Journal of Bioscience and Bioengineering
VOL. 128 No. 3, 307–315, 2019

www.elsevier.com/locate/jbiosc

Comparison of microbial community and metabolites in spontaneous fermentation of two types Daqu starter for traditional Chinese vinegar production

Hanlan Tang,[1] Hebin Liang,[1] Jiankun Song,[1] Weifeng Lin,[2] and Lixin Luo[1,*]

Guangdong Key Laboratory of Fermentation and Enzyme Engineering, School of Biology and Biological Engineering, South China University of Technology, Guangzhou 510006, China[1] and School of Food Science and Engineering, South China University of Technology, Guangzhou 510006, China[2]

Received 19 June 2018; accepted 15 March 2019
Available online 22 April 2019

Daqu starter, an important saccharifying and fermenting agent for the brewing process of traditional vinegar, is manufactured by spontaneous solid-state fermentation which routinely undergoes low or medium incubation temperature. Previous studies have demonstrated that the temperature plays a pivotal role in Daqu quality. Hence, to explore the feasibility of high temperature fermentation applied in the vinegar Daqu brewing and provide guidelines of controlling environmental parameters in traditional vinegar industries, the microbial community and metabolites of vinegar Daqu during medium-temperature and high-temperature fermentation processes (namely, MTFP and HTFP) were compared. The results indicated that the glucoamylase activity, amylase activity and microbial community showed no significant difference in the end of two batches ($P > 0.05$). Enterobacteriales, Lactobacillales, Bacillales, Saccharomycetales and Mucorales were the dominant orders during MTFP and HTFP. Redundancy analysis revealed that incubation temperature showed positive correlation with the microbial composition from days 3–14 of the fermentation process and was positively associated with the predominant phylotypes of Bacillales, Mucorales, Xanthomonadales and Rickettsiales. The acidity and moisture showed major correlations with microbial composition on day 1 of MTFP and were positively related with the predominant phylotypes of Mucorales and Lactobacillales at the order level. Moreover, higher relative contents of all volatiles were shown in the end of HTFP (13.91 mg/100 g Daqu) compared to MTFP (10.01 mg/100 g Daqu). This work illustrates high temperature (approximately 60°C) fermentation is promising to improve the vinegar Daqu flavor and shall likely contribute to preferably make traditional Daqu by modulating steerable environmental parameters.

[**Key words:** Vinegar Daqu; Temperature; Fermentation process; Microbial community; Metabolic activity]

Traditional Chinese vinegar and liquor play indispensable roles in the daily diet and chiefly depend on natural starter culture (1). Daqu, a cereal starter of traditional vinegar and Chinese liquor, consists of diverse microbes, crude enzymes and flavor compounds. Materially, it as a portion of raw materials in follow-up fermentation can influence the quality of end-products (2,3). According to the maximum incubation temperature, Daqu can be categorized into three types: low-temperature (LT) Daqu (40–50°C), medium-temperature (MT) Daqu (50–60°C) and high-temperature (HT) Daqu (60–65°C) (4). Each type of Daqu has a unique combination of microbiota dynamics, functional enzymes (e.g., glucoamylase and amylase) and special flavor (5). Traditionally, vinegar Daqu is routinely manufactured from barley and wheat following spontaneous LT and MT Daqu solid-state fermentation (SSF) which can be successively divided into six stages based on the core temperature (namely incubation temperature) of Daqu: Shangmei (stage I); Liangmei (stage II); Chaohuo (stage III); Dahuo (stage IV); Houhuo (stage V); and Chengqu (stage VI) (4).

The adjustment and adaption of the microflora develops distinct metabolic profile (such as enzyme activities and aromatic compounds) in vinegar Daqu considering the intricate physicochemical culture changes, including temperature, moisture and glucose content (3). Among them, temperature is a crucial factor for Daqu production during the fermentation process, affecting the characteristics of Daqu by microbial growth, enzyme activities and aroma profile (6). The fermentation manufacturing of conventional Daqu solely relies on natural microbial selection. Therefore, incubation temperature can alter the microorganism growth status and subsequently affects the microbial community and metabolite profile in Daqu (7,8). Moreover, enzymes secreted by bacteria and fungi in Daqu can break down macromolecules and accelerate fermentation process under definite temperature ranges despite denaturation or lower efficiency exceeding the appropriate ranges (9). The enzyme activities during the overall brewing process are sensitive to temperature changes (10). Furthermore, in different incubation temperature, microbes in Daqu can commonly convert raw materials to synthesize considerable aroma compounds with unique flavor (11). Customarily, different types of Daqu can also be divided based on the flavor of the produced products which is contributed by the aroma from Daqu during brewing process, demonstrating the type of Daqu is closely related to the flavor of end-product (12). Thus, during the SSF stages, the Daqu-making is tightly correlated with temperature. Steering the fermentation temperature is expected to improve the Daqu manufacturing technology and quality.

* Corresponding author. Tel.: +86 20 39380628; fax: +86 20 39380601.
E-mail address: btlxluo@scut.edu.cn (L. Luo).

1389-1723/$ – see front matter © 2019, The Society for Biotechnology, Japan. All rights reserved.
https://doi.org/10.1016/j.jbiosc.2019.03.011

Recent studies have indicated that the composition of the microbial communities was significantly correlated with the temperature during vinegar and liquor Daqu SSF process (13,14). The distinct microbial community were found in different types of Daqu for vinegar and Chinese liquor fermentation (6,15). These studies have illustrated that temperature functions as a decisive factor in the formation of Daqu microbial community. Moreover, the microbial activity in MT SSF process was higher comparatively to the LT SSF process; however, the microbial dynamic on HT vinegar Daqu failed to be known together (4). In addition, the dominant hydrolases in different types of Daqu have disclosed that high temperature is closely correlated the enzyme activities (16). Besides, it was reported that Daqu exhibited various volatile components and the important aroma-active components in Daqu could be related to the Daqu quality (17). Furthermore, the correlation between microbiota succession and metabolite changes in traditional Shanxi aged vinegar has been investigated (18). Nonetheless, as far as it was known, no papers have been published so far describing microbial groups and metabolites of traditional vinegar Daqu at high temperature and the relationships between temperature and succession of microbial communities during MT and HT vinegar Daqu fermentation processes in detail.

Since the temperature during the vinegar Daqu brewing process can reach the range of high temperature, the aim of this study is to explore the feasibility of high temperature fermentation applied in the vinegar Daqu brewing. For this purpose, a pilot-scale experiment with two different temperature fermentation processes (medium-temperature and high-temperature fermentation processes, MTFP and HTFP) was carried out to analyze the microbial community and metabolites profile. The correlations between the physicochemical properties and the microbial communities during the SSF process were further investigated using multivariate data analysis. This work can provide some insights in future vinegar Daqu production by modulating steerable environmental parameters, especially the fermentation temperature.

MATERIALS AND METHODS

Pilot experiment and sampling The pilot experiment was carried out in the fermentation room of a vinegar production factory located in Qishan, Shaanxi Province (34°26′N, 107°36′E) in July, which was a suitable season for vinegar Daqu production. Initially, the mixtures of ground barley and wheat (7:3, wt/wt) blended with water (40%, wt/wt) were filled into brick-shaped molds and pressed into bricks (length × width × height, 32 cm × 16 cm × 7 cm) to obtain sufficient cohesion and retain their shape after they were piled in the fermentation room. Subsequently, the shaped-bricks were incubated at an artificial control temperature for approximately 30 days (Fig. 1A). This control confined within MT and HT during manufacturing process was performed by timely ventilation and turning the bricks, a traditional method carried out by a experienced technician who has been engaged in vinegar Daqu-making for almost thirty years. The matured Daqu was for the production of traditional Qishan vinegar. Samples were taken from two comparative processes (MTFP and HTFP) and collected at the stages I-VI corresponding to day 1, 3, 6, 12, 14 and 32 of Daqu fermentation period based on temperature conversion, respectively (Fig. 1A). At each sampling event, three bricks were randomly selected from the upper, middle and lower layers, pulverized and mixed to provide an experimental Daqu powder sample. Finally, the samples were pooled into sterile stomacher bags (Stomacher Lab System, London, UK) and stored at −20°C for further analysis.

Physicochemical, enzymatic and volatile compounds analysis The continuous detection of the core temperature of vinegar Daqu was performed with a thermometer (Beijing, China) inserted into the center of the Daqu bricks and the room temperature was detected by a thermometer adhered to the wall of the incubation room. Moisture was tested by the dry-weight measurement method at 105°C. The pH of Daqu was determined on a 1:2 (w/v) ration in ultra-pure water using a Hach pH meter (Hach, Loveland, CO, USA). The method to measure the titratable acidity was titrated to the endpoint of pH 8.2 with 0.05 M NaOH (19). The reducing sugar content was determined by means of a colorimetric method using 3,5-dinitrosalicylic acid (20). The activities of glucoamylase and amylase were measured as previously described (13). Flavor compounds were analyzed by headspace-solid phase microextraction-gas chromatography–tandem mass spectrometry (HS-SPME-GC-MS). Semi-quantitative volatile compounds were carried out by adopting 2-octanol as the internal standard according to the method described by Zhang et al. (21) using the following formula: $C_2 = 100\ C_1 \times S_2/S_1$, where C_2 is the relative concentration of the analyte (mg/100 g Daqu), C_1 is the concentration of the internal standard (0.0822 mg/mL), S_2 is the relative peak area of analyte and S_1 is the peak area of internal standard.

Enumeration, DNA extraction and quantitative PCR analysis For counting the viable cells in the vinegar Daqu during the six stages of the MTFP and HTFP, the method was implemented as previously described (22). All counts were made in

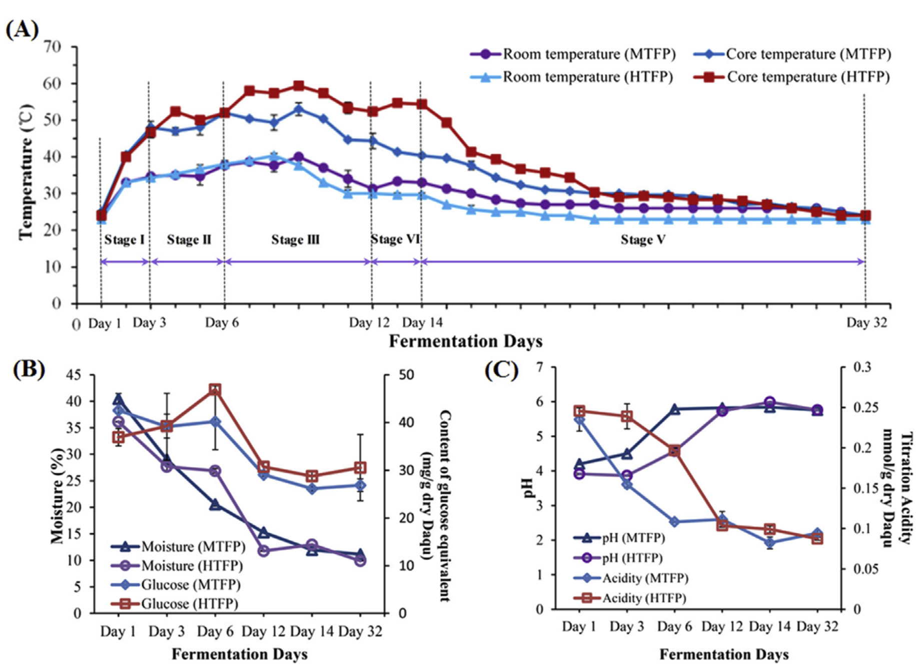

FIG. 1. Dynamics of physicochemical properties during MTFP and HTFP across all the samples. (A) Changes in room temperature and core temperature of Daqu. (B) Average content of moisture and reducing sugar (converted as glucose). (C) Changes in pH and acidity.

triplicate for each of the duplicated samples and the enumeration was performed from the plates with 20–300 colonies (23).

The pretreatment of each sample was performed as previously described (22) and microbial genomic DNA were extracted from each Daqu sample in triplicate using E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. Quantitative PCR (qPCR) analysis was conducted with a commercial kit (SYBR Premix Ex Taq II, Takara, Dalian, China) by an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The absolute quantification of the total bacteria, fungi, LAB and *Bacillus* were performed in quadruplicate, using P1/P2, Y1/Y2, Lac1/Lac2 and B1/B2 as primer pairs to amplify the bacterial 16S rRNA, fungi 18S rRNA, partial 16S rRNA of LAB and *Bacillus*, respectively (3). The qPCR reaction and amplification program were conducted as a prior study (24).

Illumina HiSeq sequencing The relative abundance and dynamic change of the bacterial and fungal community in Daqu fermentation process were systematically investigated using Illumina sequencing. The V4 regions of the bacterial rRNA genes and ITS1 regions of the fungal rRNA gene amplification were performed using the primers 515F/806R and ITS5-1737F/ITS2-2043R with Illumina barcodes, respectively (4). These amplifications were carried out in 30-μL reactions with 15 mL of Phusion HighFidelity PCR Master Mix (New England BioLabs, Ipswich, MA, USA), 0.2 mM of each primer and 10 ng DNA templates in triplicate. All PCR reactions were conducted as previously described by Ye et al. (25). PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Dusseldorf, Germany). Fungal and bacterial metagenomic libraries were constructed using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) by closely following the instructions and the index adaptors were added. The library was subjected to sequencing with a 250 bp paired-end strategy on an Illumina HiSeq2500 platform by the Beijing Novogene Bioinformatics Technology Company after the library quality was evaluated on the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system.

Processing of sequencing data Paired-end reads from the original DNA fragments were assigned to the samples based on their unique barcode and truncated by trimming off the barcode and primer sequence. Then they were merged using the FLASH program (v1.2.7) (26) and the splicing sequences were named the raw tags. Quality filtering on the raw tags was performed according to the QIIME (v1.7.0) quality-controlled process (27). The effective tags were obtained after the chimera sequences were removed using the Gold Database with the UCHIME algorithm (28). Obtained effective tags were classified into the same operational taxonomic units (OTUs) with a 97% similarity threshold using Uparse software (v7.0.1001) (29). The obtained OTUs were annotated and taxonomically assigned via GreenGene Database v. May 2013 and RDP classifier (v2.2). Alpha diversity including Chao1, Shannon, Simpson, ACE and Goods coverage were evaluated with QIIME (v1.7.0). Beta diversity estimates of unweighted unifrac were also calculated within QIIME (v1.7.0).

The raw sequences generated data from this study were submitted the GenBank Sequence Read Archive (SRA) database under accession number PRJNA316642.

Statistical analysis The statistical significance ($P \leq 0.05$) of the difference between the two batches were predicated by a one-way analysis of variance (ANOVA) with SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). Principal component analysis (PCA) was based on the relative contents of volatile compounds and conducted using ggplot2 package in R software (v3.3.2) to visualize differences in all the samples. Principal coordinate analysis (PCoA) was displayed by WGCNA package, stat packages and ggplot2 package in R software (V2.15.3). The relationship between physicochemical parameters and microbial communities in each sample during MTFP and HTFP were analyzed by redundancy analysis (RDA) operated at the order and genus level with Canoco 5.0 software.

RESULTS

Dynamics of physicochemical properties during Daqu fermentation process The dynamics of vinegar Daqu core temperature and fermentation room temperature during MTFP and HTFP are presented in Fig. 1A. The initial core temperatures of MTFP and HTFP were close to room temperature and both the core temperatures increased to nearly 45°C on day 3. Then, the core temperature of MTFP and HTFP reached the maximum incubation temperature of 53°C and approximately 60°C, respectively. Finally, the core temperature gradually decreased to room temperature in the later process of MTFP, while HTFP showed a slight increase during stage IV and then decreased to room temperature. Particularly, the core temperature during the middle stages (stage III and IV) of HTFP was higher than that of MTFP.

Fluctuations of the measured parameters were observed during MTFP and HTFP (Fig. 1B, C). No significant ($P > 0.05$) differences in the physicochemical properties were observed between integrated MTFP and HTFP. The moisture of Daqu was constantly diminished from the initial 40% to the final 11% during MTFP and reduced from 36% to 10% during HTFP (Fig. 1B). It was intriguing that the concentration of reducing sugar increased to highest of 46 mg g^{-1} dry Daqu on day 6 and further decreased to 31 mg g^{-1} dry Daqu with some fluctuation during HTFP, while it declined from primal 42 mg g^{-1} dry Daqu to 26 mg g^{-1} dry Daqu in the end of MTFP (Fig. 1B). The pH increased to approximately 5.80 and the titratable acidity decreased to about 0.10 mmol g^{-1} during days 1–12 of MTFP and HTFP (Fig. 1C). Moreover, at stages II and III, significantly higher pH and lower titratable acidity were displayed during the MTFP compared with the HTFP ($P < 0.05$).

Dynamics of functional enzyme activities during Daqu fermentation process It was indicated that the activities of functional enzyme were similar during MTFP and HTFP (Fig. 2A). The glucoamylase activity dramatically decreased from an initial 1362 mg g^{-1} h^{-1} to 487 mg g^{-1} h^{-1} and 1217 mg g^{-1} h^{-1} to 548 mg g^{-1} h^{-1} during MTFP and HTFP, respectively. The glucoamylase activity at stage IV significantly ($P < 0.05$) increased to 1179 mg g^{-1} h^{-1} during MTFP, while that significantly ($P < 0.05$) reduced to 275 mg g^{-1} h^{-1} during HTFP. Furthermore, glucoamylase activity was significantly ($P < 0.05$) higher on day 14 of MTFP than that of HTFP. Finally, the glucoamylase activity reached at 893 mg g^{-1} h^{-1} and 606 mg g^{-1} h^{-1} during MTFP and HTFP. For the amylase activity, it was significantly ($P < 0.05$) descended at stage IV of HTFP and significantly ($P < 0.05$) ascended at stage V of MTFP

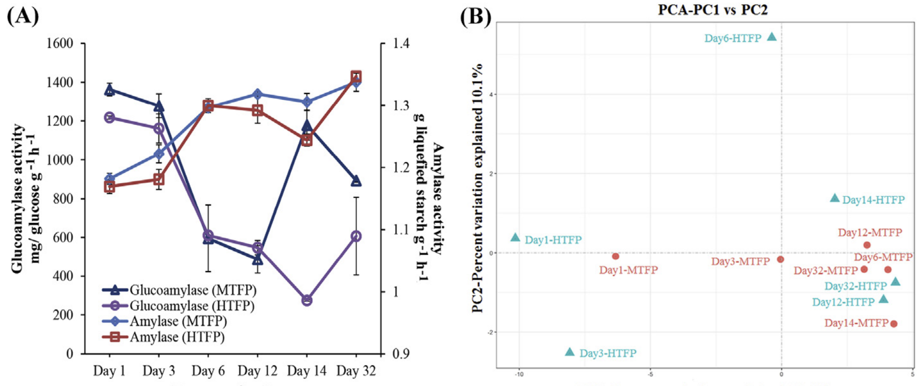

FIG. 2. (A) Dynamics of Daqu glucoamylase and amylase activities during MTFP and HTFP across all the samples. (B) Principal component analysis (PCA) based on the relative contents of volatile compounds in Daqu during MTFP and HTFP across all the samples.

and HTFP, moreover, the former on day 14 was significantly ($P < 0.05$) greater than the latter.

Dynamics of volatile compounds during Daqu fermentation process A total of 63 volatile compounds, including 13 alcohols (No. 1–13), 12 esters (No. 14–25), 7 aldehydes (No. 26–32), 8 alkanes (No. 33–40), 4 ketones (No. 41–44), 4 benzenes (No. 45–48), 3 carboxylic acids (No. 49–51), 4 pyrazines (No. 52–55), and 8 others aromatic compounds (No. 56–63), were identified by HS-SPME-GC-MS in vinegar Daqu samples during the MTFP and HTFP (Tables 1 and S1). Higher relative contents of all flavor compounds were presented on all sample days of HTFP compared to MTFP except for day 14 (Table 1). Moreover, the higher relative contents of alcohols and lower contents of pyrazines were exhibited at end of HTFP than MTFP. The relative contents of alcohols during MTFP dramatically increased to the highest of 12.92 mg/100 g on day 6 and ended with 6.09 mg/100 g; while it during HTFP decreased to 5.66 mg/100 g on day 14 and reached 10.70 mg/100 g on day 32. Nonetheless, the total contents of pyrazines during MTFP and HTFP increased to 1.11 mg/100 g and 0.29 mg/100 g in the end, respectively. In addition, isoamyl alcohol, phenethyl alcohol, 2,3-butanediol, hexyl alcohol, benzyl alcohol, ethyl acetate, acetoin and tetramethylpyrazine were detected at the end of MTFP and HTFP and regarded as the major volatile compounds that contributed desirable aroma components to the matured vinegar Daqu (Table S1). Furthermore, higher relative contents of ethanol, 2,3-butanediol and hexyl alcohol were indicated in matured HT Daqu than the MT Daqu. The PCA analysis indicated a high similarity from days 6–32 of MTFP and days 12–32 of HTFP (Fig. 2B). Moreover, the relative contents of the aroma compounds were inclined to be stable at the late stages of MTFP and HTFP.

Dynamics of viable microbial counts and gene copies during Daqu fermentation process Changes in the viable microbial counts were monitored during the six stages of MTFP and HTFP (Fig. 3). During MTFP and HTFP, the average bacterial and fungal counts of samples on day 1 were rather low and were below 6 Log cfu/g (Fig. 3A, C), but the count of LAB was high with more than 8 Log cfu/g (Fig. 3B). Nevertheless, the total viable numbers of bacteria in the LB and fungi in the PDA significantly ($P < 0.05$) increased at stage I of MTFP and HTFP. In addition, the amount of LAB at stages I and II of HTFP was significantly ($P < 0.05$) lower than that of MTFP. Moreover, at stage IV, the viable bacteria and LAB significantly decreased during HTFP, meanwhile, bacteria significantly increased while LAB significantly decreased in MTFP ($P < 0.05$).

The gene copies of total bacteria, fungi, LAB and *Bacillus* during vinegar Daqu MTFP and HTFP were conducted by qPCR (Fig. 4). The gene copies of the total bacteria, LAB and *Bacillus* significantly ($P < 0.05$) increased at stage IV and then declined until the end of MTFP. In contrast, those of HTFP significantly ($P < 0.05$) decreased at stage IV and then increased to the end of fermentation. Furthermore, significantly ($P < 0.05$) higher gene copies were found for these three microbes on day 14 of the MTFP compared with the HTFP. Compared with MTFP, the fungi gene copies at stages IV and V of HTFP significantly ($P < 0.05$) decreased (Fig. 4D). Higher levels of fungi gene copies were exhibited at the final phase of MTFP than HTFP.

Analysis of high-throughput sequencing data by Illumina HiSeq For bacterial communities, a sum of 660,459 high-quality sequences from all the samples were acquired after the removal of low-quality reads and chimeras (Table S2). Those effective bacterial tags were assigned to range from 454 to 919 OTUs for each sample. For fungal communities, a total of 755,590 high-quality sequences were obtained, and each sample contained 46,809 to 71,887 effective tags with different engendered OTUs ranging from 66 to 301. In addition, the rarefaction curves were inclined to the saturation plateau and the coverage of the good-quality sequences across each sample was higher than 99%, which indicated that the sequencing depth could cover the microbial diversity (Fig. S1 and Table S2). For the bacterial richness and diversity, the sample from day 14 of MTFP possessed a higher Chao1 indexs compared with HTFP (Table S2), indicating a higher species richness was observed in MTFP. Different Shannon indexes were observed and the diversity of the two fermentation processes decreased in MTFP and HTFP. However, for fungal richness and diversity (Table S2), the Chao1 and Shannon indexes during MTFP and HTFP were enhanced, with obviously higher richness and diversity during MTFP than HTFP observed from days 14–32.

Generally, the evolution of microbial communities during MTFP and HTFP were different at the order (averaging at 97% and 72% of total bacterial and fungal sequences, respectively) and genus (averaging at 14% and 72% of total bacterial and fungal sequences, respectively) levels (Fig. 5). There were no significant differences ($P > 0.05$) in the microbial communities during entire MTFP and HTFP. Enterobacteriales, Lactobacillales, Bacillales, Saccharomycetales and Mucorales were the dominant orders in the whole two fermentation processes (Fig. 5A). The prevailing genera during MTFP and HTFP were *Saccharomycopsis*, *Rhizomucor* and *Wickerhamomyces* (Fig. 5B). Highly similar successional dynamics of microbial communities was observed during vinegar Daqu MTFP and HTFP at order and genus levels. The trend of Lactobacillales' and *Wickerhamomyces*' relative abundance was gradually declined during the whole MTFP and HTFP. Moreover, the relative abundance of Bacillales, *Saccharomycopsis* and *Rhizomucor* increased at stage I of MTFP and HTFP. For Mucorales, the trends of relative abundance increased during days 1–14 of HTFP and days 1–12 of MTFP, followed by a rapid decline to the end of both fermentation. The relative abundances of Eurotiales were higher on day 12 of the MTFP and during days 12–14 (at stage IV) of HTFP compared with other period of fermentation processes. Meanwhile, *Leuconostoc* and *Pediococcus* were the predominant genera detected within the

TABLE 1. The relative contents of volatile compounds in Daqu during MTFP and HTFP across all the samples by HS-SPME-GC-MS (mg/100 g Daqu).

Volatile compounds [number]	Day 1-MTFP	Day 3-MTFP	Day 6-MTFP	Day 12-MTFP	Day 14-MTFP	Day 32-MTFP	Day 1-HTFP	Day 3-HTFP	Day 6-HTFP	Day 12-HTFP	Day 14-HTFP	Day 32-HTFP
Alcohols [13]	11.19	12.02	12.92	7.49	8.03	6.09	8.07	14.77	11.49	11.08	5.66	10.70
Esters [12]	9.89	4.94	0.09	0.55	0.41	0.53	16.70	14.70	10.24	0.76	0.24	0.59
Aldehydes [7]	2.09	0.85	0.30	0.50	0.40	0.33	1.55	1.52	1.43	0.26	0.28	0.33
Alkanes [8]	nd	0.20	0.24	0.06	0.38	0.64	nd	nd	nd	0.81	0.37	0.28
Ketones [4]	nd	0.67	0.68	0.42	1.59	0.36	nd	1.64	0.71	2.25	0.59	0.27
Benzenes [4]	nd	0.16	0.51	0.49	0.55	0.54	nd	0.18	0.25	0.11	0.26	0.36
Carboxylic acids [3]	1.03	0.83	0.25	0.34	0.55	0.41	nd	nd	0.63	0.61	0.19	0.63
Pyrazines [4]	nd	nd	0.18	0.39	6.53	1.11	nd	nd	nd	0.13	nd	0.29
Others [8]	nd	nd	0.94	0.06	1.27	nd	nd	0.72	0.19	nd	0.18	0.46

The numbers in brackets represent the specific numbers of types belonging to these category volatile substances. nd, not detected.

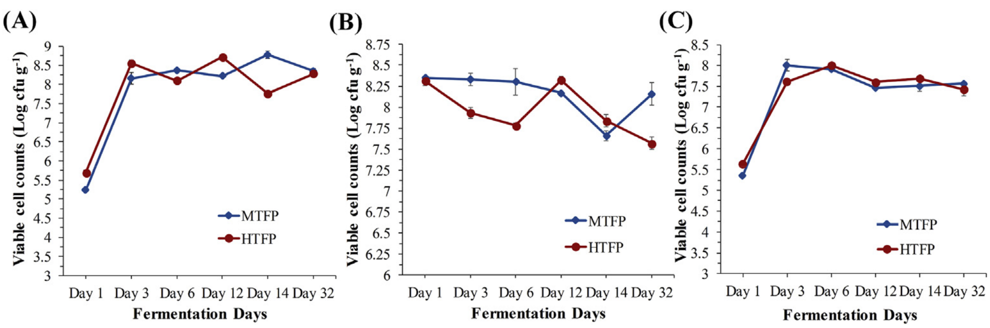

FIG. 3. Dynamics of viable microbial counts in Daqu during MTFP and HTFP across all the samples. (A) Total bacteria, (B) LAB and (C) fungi.

first three days of fermentation and were retrieved afterward at low frequencies until the end of fermentation.

Nevertheless, there were some discrepancy at different stages. The relative abundance of Enterobacteriales was slightly higher in days 3–14 of MTFP than that of HTFP, while it was contrary in the end of MTFP and HTFP (77.37% of the total sequences in the end of MTFP, while 83.71% in the end of HTFP). Moreover, the relative abundance of Lactobacillales at each stage of HTFP was slightly greater than that of MTFP. For Bacillales, its relative abundance gradually decreased in days 3–12 of MTFP and finally reached to 7.18% of the total sequences; while its relative abundance during HTFP reached the highest at 10.89% and remained at 3.84%. Actually, the relative abundance of Saccharomycetales first showed an upward trend in the initial 6 days, then sharply decreased from days 12–14 and ended up with 16.32% of the total sequences during HTFP, while its relative abundance in MTFP rapidly increased from days 12–32 and ended up with 75.40%. Besides, the relative abundance of Mucorales decreased from 64.51% to 43.28% at stage IV of MTFP, whereas increased from 28.77% to 80.25% at this stage of HTFP. For *Saccharomycopsis*, the relative abundance raised to the highest abundance of 52.57% on day 3 of MTFP and of 85.10% on day 6 of HTFP. Subsequently, the relative abundance of *Saccharomycopsis* during MTFP decreased from days 3–12 and the following days increased to 42.06%; while it during HTFP showed a decreased tendency (declined from 52.86% to 5.68% at stage IV) until the end of fermentation and ended up with 13.04%. Moreover, the relative abundance of *Rhizomucor* had similar successional dynamics to *Saccharomycopsis* and reached the highest at 62.32% on day 12 of MTFP and 56.49% on day 14 of HTFP. *Wickerhamomyces* had the highest relative abundance on the first day during MTFP and HTFP, then reached the lowest at 0.64% on day 12 and 0.62% on day 14, and ended up with 6.23% and 3.26%, respectively.

Based on 16S rRNA and ITS gene amplicons, an Unifrac distance-based weighted PCoA was conducted to evaluate similarities and differences in the microbiota among the different samples. Comparing the MTFP and HTFP, clusters tended to form from days 3–32 for bacterial (Fig. S2A) communities and only on day 1 and day 6 for fungal (Fig. S2B) communities, respectively.

Relationships between microbial composition and physicochemical properties during Daqu fermentation process Microbial community and physicochemical properties interact at different stages of vinegar Daqu production in a complex way. The potential relationship between measurable parameters and microbiota composition at the order (Fig. 6A) and genus (Fig. 6B) levels were discerned by the RDA. The core temperature

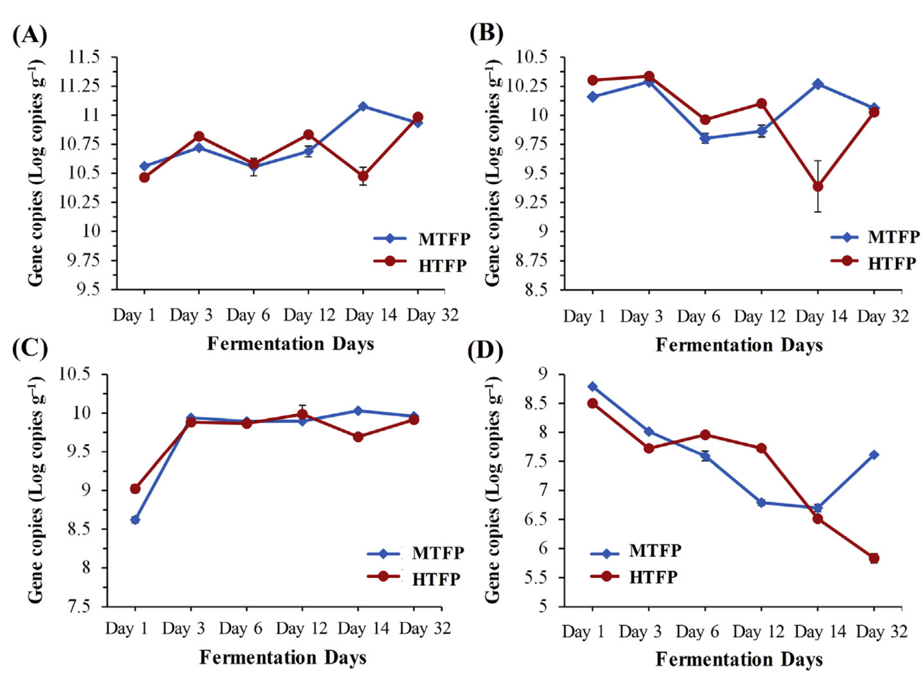

FIG. 4. Dynamics of gene copies of total bacteria (A), LAB (B), *Bacillus* (C), and fungi (D) in Daqu during MTFP and HTFP across all the samples.

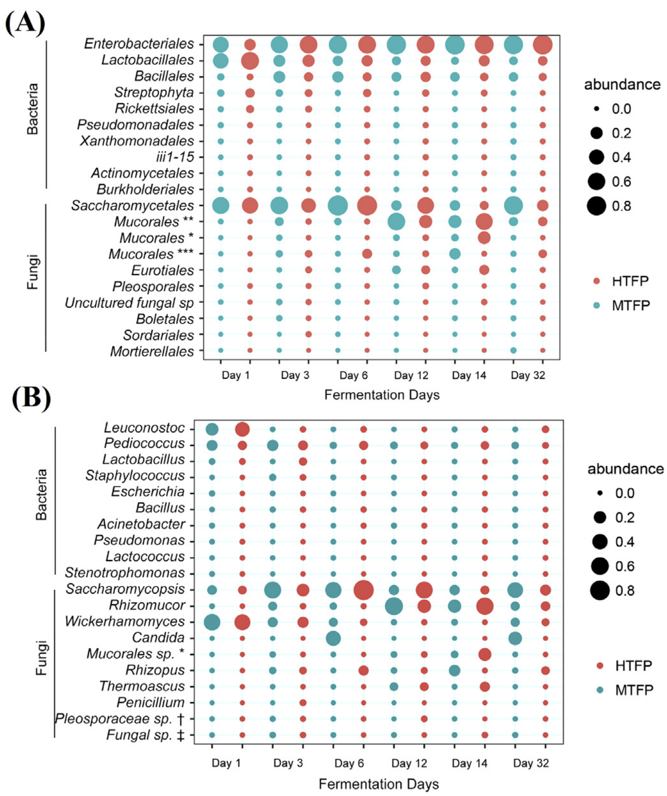

FIG. 5. Succession of microbial community in Daqu during MTFP and HTFP across all the samples. The distribution of major order in the bacterial and fungal communities (A). The distribution of major genus in the bacterial and fungal communities (B). The taxonomy: *, IS–s-*Mucorales* sp.-*Mucorales*; **, IS–s-*Rhizomucor pusillus-Mucorales*; ***, IS–s-*Rhizopus arrhizus* var. arrhizus-*Mucorales*; †, Un–s-*Pleosporaceae* sp. RS_5; ‡, Un–s-fungal sp. 38 CC 06_28.

showed a positive correlation with the microbial composition from days 3–14, and was positively related to the dominant phylotypes of Bacillales, Mucorales and Xanthomonadales at the order level (Fig. 6A) and *Staphylococcus*, *Mucorales* sp., *Thermoascus* and *Rhizopus* at the genus level (Fig. 6B). Nevertheless, it showed a negative correlation with the microbial composition at stages I and V, and was negatively associated with primary phylotypes of Pseudomonadales and Enterobacteriales at the order level and *Acinetobacter* and *Lactococcus* at the genus level. Furthermore, the relationship between core temperature and microbial composition switched from day 12 of the MTFP and HTFP.

Acidity, glucose content and moisture showed positive associations with the microbiota composition at stage I and II. However, the acidity and moisture showed major correlations with microbial composition on day 1 of MTFP and were positively related with the predominant phylotypes of Mucorales* (namely, IS–s-*Mucorales* sp.-*Mucorales*) and Lactobacillales at the order level and *Rhizomucor*, *Wickerhamomyces*, *Leuconostoc* and *Pediococcus* at the genus level. Moreover, the glucose content showed strong correlation with the microbial composition at stage II of HTFP and positively correlated with the predominant phylotypes of Streptophyta, Rickettsiales and Xanthomonadales at the order level and *Rhizopus*, *Lactobacillus* and *Pleosporaceae* sp. at the genus level. Furthermore, the pH showed a positive correlation with the microbial composition at stage IV and was positively associated with the dominant phylotypes of Enterobacteriales, Saccharomycetales and Actinomycetales at the order level and *Lactococcus*, *Saccharomycetales* sp., *Candida* and *Bacillus* at the genus level.

DISCUSSION

As operated, the profile of vinegar Daqu monitored by a thermometer conformed to characteristic MT and HT Daqu SSF process (Fig. 1A) (13,30). The calorigenesis of the early stages was derived from rapid microbial propagation, which was verified by the results of biomass dynamics (Figs. 3 and 5). Moreover, the core temperature was the most pivotal factor at the stage II-IV impacting the microbial composition and higher relative abundance of Eurotiales and Bacillales thrived on higher core temperature by the RDA analysis (13) (Fig. 6). In addition, more gene copies of acid-producing bacteria and the higher relative abundance of Lactobacillales (e.g., *Leuconostoc* and *Pediococcus*) positively responded to the acidity were exhibited in the II-III stages of HTFP explaining higher titratable acidity during the two stages of HTFP compared with MTFP Figs. 4B, 5A and 6 (24). This result implied that the high temperature fermentation altered the relative composition of microbes and metabolic activity, consequently changing the physicochemical parameters (acidity and pH) during Daqu fermentation process. In addition, the RDA results implied that microbial structure transition occurred at stage IV under a series of environmental stresses by the distribution of vinegar Daqu samples, and similar

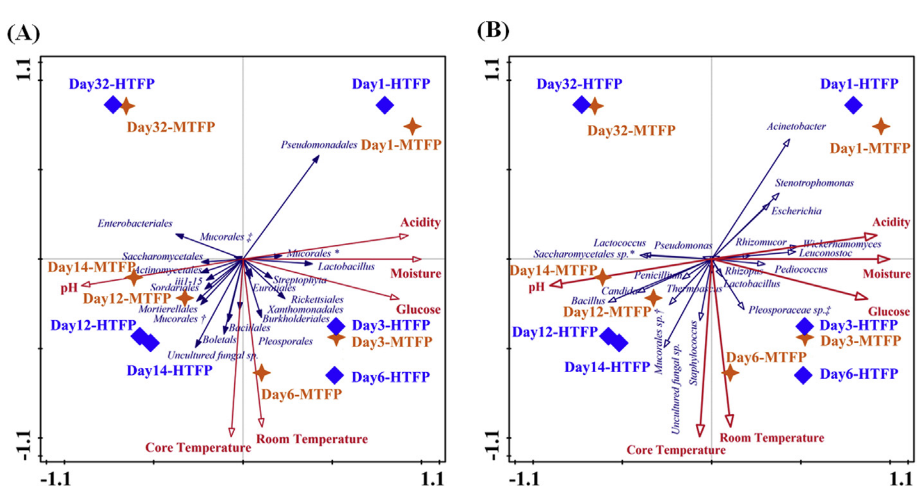

FIG. 6. RDA correlation with microbial community structure acquired by Illumina HiSeq sequencing and measurable parameters in Daqu during MTFP and HTFP at levels of order (A) and genus (B). The taxonomy: *, IS–s-*Mucorales* sp.-*Mucorales*; **, IS–s-*Rhizomucor pusillus-Mucorales*; ***, IS–s-*Rhizopus arrhizus* var. arrhizus-*Mucorales*; †, Un–s-*Pleosporaceae* sp. RS_5; ‡, Un–s-fungal sp. 38 CC 06_28.

distinct microbial composition transitions were also reported during the fermentation process (31). This illustrated that environmental variables functioned as the driver promoting the dynamics of microbial community during Daqu fermentation process (14,32).

Enzyme activities and volatile compounds are part and parcel components in Daqu admittedly (32,33). Glucoamylase and amylase endowed with liquefaction and saccharification abilities to Daqu. In the present study, the filamentous fungus propagated (Fig. 5) and metabolized to secrete glucoamylase inducing significant increase in glucoamylase activity at stage IV of MTFP (Fig. 2A) (34). The plausible reason of the decreased activity during this stage of HTFP was that heat-labile fungi (e.g., *Rhizopus*, *Aspergillus*, *Saccharomycopsis*) which contribute to the saccharifying power were inhibited and enzymes were inactivated by high temperature fermentation (22). In particular, higher glucoamylase and amylase activities of Daqu in MTFP than HTFP occurred on day 14 (Fig. 1A), indicating that temperature was too high to maintain the activities of glucoamylase and amylase. Rather, the liquefaction was not only related to filamentous fungus but also associated with *Bacillus* that possesses a high temperature tolerance and a strong ability to hydrolyze starch (35). The glucoamylase activity and amylase activity of two types matured vinegar MT and HT Daqu were in the appropriate ranges to guarantee a certain number of fermentable sugar during acetic acid fermentation process (3,24). Furthermore, no significant difference was found in enzyme activities at the end of MTFP and HTFP, demonstrating that the two types of matured vinegar Daqu in enzyme activities were similar under experimental temperature. *Saccharomycopsis* was interrelated to the production of alcohol via the Ehrlich metabolic pathway on account of rich amino acids in the raw materials (wheat), conferring vinegar flower, sweet flavor and more soft tastes (36). Consequently, that higher relative contents of alcohols presented in matured HT vinegar Daqu could be explained by the higher relative abundance of *Saccharomycopsis* during HTFP (Fig. 5B and Table 1). 2,3-butanediol and isoamyl alcohol (with floral and fruity aromas) endowed vinegar with desirable flavor and predominated in both fermentation processes, as was coincident with previous reports (37–39). However, the matured vinegar MT Daqu exhibited richer relative contents in pyrazines (Table 1), which provide roasted, milky, nutty and toasty aromas and are the major bioactive components in vinegar (32). The plausible explanation may be that the formation of pyrazine compounds was also related bioactivities of microbial metabolisms during fermentation (21). The higher abundance of Bacillales with the ability of pyrazines production (40) contributed to the more pyrazines in matured MT Daqu (Fig. 5A). Furthermore, a higher relative content of ethyl acetate (with rosy, floral, and fruity aromas) as the main ester was detected at the final stages of MTFP than that of HTFP, which was consistent with the relative abundance dynamics of ester-producing yeast (mainly *Wickerhamomyces* and *Candida*) (Fig. 5B). Hexanal (with green grass aroma) played an important role in forming the Daqu flavor, and a richer hexanal was detected as the major aldehyde in matured HT Daqu compared with the MT Daqu (17). Moreover, a majority of the volatiles in vinegar Daqu could be detected in the acetic acid fermentation process (41). Furthermore, although the PCA analysis indicated small differences that were present in the flavor materials at the final MTFP and HTFP, higher relative contents of all flavor compounds were shown in matured HT vinegar Daqu. The HT vinegar Daqu as starter culture and a portion of raw materials in follow-up fermentation could be able to improve the vinegar Daqu flavor. This illustrated the HT was promising for forming more content aroma substances than MT and affecting the quality of end-product (42).

During the MTFP and HTFP of vinegar Daqu, the low bacterial and fungal counts and high LAB counts on day 1 indicated that LAB was the dominant viable microbes during initial fermentation stage with low temperature and acidity. Bacteria and fungi multiplied in Daqu media and increased approximately 3 orders of magnitude during stage I of MTFP and HTFP (Fig. 3A, C). Thereafter, the population level of the fungi during MTFP declined until the final stage, as previously shown in a Fen-Daqu study (2). The gene copies of total bacteria and fungi is the characteristic of microbial biomass which is crucial indicator for assessing the Daqu since production of enzyme depends on microbial biomass (4). Moreover, LAB and *Bacillus* as functional microbes play key roles in flavor formation during cereal starter fermentation and are related to thermo-labile and thermophilic microbes, respectively. Hence, the total bacteria, fungi, LAB and *Bacillus* during vinegar Daqu MTFP and HTFP were quantified by qPCR. Among the gene copies of bacteria, LAB and *Bacillus* showed poles apart at stage IV of MTFP and HTFP (Fig. 4A–C), indicating LAB have a low tolerance to high heat stress. Thermophilic bacteria (e.g., *Bacillus*) could breed at this stage during the fermentation process and a too high temperature at that time ($>55°C$) might not conducive to this bacterial growth (43,44). Moreover, the fungal gene copies at the final stage of MTFP were significantly higher than that of HTFP, and the obvious difference in the abundance of Saccharomycetales including *Saccharomycopsis*, *Wickerhamomyces* and *Candida* during HTFP and MTFP (Fig. 5), which demonstrated that the fungal group were negatively responded the high temperature fermentation (6). Conclusively, even though there was significant decline in the biomass of bacteria, LAB, *Bacillus* and fungi were exhibited at stage IV of HTFP, the biomass of the first three were no significant ($P > 0.05$) at end of the HTFP and MTFP.

Understanding the microbial diversity and dynamics involved in MT and HT vinegar starter provides important and deep insights of intricate fermentation system. Throughout the whole fermentation processes, the dominant orders Enterobacteriales, Lactobacillales, Bacillales, Saccharomycetales and Mucorales were widely distributed in various cereal starters (Fig. 5A) (3,24). This demonstrated the deterministic microbial community assemblage by taxonomic relatedness (45). Intriguingly, the relative abundance of Enterobacteriales peaked after HTFP loading the evidence for heat-resistant of Enterobacteriales and Enterobacteriales might be suitable to propagate on the condition of HT fermentation (46). In addition, various genera of LAB contributing to favor formation were identified in both vinegar Daqu starters, including *Leuconostoc*, *Pediococcus*, *Lactobacillus* (a dominated genus in vinegar) (24,47), *Streptococcus* and *Lactococcus* (Fig. 5B), which indicated that these LAB adapted the physicochemical properties of Daqu media. In particular, the 2,3-butanediol-producing bacteria *Paenibacillus* was detected during the whole fermentation despite low abundance, and the highest abundance that occurred was during HTFP, which might explain the higher relative content of 2,3-butanediol in matured HT vinegar Daqu (48). Moreover, *Pseudomonas* had a good ability to produce alcohols, whose abundance was the same as *Paenibacillus*, elucidating the higher relative contents of alcohols in matured HT vinegar Daqu (49). For fungi, *Saccharomycopsis* was the most commonly isolated yeast in those vinegar Daqu samples and previous study has described that it has strong glucoamylase and amylolytic activity in wheat bran koji and Korean nuruk (both are starter) (50). Meanwhile, this study suggested that the higher bacterial and fungal richness and diversity were observed during the MTFP compared with the HTFP (Table S2). However, there were no significant differences in the microbial composition between the entire MTFP and HTFP according to the P value (> 0.05). Therefore, the microbial composition in matured HT Daqu resembled that both in matured MT Daqu in this study and our previous studies which have been applied for practical vinegar production (24,41).

To the best of our knowledge, this work is the first to report two types of MT and HT vinegar Daqu from a comprehensive perspective.

No significant difference ($P > 0.05$) was found in the enzyme activities and microbial composition in the end of vinegar Daqu MTFP and HTFP despite subsistent marked difference between the two batches at some stages of fermentation. The microbial structure transition occurred at stage IV under intricate environmental stresses, demonstrating that environmental variables functioned as the driver promoting the dynamics of microbial community during Daqu fermentation process. Although the flavor compounds reach conformity during the later stages of fermentation in this study, a higher relative content of all flavor compounds was displayed and the microbial community might be significantly influenced during HTFP compared with MTFP. In conclusion, high temperature (approximately 60°C) fermentation was promising to produce the Daqu applied for high quality vinegar fermentation. This work might be conducive to preferably make traditional Daqu by modulating steerable environmental parameters, and further investigation concerning microbial succession and metabolite changes during acetic acid fermentation with the addition of HT vinegar Daqu need to be performed to stabilize the quality of the end-product.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.03.011.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (grant 31771962). The authors declare no conflict of interest.

References

1. **Brandt, M. J.:** Starter cultures for cereal based foods, Food Microbiol., **37**, 41–43 (2014).
2. **Zheng, X. W., Yan, Z., Nout, M. J., Smid, E. J., Zwietering, M. H., Boekhout, T., Han, J. S., and Han, B. Z.:** Microbiota dynamics related to environmental conditions during the fermentative production of Fen-Daqu, a Chinese industrial fermentation starter, Int. J. Food Microbiol., **182–183**, 57–62 (2014).
3. **Li, P., Lin, W., Liu, X., Wang, X., Gan, X., Luo, L., and Lin, W. T.:** Effect of bioaugmented inoculation on microbiota dynamics during solid-state fermentation of Daqu starter using autochthonous of *Bacillus*, *Pediococcus*, *Wickerhamomyces* and *Saccharomycopsis*, Food Microbiol., **61**, 83–92 (2017).
4. **Li, P., Lin, W., Liu, X., Wang, X., and Luo, L.:** Environmental factors affecting microbiota dynamics during traditional solid-state fermentation of Chinese Daqu starter, Front. Microbiol., **7**, 1237 (2016).
5. **Zhang, L., Wu, C., Ding, X., Zheng, J., and Zhou, R.:** Characterisation of microbial communities in Chinese liquor fermentation starters Daqu using nested PCR-DGGE, World J. Microbiol. Biotechnol., **30**, 3055–3063 (2014).
6. **Zheng, X.-W., Yan, Z., Nout, M. R., Boekhout, T., Han, B.-Z., Zwietering, M. H., and Smid, E. J.:** Characterization of the microbial community in different types of Daqu samples as revealed by 16S rRNA and 26S rRNA gene clone libraries, World J. Microbiol. Biotechnol., **31**, 199–208 (2015).
7. **Du, H. and Xu, Y.:** Determination of the microbial origin of geosmin in Chinese liquor, J. Agric. Food Chem., **60**, 2288–2292 (2012).
8. **Du, H., Lu, H., Xu, Y., and Du, X.:** Community of environmental streptomyces related to geosmin development in Chinese liquors, J. Agric. Food Chem., **61**, 1343–1348 (2013).
9. **Singhania, R. R., Patel, A. K., Soccol, C. R., and Pandey, A.:** Recent advances in solid-state fermentation, Biochem. Eng. J., **44**, 13–18 (2009).
10. **Li, P., Aflakpui, F. W. K., Yu, H., Luo, L., and Lin, W. T.:** Characterization of activity and microbial diversity of typical types of Daqu for traditional Chinese vinegar, Ann. Microbiol., **65**, 2019–2027 (2015).
11. **Couto, S. R. and Sanromán, M.Á.:** Application of solid-state fermentation to food industry—a review, J. Food Eng., **76**, 291–302 (2006).
12. **Pang, X. N., Han, B. Z., Huang, X. N., Zhang, X., Hou, L. F., Cao, M., Gao, L. J., Hu, G. H., and Chen, J. Y.:** Effect of the environment microbiota on the flavour of light-flavour Baijiu during spontaneous fermentation, Sci. Rep., **8**, 3396 (2018).
13. **Li, P., Liang, H., Lin, W. T., Feng, F., and Luo, L.:** Microbiota dynamics associated with environmental conditions and potential roles of cellulolytic communities in traditional Chinese cereal starter solid-state fermentation, Appl. Environ. Microbiol., **81**, 5144–5156 (2015).
14. **Xiao, C., Lu, Z. M., Zhang, X. J., Wang, S. T., Ao, L., Shen, C. H., Shi, J. S., and Xu, Z. H.:** Bio-heat is a key environmental driver shaping the microbial community of medium-temperature Daqu, Appl. Environ. Microbiol., **83**, e01550-17 (2017).
15. **Wang, H. and Xu, Y.:** Effect of temperature on microbial composition of starter culture for Chinese light aroma style liquor fermentation, Lett. Appl. Microbiol., **60**, 85–91 (2015).
16. **Liu, J., Chen, J., Fan, Y., Huang, X., and Han, B.:** Biochemical characterisation and dominance of different hydrolases in different types of Daqu - a Chinese industrial fermentation starter, J. Sci. Food Agric., **98**, 113–121 (2017).
17. **Zhang, C., Ao, Z., Chui, W. Q., Shen, C., Tao, W., and Zhang, S.:** Characterization of the aroma-active compounds in Daqu: a tradition Chinese liquor starter, Eur. Food Res. Technol., **234**, 69–76 (2012).
18. **Nie, Z., Zheng, Y., Xie, S., Zhang, X., Song, J., Xia, M., and Wang, M.:** Unraveling the correlation between microbiota succession and metabolite changes in traditional Shanxi aged vinegar, Sci. Rep., **7**, 1294 (2017).
19. **Wu, J. J., Ma, Y. K., Zhang, F. F., and Chen, F. S.:** Biodiversity of yeasts, lactic acid bacteria and acetic acid bacteria in the fermentation of "Shanxi aged vinegar", a traditional Chinese vinegar, Food Microbiol., **30**, 289–297 (2012).
20. **Miller, G. L.:** Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem., **31**, 426–428 (1959).
21. **Zhang, L., Huang, J., Zhou, R., and Wu, C.:** Evaluating the feasibility of fermentation starter inoculated with *Bacillus amyloliquefaciens* for improving acetoin and tetramethylpyrazine in Baoning bran vinegar, Int. J. Food Microbiol., **255**, 42–50 (2017).
22. **Li, P., Li, S., Cheng, L., and Luo, L.:** Analyzing the relation between the microbial diversity of DaQu and the turbidity spoilage of traditional Chinese vinegar, Appl. Microbiol. Biotechnol., **98**, 6073–6084 (2014).
23. **Zheng, X. W., Yan, Z., Han, B. Z., Zwietering, M. H., Samson, R. A., Boekhout, T., and Robert Nout, M. J.:** Complex microbiota of a Chinese "Fen" liquor fermentation starter (Fen-Daqu), revealed by culture-dependent and culture-independent methods, Food Microbiol., **31**, 293–300 (2012).
24. **Gan, X., Tang, H., Ye, D., Li, P., Luo, L., and Lin, W.:** Diversity and dynamics stability of bacterial community in traditional solid-state fermentation of Qishan vinegar, Ann. Microbiol., **67**, 703–713 (2017).
25. **Ye, D., Liang, H., Zhou, W., Yan, J., Zhou, S., and Luo, L.:** Total and active microbial communities in a full-scale system treating wastewater from soy sauce production, Int. Biodeterior. Biodegradation, **123**, 206–215 (2017).
26. **Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A.:** PEAR: a fast and accurate Illumina Paired-End reAd mergeR, Bioinformatics, **30**, 614–620 (2014).
27. **Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., and other 17 authors:** QIIME allows analysis of high-throughput community sequencing data, Nat. Methods, **7**, 335–336 (2010).
28. **Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., Ciulla, D., Tabbaa, D., Highlander, S. K., Sodergren, E., and other 6 authors:** Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons, Genome Res., **21**, 494–504 (2011).
29. **Edgar, R. C.:** UPARSE: highly accurate OTU sequences from microbial amplicon reads, Nat. Methods, **10**, 996–998 (2013).
30. **Zheng, X. W., Tabrizi, M. R., Nout, M. J. R., and Han, B. Z.:** Daqu – a traditional Chinese liquor fermentation starter, J. Inst. Brew., **117**, 82–90 (2012).
31. **Hu, X., Du, H., Ren, C., and Xu, Y.:** Illuminating anaerobic microbial community and cooccurrence patterns across a quality gradient in Chinese liquor fermentation pit muds, Appl. Environ. Microbiol., **82**, 2506–2515 (2016).
32. **Wang, P., Wu, Q., Jiang, X., Wang, Z., Tang, J., and Xu, Y.:** *Bacillus licheniformis* affects the microbial community and metabolic profile in the spontaneous fermentation of Daqu starter for Chinese liquor making, Int. J. Food Microbiol., **250**, 59–67 (2017).
33. **Kim, O., Magnus, B., and Gunnar, L.:** A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks, Biotechnol. Biofuels, **1**, 7 (2008).
34. **Kwon, M. J., Jørgensen, T. R., Nitsche, B. M., Arentshorst, M., Park, J., Ram, A. F., and Meyer, V.:** The transcriptomic fingerprint of glucoamylase over-expression in *Aspergillus niger*, BMC Genomics, **13**, 701 (2012).
35. **Asgher, M., Asad, M. J., Rahman, S. U., and Legge, R. L.:** A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing, J. Food Eng., **79**, 950–955 (2007).
36. **Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T., and Dickinson, J. R.:** The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism, Appl. Environ. Microbiol., **74**, 2259–2266 (2008).
37. **Yu, Y.-J., Lu, Z.-M., Yu, N.-H., Xu, W., Li, G.-Q., Shi, J.-S., and Xu, Z.-H.:** HS-SPME/GC-MS and chemometrics for volatile composition of Chinese traditional aromatic vinegar in the Zhenjiang region, J. Inst. Brew., **118**, 133–141 (2012).
38. **Xiao, Z. B., Dai, S. P., Niu, Y. W., Yu, H. Y., Zhu, J. C., Tian, H. X., and Gu, Y. B.:** Discrimination of Chinese vinegars based on headspace solid-phase microextraction-gas chromatography mass spectrometry of volatile compounds and multivariate analysis, J. Food Sci., **76**, C1125–C1135 (2011).
39. **Wu, L.-H., Lu, Z.-M., Zhang, X.-J., Wang, Z.-M., Yu, Y.-J., Shi, J.-S., and Xu, Z.-H.:** Metagenomics reveals flavour metabolic network of cereal vinegar microbiota, Food Microbiol., **62**, 23–31 (2017).

40. **Zhu, B.-F., Xu, Y., and Fan, W.-L.:** High-yield fermentative preparation of tetramethylpyrazine by *Bacillus* sp. using an endogenous precursor approach, J. Ind. Microbiol. Biotechnol., **37**, 179−186 (2010).
41. **Li, S., Li, P., Liu, X., Luo, L., and Lin, W.:** Bacterial dynamics and metabolite changes in solid-state acetic acid fermentation of Shanxi aged vinegar, Appl. Microbiol. Biotechnol., **100**, 4395−4411 (2016).
42. **Su, Y., Yang, L., Hui, L., Yuan-Yuan, G., Ming-Juan, Z., Chun-Hui, X., Ling, X., and Chi, C.:** Bacterial communities during the process of high-temperature Daqu production of roasted sesame-like flavour liquor, J. Inst. Brew., **121**, 440−448 (2015).
43. **Darsonval, M., Msadek, T., Alexandre, H., Grandvalet, C., and Elkins, C. A.:** The antisense RNA approach: a new application forIn VivoInvestigation of the stress response of *Oenococcus oeni*, a wine-associated lactic acid bacterium, Appl. Environ. Microbiol., **82**, 18−26 (2016).
44. **Huang, S., Yang, Y., Fu, N., Qin, Q., Zhang, L., and Chen, X. D.:** Calcium-aggregated milk: a potential new option for improving the viability of lactic acid bacteria under heat stress, Food Bioprocess Technol., **7**, 3147−3155 (2014).
45. **Griffin, J. S. and Wells, G. F.:** Regional synchrony in full-scale activated sludge bioreactors due to deterministic microbial community assembly, ISME J., **11**, 500−511 (2017).
46. **Mercer, R. G., Walker, B. D., Yang, X., Mcmullen, L. M., and Gänzle, M. G.:** The locus of heat resistance (LHR) mediates heat resistance in *Salmonella enterica*, *Escherichia coli* and *Enterobacter cloacae*, Food Microbiol., **64**, 96−103 (2017).
47. **Huang, Y., Yi, Z., Jin, Y., Zhao, Y., He, K., Liu, D., Zhao, D., He, H., Luo, H., Zhang, W., Fang, Y., and Zhao, H.:** New microbial resource: microbial diversity, function and dynamics in Chinese liquor starter, Sci. Rep., **7**, 14577 (2017).
48. **Häßler, T., Schieder, D., Pfaller, R., Faulstich, M., and Sieber, V.:** Enhanced fed-batch fermentation of 2, 3-butanediol by *Paenibacillus polymyxa* DSM 365, Bioresour. Technol., **124**, 237−244 (2012).
49. **Liu, S. P., Mao, J., Liu, Y. Y., Meng, X. Y., Ji, Z. W., Zhou, Z. L., and Ai-lati, A.:** Bacterial succession and the dynamics of volatile compounds during the fermentation of Chinese rice wine from Shaoxing region, World J. Microbiol. Biotechnol., **31**, 1907−1921 (2015).
50. **Carroll, E., Trinh, T. N., Son, H., Lee, Y. W., and Seo, J. A.:** Comprehensive analysis of fungal diversity and enzyme activity in nuruk, a Korean fermenting starter, for acquiring useful fungi, J. Microbiol., **55**, 357−365 (2017).