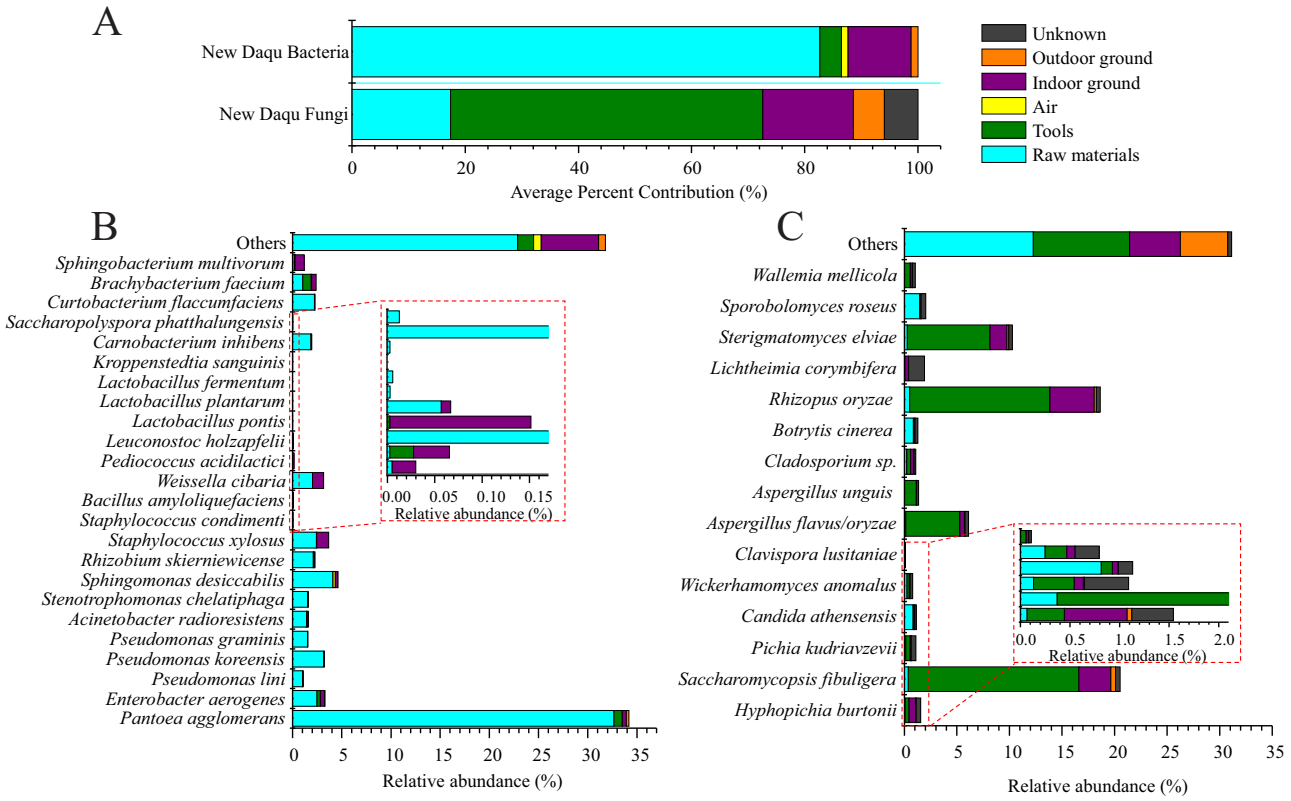

Fig. 6. SourceTracker results highlight the percentages of inferred sources of bacterial and fungal communities in new Daqu. (A) Average percent contribution of source communities. (B) Source tracking analysis of bacterial communities in new Daqu. (C) Source tracking analysis of fungal communities in new Daqu.

Among the microorganisms detected in Daqu, we found the mature Daqu was dominated by fungal and bacterial communities that can produce flavor components, saccharifying enzyme or hydrolytic enzyme, such as *Saccharomycopsis fibuligera*, *Pichia kudriavzevii*, *Rhizopus oryzae*, *Aspergillus flavus/oryzae*, *Bacillus amyloliquefaciens* and various lactic acid bacteria (Fig. 3 and Fig. 4). *Saccharomycopsis fibuligera*, *Rhizopus oryzae*, *Aspergillus flavus/oryzae* and *Bacillus amyloliquefaciens* are known to produce extracellular proteolytic and saccharolytic enzymes with high activities (Huang et al., 2017; Li et al., 2017). *Pichia kudriavzevii* is the major fermentation functional fungi during liquor fermentation, which can utilized sucrose and glucose to produce various aromatic compounds, including ethanol, ethyl acetate and

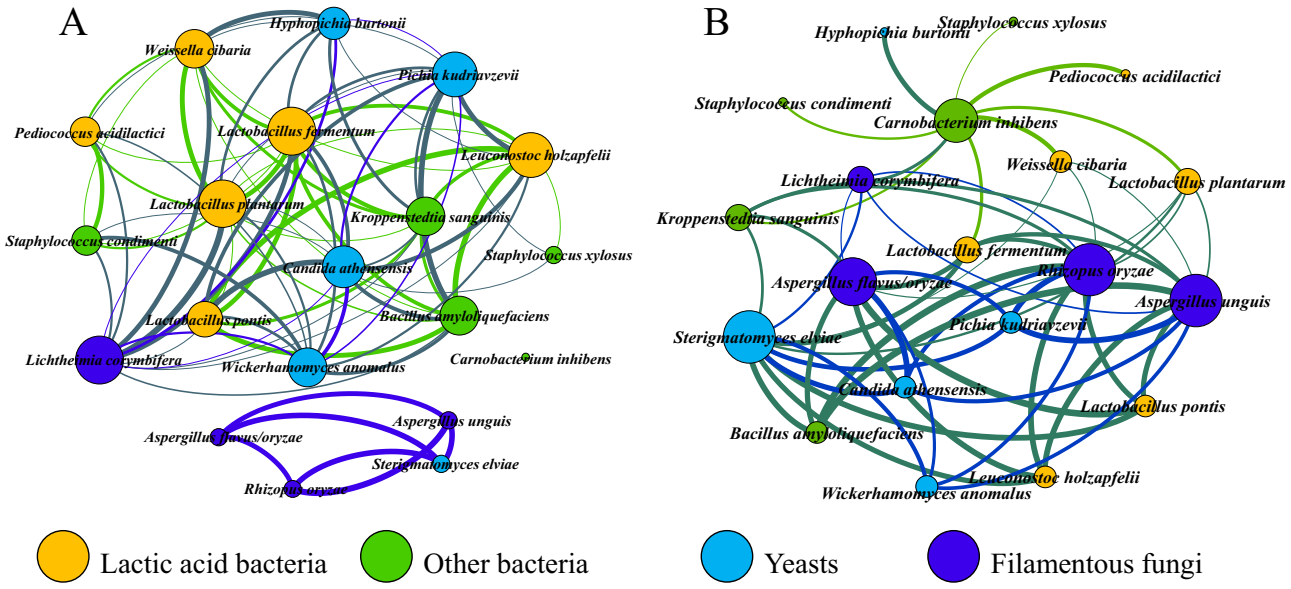

Fig. 7. Co-occurrence network of microbial communities in Daqu based on Spearman correlation analysis. A connection stands for a significant ($p < 0.05$) and strong positive (A, Spearman's $\rho > 0.6$) or negative (B, Spearman's $\rho < -0.6$) correlation. Size of each node is proportional to the number of connections. Color of each node is proportional to the types of microorganisms. Lactic acid bacteria, yellow; Other bacteria, green; Yeasts, wathet blue; Filamentous fungi, blue. The thickness of edge is proportional to the absolute value of Spearman's correlation coefficients. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4-hydroxy-2-butanon (Li et al., 2016a; Li et al., 2011). Lactic acid bacteria can produce a large amount of lactic acid, which is the precursor of ethyl lactate, and can enhance the mellow feeling of Chinese liquor (Gao et al., 2014). Moreover, High concentrations of lactic acid in Daqu resulted in acidic stress, which can inhibit the microorganisms without acid-tolerant ability (Wang et al., 2017). Interestingly, we found most of these fermentation functional microorganisms are not abundant in new Daqu, especially for the bacterial communities. The new Daqu was dominated by Gammaproteobacteria and Alphaproteobacteria which have been reported as pathogenic bacteria (Garrity et al., 2015). The change of microbial community structure in Daqu also showed that the Daqu-making process was a method of natural selection, which could select the beneficial microbes for liquor fermentation from the complex environmental microorganisms.

SourceTracker, a Bayesian probability tool (Knights et al., 2011), was used to identify the likely source of fermentation functional microorganisms in Daqu. SourceTracker has been widely used as an important tools of microbial source tracking in many fields, including food fermentation(Bokulich et al., 2015), meat processing (Stellato et al., 2016) and home environments (Lax et al., 2014). We found that tools and indoor ground were the major sources of fungal communities in both of new and mature Daqu, which contributed most of *Saccharomycopsis fibuligera*, *Pichia kudriavzevii*, *Rhizopus oryzae*, *Sterigmatomyces elviae*, *Aspergillus flavus/oryzae*, *Hyphopichia burtonii* and *Lichtheimia corymbifera* in Daqu (Fig. 6). These results filled the blank of previous research, which proposed that the raw materials was a source of both bacterial and fungal communities in Daqu (Liu et al., 2010). SourceTracker also showed tools mainly contributed filamentous fungi to new Daqu, which can produce massive saccharifying enzyme and hydrolytic enzyme, including *Rhizopus oryzae*, *Sterigmatomyces elviae* and *Aspergillus flavus/oryzae*. However, the abundance of *Rhizopus oryzae*, *Sterigmatomyces elviae* and *Aspergillus flavus/oryzae* declined during Daqu-making process. The mature Daqu was dominated by *Hyphopichia burtonii*, *Saccharomycopsis fibuligera*, *Pichia kudriavzevii* and *Lichtheimia corymbifera*, which also originated from indoor ground and tools (Figs. 4A and 6C). It was suggested that environments (indoor ground and tools), but not raw materials, were the main sources of fungal communities in Daqu.

Furthermore, we found the bacterial communities detected in Daqu were widely distributed in the raw materials and Daqu-making environments (Fig. 3A). SourceTracker showed the bacterial communities in Daqu mainly originated from raw materials (Fig. 6A and B). Raw materials mainly contributed Gammaproteobacteria (*Pantoea agglomerans*, *Enterobacter aerogenes* and *Pseudomonas koreensis*, etc.) and Alphaproteobacteria (*Sphingomonas desiccabilis*, etc.) to new Daqu (Fig. 6B), which had been widely detected as endophytic bacteria in wheat (raw materials of Daqu) (Patel and Archana, 2017). Previous study based on culture method also indicated the strains isolated from raw materials were the same with those isolated from high-temperature Daqu (Liu et al., 2010). Generally, *Pantoea* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Sphingomonas* spp. were only abundant at the early stage of Daqu fermentation, and the fermentation function of these genera in Daqu is still unclear (Su et al., 2015). However, the Gammaproteobacteria and Alphaproteobacteria originated from raw materials declined in mature Daqu, but the Bacilli [*Staphylococcus xylosus*, *Weissella cibaria*, *Lactobacillus pontis* and *Lactobacillus plantarum* from raw materials, and *Bacillus amyloliquefaciens*, *Pediococcus acidilactici* and *Lactobacillus fermentum* from environments (tools and indoor ground)] became dominant in mature Daqu (Figs. 3A and 6B). *Staphylococcus* spp., *Bacillus* spp., *Lactobacillus* spp., *Weissella* spp. and *Pediococcus* spp. had been detected in many studies about Daqu and other starters, and are also fermentation functional bacteria during liquor fermentation (Li et al., 2015; Zheng et al., 2012; Zheng et al., 2014). *Staphylococcus* spp. can produce aromatic compounds such as 3-methyl-1-butanol and acetoin, which play an important role in fermentation process of Chinese liquor (Søndergaard and Stahnke, 2002). *Lactobacillus* spp., *Weissella* spp. and *Pediococcus* spp. can produce lactic acid in liquor-making process, and lactic acid is the precursor of ethyl lactate, which can enhance the mellow feeling of Chinese liquor (Li et al., 2011; Zhang et al., 2005). *Bacillus* spp. can produce various enzymes, such as amylases, proteases, and lipases in *Daqu* (Huang et al., 2017). The succession from Gammaproteobacteria and Alphaproteobacteria to Bacilli has also been detected during wheat sourdough preparation (Alfonzo et al., 2017; Ercolini et al., 2013).

However, how these diverse microbial communities in new Daqu success to the fermentation functional communities in mature Daqu? To

investigate this question, network analysis was applied to analyze the interactions among microorganisms in Daqu (Fig. 7). We found that the species from Bacilli (mainly lactic acid bacteria) were positive related with yeasts, but negative related with the filamentous fungi. This might be attributed to two reasons, i) the lactic acid bacteria in Daqu could rapidly produce lactate, and the high concentrations of lactate could inhibit the Gammaproteobacteria and Alphaproteobacteria without acid-resistant properties in the Daqu-making process (Li et al., 2011). ii) The declined moisture (declined from about 55% to 10%) and high temperature (55–60 °C) also inhibits the microorganisms those need high moisture and without thermotolerant ability (Li et al., 2015). Thus, only the microorganisms that are tolerant of high concentrations of lactate, high-temperature (> 45 °C) and low moisture existed through to the end of fermentation process.

In conclusion, this study highlights that Daqu-making environments (indoor ground and tools) were the main sources of fungal communities in Daqu, whereas raw materials mainly contributed bacterial communities to Daqu. This study also shows that the interactions among microorganisms from raw materials and environments drives the change of microbial community structure in Daqu, which is beneficial to screen fermentation functional microorganisms during Daqu-making process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.02.020.

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