Ann Microbiol (2017) 67:703–713
DOI 10.1007/s13213-017-1299-6

ORIGINAL ARTICLE

Diversity and dynamics stability of bacterial community in traditional solid-state fermentation of Qishan vinegar

Xing Gan[1] · Hanlan Tang[1] · Dongdong Ye[1] · Pan Li[1] · Lixin Luo[1] · Weifeng Lin[2]

Received: 20 August 2017 / Accepted: 3 September 2017 / Published online: 16 September 2017

Abstract Qishan vinegar is a typical Chinese fermented cereal product that is prepared using traditional solid-state fermentation (SSF) techniques. The final qualities of the vinegar produced are closely related to the multiple bacteria present during SSF. In the present study, the dynamics of microbial communities and their abundance in Daqu and vinegar *Pei* were investigated by the combination of high throughput sequencing and quantitative PCR. Results showed that the *Enterobacteriales* members accounted for 94.7%, 94.6%, and 92.2% of total bacterial sequences in Daqu Q3, Q5, and Q10, respectively. Conversely, *Lactobacillales* and *Rhodospirillales* dominated during the acetic acid fermentation (AAF) stage, corresponding to the quantitative PCR results. *Lactobacillus*, *Acetobacter*, *Weissella*, *Leuconostoc* and *Bacillus* were the dominant and characteristic bacterial genera of Qishan vinegar during AAF process. Redundancy analysis suggested that *Lactobacillales* and *Rhodospirillales* had a positive correlation with humidity and acidity, respectively. These results confirmed that the bacterial community structure could be affected by physiochemical factors, which determined the unique bacterial composition at different fermentation stages and showed batch-to-batch consistency and stability. Therefore, the conformity of bacterial community succession with physiochemical parameters guaranteed the final quality of Qishan vinegar products. This study provided a scientific perspective for the uniformity and stability of Qishan vinegar, and might aid in controlling the manufacturing process.

Keywords Bacterial composition · Physiochemical parameters · Uniformity · Chinese Qishan vinegar

Introduction

Vinegar—a fermented food—is a common and important condiment with a unique flavor, nutritional value, and health benefits (Liljeberg and Björck 1998; Xu et al. 2011a). It is produced using solid-state fermentation (SSF) technology, mainly in Asian countries, or using submerged fermentation technology, mainly in European countries (Morales et al. 2001; Tesfaye et al. 2002; Ooijkaas 2004). Chinese Qishan vinegar is a typical cereal vinegar that uses SSF technology in an open environment that allows various microbial communities to participate in the fermentation process. In China, the annual production of vinegar is approximately 3.5 million metric tons, with average per capita consumption of vinegar of ~2.3 kg (Li et al. 2014). Recently, SSF has gained renewed attention not only because of its low-energy requirement associated with high-product yield but also because of its low waste water production and low risks of microbial contamination (Thomas et al. 2013).

The famous traditional Chinese Qishan vinegar is brewed from cereals such as wheat, barley, corn, wheat bran and rice hull using a solid-state acetic acid fermentation (AAF) technique with the addition of a Daqu starter. Daqu, mixed with barely, wheat, and peas, contains diverse functional microbes

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13213-017-1299-6) contains supplementary material, which is available to authorized users.

✉ Lixin Luo
btlxluo@scut.edu.cn

✉ Weifeng Lin
linwf@scut.edu.cn

[1] Guangdong Key Laboratory of Fermentation and Enzyme Engineering, School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China

[2] School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China

and enzymes. It is the primary saccharifying and liquefying agent, and can accelerate and steer the fermentation process (Li et al. 2015a; Zhu et al. 2017). The raw materials are added with water to initiate alcohol fermentation. After alcohol fermentation, vinegar *Pei*, a mixture of Daqu, wheat bran, and rice hull, is added leading to the AAF stage, which is considered the most important step during the Qishan vinegar brewing process. The mixture is then stirred mechanically daily for approximately 19 days. In contrast to the Qishan vinegar brewing process, stirring of Tianjin Duliu Mature Vinegar takes up to 30 days, and only the vinegar *Pei* in the upper half layer is stirred during the AAF process (Nie et al. 2013). On the other hand, Zhenjiang aromatic vinegar uses the day 7 vinegar *Pei* as a starter to initiate the AAF process (Wang et al. 2015).

SSF is driven by a succession of complex microbial communities (Li et al. 2015b). The quality of vinegar products is determined by numerous factors, particularly the microbiota involved in fermentation (Liu et al. 2011). Some studies have analyzed microbial communities only during the Daqu starter fermentation stage or AAF stage (Zheng et al. 2014; Li et al. 2015a, 2016a). Given that the microbial associations between starter and AAF stages are still poorly understood (Nie et al. 2013; Wang et al. 2015), it is necessary to consider them together. Moreover, the dynamics of physiochemical properties during vinegar fermentation eventually affect the quality of the final vinegar product (Kim et al. 2012). Therefore, it is essential to analyze the potential link between Daqu starter and vinegar *Pei*, and to explore the batch-to-batch consistency of the entire traditional SSF process.

Recently, the culture-dependent and PCR-DGGE methods have been used widely to study microbiota ecology (González-Arenzana et al. 2013; Ivone et al. 2013; Li et al. 2016b). However, culture-dependent methods are ineffective (Trcek et al. 2016), and low-abundant organisms could not be detected by PCR-DGGE and other conventional molecular techniques (Zhong et al. 2015; Liang et al. 2016). With the development of molecular technology, the second-generation sequencing technology of high-throughput sequencing has become a useful culture-independent method for the in-depth quantitative analysis of complex microbiota (Trcek et al. 2016). This method has been used to investigate microbial diversity in various fermented food ecosystems, such as Zhenjiang vinegar (Wang et al. 2015), Moutai-flavor Daqu (Wang et al. 2017), Chinese Luzhou-flavor liquor (Sun et al. 2016) and milk (Bokulich et al. 2015).

In this study, Illumina HiSeq sequencing and quantitative polymerase chain reaction (qPCR) analyses were utilized to trace bacterial dynamics and biomass in three batches of matured Daqu starter and following a solid-state AAF process. Physiochemical parameters were also determined during the fermentation of Qishan vinegar. Statistical analysis was conducted to compare the similarities and differences in the bacterial community, and to explore the correlations between physiochemical parameters and bacterial community. This study can provide a better understanding into the structure and dynamic changes of microbial community during the SSF process of Qishan vinegar. The results will also help to understand the potential link between Daqu starter and vinegar *Pei* and to explore the batch-to-batch uniformity of the traditional SSF process.

Materials and methods

Sampling

Three individual batches during the vinegar brewing process were obtained from a vinegar factory in Qishan, Shaanxi Province (N34°26′, E107°36′). Daqu blocks, which comprised approximately 70% barley and 30% wheat with the addition of 36–37% water, were piled layer-by-layer in fermentation rooms. At the end of Daqu fermentation, three matured Daqu starters, named Q3, Q5, and Q10, were selected from different Daqu fermentation rooms 3, 5, and 10, respectively. The starters were correspondingly added to an AAF process in three individual batches (Batch T3, Batch T5, and Batch T10). To trace the solid-state AAF process that was conducted with a 16-m^3 working volume, vinegar *Pei* samples were randomly selected on days 1, 9, and 19 from three individual batches during AAF. In order to obtain sufficient representation of the samples, Daqu blocks were randomly selected in triplicate from the upper, middle, and lower locations, and then ground, mixed, and pooled into sterile plastic bags. Vinegar *Pei* samples were also collected randomly in triplicate at equal depths of the fermentation culture (approximately 15 cm from the upper surface) to avoid edge effects and to collect the most unbiased samples. The samples were then divided into three groups, including T3 (Q3, P3.1, P3.9, and P3.19), T5 (Q5, P5.1, P5.9, and P5.19) and T10 (Q10, P10.1, P10.9, and P10.19), transferred and stored immediately at −20 °C until further analysis.

Physiochemical analysis of Daqu and vinegar *Pei*

The humidity of Daqu and vinegar *Pei* samples was measured with the dry weight measurement method. Total acids were detected using 0.05 mol/L NaOH with a titration endpoint of pH 8.2 (Wu et al. 2012). The pH was detected directly with a pH meter (Sartorious PB-10, Göttingen, Germany). Amino acid nitrogen content was determined by the formaldehyde titration method (Li et al. 2015a). In addition, glucoamylase and amylase activities in Daqu samples were also measured. Glucoamylase activity was determined by the iodometric method, and the colorless end-point titration was identified by 0.05 M sodium thiosulphate. Amylase activity was

measured by general methods (Ministry of Light Industry of China 1993).

Metagenomic DNA extraction and qPCR analysis

Two grams of Daqu and vinegar *Pei* were pretreated for total DNA extraction, the pellets were resuspended and washed twice using phosphate-buffered saline (PBS, pH 7.0), and then centrifuged at 2000 *g* for 5 min (Li et al. 2014). Afterward, DNA extraction was performed using the Soil DNA Kit (Omega Bio-Tek, Norcross, GA) in accordance with the manufacturer's instructions. The qPCR analysis was performed using a commercial kit mixture (SYBR® *Premix Ex Taq*™ II, Takara, Dalian, China). The primer pairs P1/P2, Lac1/Lac2, Ace1/Ace2, and B1/B2 were used to quantify the biomass of total bacteria, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and *Bacillus*, respectively (Xu et al. 2011b). The qPCR reaction mixture consisted of 12.5 μL SYBR Premix Ex Taq (Takara), 0.5 μL forward primer, 0.5 μL reverse primer, 2 μL of 10-fold dilution DNA template and 9.5 μL RNA free water. The amplification program was the same for total bacteria, LAB, AAB and *Bacillus*, and consisted of 1 cycle of 94 °C for 5 s, then 40 cycles of 94 °C for 5 s, 55 °C for 34 s, and 72 °C for 30 s. The fluorescence signal of qPCR was collected during the annealing stage. Melting analysis and agarose gel electrophoresis were conducted at the end of amplification cycle to determine specificity of PCR products. All reactions were performed in quadruplicate in 96-well plates by use of an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The amplification efficiency and correlation (R^2) for the amplification of specific genes of bacteria, LAB, AAB and *Bacillus* were 101.2% and 0.991, 99.7% and 0.993, 99.3% and 0.990, 100.8% and 0.998, respectively.

Illumina HiSeq sequencing of complex bacterial populations

Based on the Illumina HiSeq sequencing platform, DNAs were sequenced by targeting the V4 hypervariable regions of 16S rDNA, which were amplified using the specific primers 515F/806R with Illumina barcodes (Peiffer et al. 2013; Moreau et al. 2014). The 515F/806R primer pair (V4 region) yields more diversity (Peiffer et al. 2013), and has been used to determine bacterial diversity in microbial fuel cells (Gao et al. 2014) and wastewater treatment (Yang et al. 2016). All PCR reactions were performed in triplicate using 30 μL reaction mixtures that contained 15 μL Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England BioLabs, Ipswich, MA), 0.2 μM of each primer, and 10 ng DNA templates. Thermocycling conditions were set as follows: initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 60 s, and final elongation at 72 °C for 5 min. The negative control was treated to confirm contamination. Afterward, the PCR products were mixed in equimolar ratios and mixture PCR product were purified with Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing libraries were constructed using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA) following the manufacturer's recommendations, and index adaptors were added. Library quality was assessed with Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA) and the Agilent Bioanalyzer 2100 system. Finally, the library was sequenced using Illumina HiSeq 2500, and approximately 250 bp paired-end reads were generated.

Data processing and analysis

After sequencing, paired-end reads were assigned to samples based on their unique barcode, and merged using FLASH, which was designed to obtain raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags in accordance with the QIIME (V1.7.0) quality control process (Bokulich et al. 2013). The tags were compared with the reference database (Gold database). Chimera sequences were removed by using the UCHIME algorithm to obtain effective tags (Edgar 2013). Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs), and analysis was performed using Uparse software (Uparse v7.0.1001). Representative sequences were selected for each RDP (Ribosomal Database Project), and multiple sequence alignment was performed using MUSCLE software (Version 3.8.31). The QIIME suite of programs was used to calculate alpha diversity indices, including Shannon, Simpson, Chao1, ACE and Goods coverage as well as beta diversity. In addition, principal components analysis (PCA) of microbial community composition in all samples were conducted in the R Packages, Version 3.3.2 (https://www.r-project.org/), and redundancy analysis (RDA) was conducted using Conoco for Windows version 4.5 software to assess the correlations between physiochemical parameters and bacterial community composition. Finally, analysis of similarities (ANOSIM) and multi-response permutation procedure (MRPP) were further utilized to compare the similarities and differences in microbial community between any two batches during SSF of the vinegar brewing process.

Nucleotide sequence accession numbers

Sequencing results are available through the GenBank sequence read archive (SRA) database under the accession number PRJNA347602.

Table 1 Physicochemical properties in Daqu and vinegar *Pei*

Sample name	Q3	P3.1	P3.9	P3.19	Q5	P5.1	P5.9	P5.19	Q10	P10.1	P10.9	P10.19
pH	6.12	4.61	3.92	3.79	6.06	4.77	3.95	3.90	5.88	4.91	3.96	3.86
Titratable acidity g/100 g	0.83	1.15	4.46	5.75	0.76	1.09	3.77	5.33	0.57	1.04	3.78	5.90
Humidity (%)	9.50	55.24	58.86	58.82	9.27	52.51	59.39	58.39	9.65	52.92	61.21	61.22
Amino acid nitrogen mg/g	3.53	0.99	3.54	4.09	3.46	1.12	2.86	3.66	2.49	1.21	3.01	3.47

Results and discussion

Physiochemical parameters of the three batches

Physiochemical parameters change dynamically with microbial growth during SSF, and contribute to the quality of the final vinegar product (Morales et al. 2001). The key physiochemical properties, including pH, titratable acidity, humidity, and amino acid nitrogen, are summarized in Table 1. The pH values of Daqu Q3, Q5 and Q10 were 6.12, 6.06 and 5.88, respectively. The titratable acidity values were 0.83, 0.76, and 0.57 g/100 g in Daqu Q3, Q5, and Q10, respectively. The humidity of Daqu Q3, Q5 and Q10 were 9.50, 9.27, and 9.65%, respectively, and were similar to that of the final humidity of Fen-Daqu, in which humidity declined constantly during Daqu fermentation (Zheng et al. 2014). The contents of amino acid nitrogen in Daqu Q3, Q5 and Q10 were 3.53 mg/g, 3.46 mg/g, and 2.49 mg/g. As shown in Fig. 1, the activities of glucoamylase and amylase in Daqu were also measured. The glucoamylase activity in Daqu Q3, Q5 and Q10 were 701.70, 485.18, and 788.66 mg glucose g^{-1} h^{-1}, respectively. The Daqu samples had closely similar amylase activities of 1.33, 1.32 and 1.32 g liquefied starch g^{-1} h^{-1}. Overall results indicated that there were no obvious differences between the three Daqu samples.

As shown in Table 1, the pH values of vinegar *Pei* samples were lower than those of Daqu samples, and decreased successively during the AAF process. The pH variations during the AAF process were different from those of Tianjin Duliu mature vinegar, for which the changes in pH were divided into two phases, before and after 15 days, whereby, in both phases, the change in pH tendency was similar (Nie et al. 2013). The results indicated that different vinegar brewing techniques could cause different trends in physiochemical parameters, thus leading to the differences in final vinegar products. At the end of the AAF process, the pH of vinegar *Pei* P3.19, P5.19, and P10.19 decreased to 3.79, 3.90 and 3.86, respectively. As shown in Table 1, the titratable acidity of vinegar *Pei* increased during AAF process. At the end of AAF, vinegar *Pei* P3.19, P5.19, and P10.19 had titratable acidity values of 5.57, 5.33, and 5.90 g/100 g, respectively. During this process, the constant decrease in pH values and subsequent increase in the titratable acidity of vinegar *Pei* were due mainly to the accumulation of organic acids, including acetic acid and lactic acid—the dominant flavor components in vinegar (Liu et al. 2011). Humidity values of all vinegar *Pei* samples ranged from 52% to 62%, which favored the growth and metabolism of the dominant bacteria, including LAB and AAB, which require relatively higher moisture in order to maintain viability and to initiate the AAF process (Kittelmann et al. 1989). The amino acid nitrogen content of vinegar *Pei*, however, increased continuously during AAF. The amino acid nitrogen content on the last day of vinegar *Pei*, P3.19, P5.19, and P10.19 reached 4.09 mg/g, 3.66 mg/g, and 3.47 mg/g, respectively. The amino acid nitrogen contents of mature Daqu samples on the other hand, were higher than those of day 1 vinegar *Pei* but lower than those of day 19 vinegar *Pei*. This result suggested that microbes could metabolize and accumulate more amino acids in vinegar *Pei* during AAF than in mature Daqu.

In all, there were no significant differences ($P > 0.05$) in pH, titratable acidity, humidity, and amino acid nitrogen content between any two batches of Daqu and vinegar *Pei*. This is depicted in box-and-whisker plots in Fig. S1, as well as in the one-way analysis of variance among different batches (Fig. S1). The different batches could therefore be said to have highly similar physiochemical parameters.

Biomass dynamics by qPCR

Gene copies representing total bacteria, LAB, AAB, and *Bacillus* in Daqu and vinegar *Pei* samples were quantified by qPCR. The results are shown in Fig. 2. The total bacteria count in Daqu Q3, Q5 and Q10 were 9.89, 9.78, and 9.95 Log copies/g, respectively, in which LAB accounted for 8.75, 8.75 and 8.83 Log copies/g, respectively, i.e., higher than that of

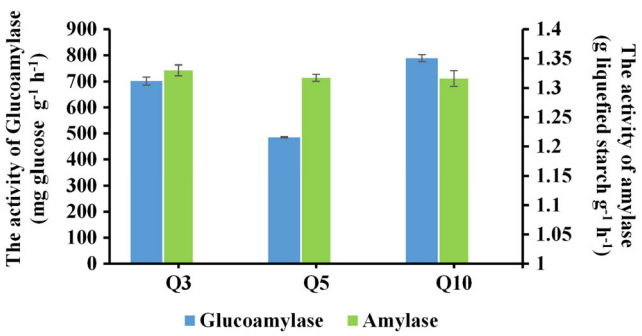

Fig. 1 Glucoamylase and amylase activities in Daqu samples

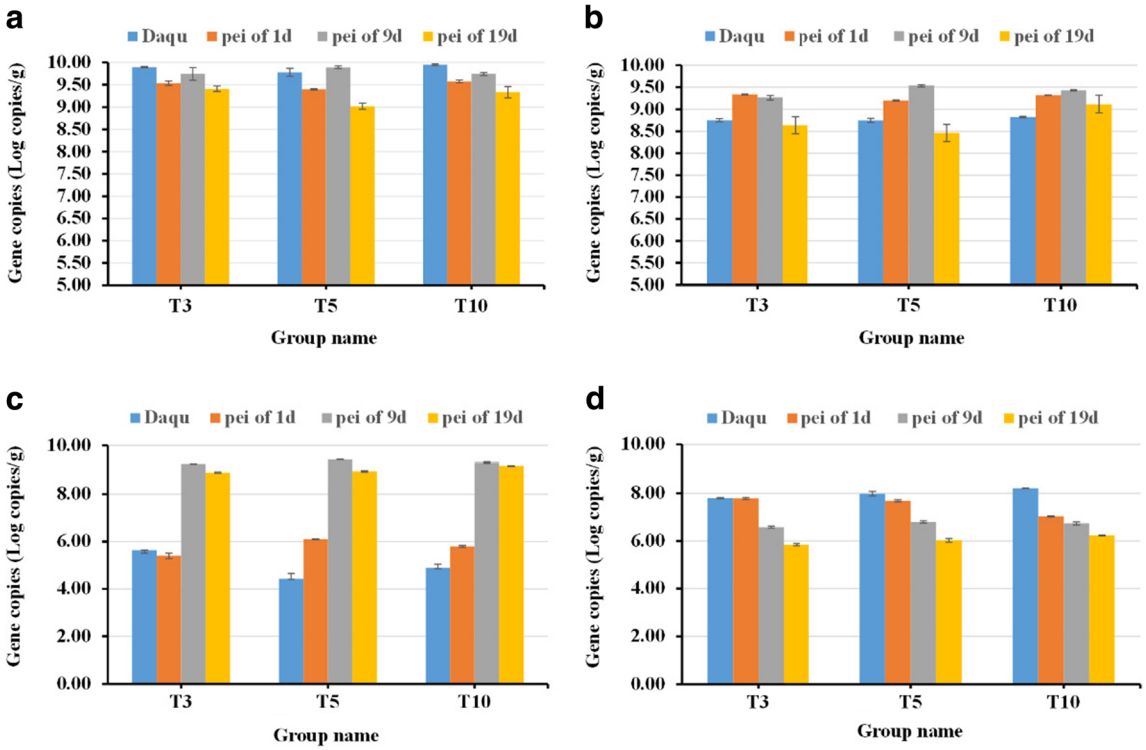

Fig. 2 Dynamics of quantification of **a** total bacteria, **b** lactic acid bacteria (LAB), **c** acetic acid bacteria (AAB), and **d** *Bacillus* abundance during fermentation process in three batches

Bacillus (Fig. 2), while the counts for AAB were characterized by only 5.63, 4.43 and 4.90 Log copies/g in Q3, Q5 and Q10, respectively. This indicated that high temperatures during Daqu fermentation could not favor AAB growth (Li et al. 2014). Overall, the gene copies of total bacteria, LAB, AAB, and *Bacillus* were not distinctly different among the three Daqu samples.

During the AAF process, the dynamics of total bacterial biomass in vinegar *Pei* were similar. The total bacterial biomass increased slightly from day 1 vinegar *Pei* to day 9 vinegar *Pei* and slightly decreased in day 19 vinegar *Pei* reaching 9.41, 9.02 and 9.33 Log copies/g in P3.19, P5.19 and P10.19, respectively. The change in LAB biomass was similar to that in total bacteria, and its biomass peaked at 9.27, 9.54 and 9.43 Log copies/g in P3.9, P5.9, and P10.9, respectively. This showed that LAB occupied a higher percentage of total bacterial species and played a vital role in AAF. LAB are a group of microorganisms that play significant roles in several fermented ecosystems, and can also be found in, for example, liquor, cocoa bean, sausages, and pig manure biodegradation fermentation systems (Fraqueza 2015; Sun et al. 2016; Visintin et al. 2016; Chen et al. 2017). Biomass of AAB increased dramatically during AAF, peaked in day 9 vinegar *Pei*, and finally decreased slightly in day 19 vinegar *Pei*. This confirmed that AAB, which dominated at the later stages of AAF, also plays an important role in vinegar fermentation. LAB and AAB are the two dominant bacterial groups involved in flavor production during the AAF process, and have a significant influences on the taste of vinegar (Wu et al. 2016). The biomass of *Bacillus* species, however, showed a completely different trend compared to that of total bacteria, AAB, and LAB. Biomass of *Bacillus* species decreased gradually during AAF, reaching 5.84, 6.03, and 6.23 Log copies/g in P3.19, P5.19, and P10.19, respectively. Similar trends in *Bacillus* biomass in the three batches indicated that *Bacillus* could not thrive in acidic environments. Conversely, AAB and LAB dominated during AAF fermentation and could survive in high-acidity and low-pH conditions. The biomass dynamics of LAB, AAB, and *Bacillus* in the three batches, however, showed similar trends during the vinegar fermentation process.

Bacterial community composition and dynamics by Illumina HiSeq sequencing

Microbial diversity and dynamics during the fermentation process were analyzed by Illumina HiSeq sequencing. A total number of 699,204 raw tags was obtained from 12 tagged Daqu and vinegar *Pei*. After filtering the low-quality reads and detecting chimera, a total number of 676,786 effective tags was acquired. Each sample ranged from 49,584 to 65,876 effective tags with different phylogenetic OTUs, which ranged from 89 to 650 via 97% sequence identity cut-off. The alpha diversity indices (Table 2), including number of

Table 2 Observed 16S rDNA Illumina Hiseq sequencing results and alpha diversity indices in samples

Sample	No. of							Alpha diversity					
	Raw tags	Clean tags	Effective tags	Base	Q20 (100%)[a]	Q30 (100%)[b]	GC (100%)[c]	Effective (100%)	OTU (97%)	Shannon (97%)	Simpson (97%)	Chao (97%)	ACE (97%)	Goods coverage
Q3	52,298	51,466	51,078	12,912,014	98.86	97.68	56.48	96.70	92	1.825	0.613	83.000	86.925	0.999
P3.1	57,832	56,833	55,436	13,969,988	98.71	97.42	52.79	94.50	163	4.231	0.916	147.882	155.247	0.999
P3.9	57,257	56,501	55,696	14,081,574	98.98	97.93	51.29	96.43	123	2.837	0.765	109.091	115.195	0.999
P3.19	59,029	58,128	57,504	14,538,507	98.82	97.63	50.82	96.46	119	2.359	0.670	97.200	105.752	0.999
Q5	50,744	49,966	49,584	12,535,599	98.89	97.72	56.31	96.76	89	1.849	0.612	84.600	85.594	0.999
P5.1	66,652	65,372	64,136	16,140,192	98.64	97.26	52.76	94.64	158	3.983	0.888	162.545	153.520	0.999
P5.9	58,350	57,410	55,854	14,120,356	98.81	97.60	52.63	94.54	650	4.277	0.841	640.632	659.751	0.994
P5.19	51,988	51,304	50,622	12,798,721	99.00	97.97	52.11	96.45	96	2.306	0.707	84.750	86.156	1.000
Q10	67,455	66,385	65,876	16,651,036	98.76	97.45	55.65	96.71	299	1.713	0.487	227.722	254.241	0.998
P10.1	62,675	61,530	59,758	15,066,458	98.68	97.32	51.63	93.97	423	4.123	0.880	396.312	429.888	0.996
P10.9	56,408	55,613	54,394	13,749,095	98.80	97.55	52.71	95.51	375	2.929	0.762	456.800	489.181	0.995
P10.19	58,516	57,677	56,848	14,371,036	98.82	97.62	52.16	96.20	329	2.739	0.764	286.961	317.021	0.997

[a] 99% accuracy of effective tags

[b] 99.9% accuracy of effective tags

[c] GC content of effective tag

OTUs, Shannon, Simpson, Chao, ACE, and Goods coverage, suggested that the diversity and richness of bacterial communities varied during the fermentation. The rarefaction curves analysis showed a clear-cut asymptotic plateau, which indicated adequate sampling and sequencing efforts (Fig. S2).

Similar and successional dynamics of bacterial communities at the order level were exhibited during the batch-to-batch fermentation process (Fig. 3a). The ten most dominant orders constituted 85.2–99.9% of all sequences. *Enterobacteriales* were the most predominant bacteria order and constituted 94.7, 94.6, and 92.2% of all sequences in Daqu Q3, Q5, and Q10, respectively, which was in line with RDA analysis (Fig. 5). Members of *Enterobacteriales* were also discovered in other Daqu samples; these bacteria can adapt to a wide range of temperature and moisture conditions (Carranza et al. 2010; Gou et al. 2015; Li et al. 2015b). It is therefore likely that *Enterobacteriales* members play a crucial role in Daqu formation, although their specific functions remain unclear at present, and requires further studies. The phylotypes of *Lactobacillales* (<4.4% of total Daqu sequences), *Rhodospirillales* (<0.2% of total Daqu sequences), *Bacillales* (<2.1% of total Daqu sequences), *Pseudomonadales* (<0.7 of total Daqu sequences), *Rhizobiales* (<0.1% of total Daqu sequences) *Clostridiales* (<0.3% of total sequences), *Xanthomonadales* (<0.2% of total Daqu sequences) were also found in Daqu samples. *Lactobacillales* members were the main contributors of lactic, which is known to improve the soft taste of vinegar (Unden and Zaunmüller 2009). *Rhodospirillales* and *Bacillales* correlated highly with the formation of acetate esters (Li et al. 2016a). Bacterial communities at genus level in Daqu Q3, Q5 and Q10 were also observed (Fig 3b). *Bacillus*, *Escherichia-Shigella*, *Lactobacillus*, *Pseudomona, Weissella, Acinetobacter,* and *Acetobacter* genera were detected with rare distribution in all Daqu samples. Unlike in the Daqu samples, in Duliu vinegar, *Streptomyces* were highly abundant in Duliu-Daqu; members of this genus can be used for isolating enzymes (Nie et al. 2013). This suggests that differences in the microbial communities could contribute to the differences in Daqu. It has been observed that multispecies participate in Daqu fermentation, and each individual species might have specific functions in Daqu fermentation, with their synergistic effect contributing to the final quality of the Daqu. Largely, the three Daqu samples had highly similar bacterial communities. The quality and uniformity of Daqu greatly affects the quality and stability of the later stages of AAF, and eventually influences the quality and uniformity of the final vinegar products (Li et al. 2014). Therefore, the uniformity of bacteria in different Daqu batches ensures the consistency of AAF and the quality of the vinegar products.

For bacterial communities at order level in vinegar *Pei*, *Enterobacteriales* groups were retrieved at a low frequency (<3.2% of total vinegar *Pei* sequences) during AAF (Fig. 3a).

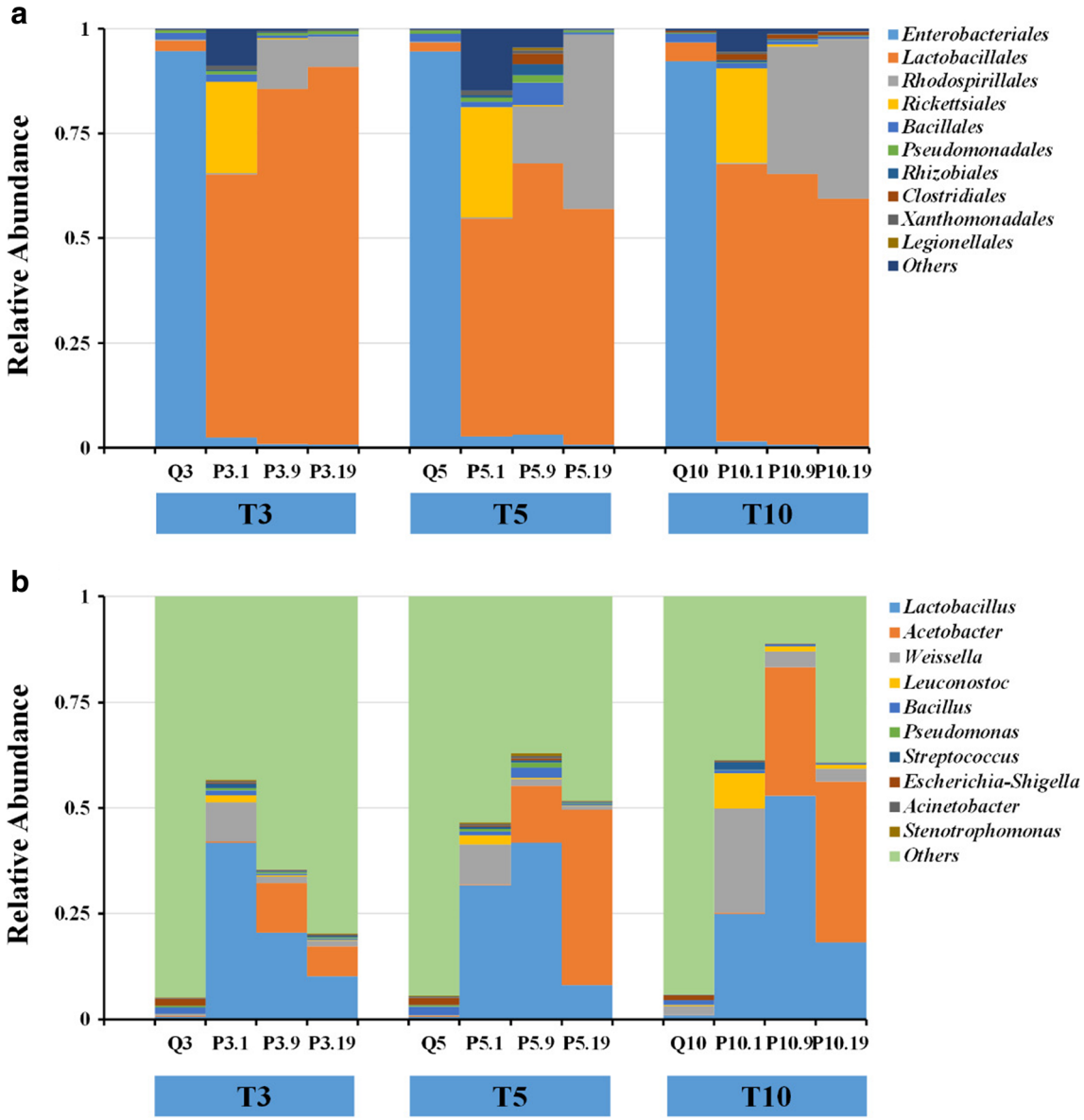

Fig. 3 Dynamics of relative abundance of **a** major bacteria orders and **b** genera in samples obtained by Illumina HiSeq Sequencing, respectively

Stressful conditions, such as acidic stress, could negatively affect *Enterobacteriales*; similar results have been reported (Li et al. 2016a). In addition, members of *Enterobacteriales*, *Enterobacter*, *Escherichia* and *Pantoea*, could also be found in Tianjin Duliu aged vinegar and Zhenjiang aromatic vinegar, which are the typical cereal vinegar in China (Nie et al. 2013; Peng et al. 2015; Wang et al. 2016). It is therefore obvious that *Enterobacteriales* is commonly present during AAF in cereal vinegar but might play a comparatively lesser role in the final product. Notably, the relative abundance of *Lactobacillales* in vinegar *Pei* P3.1, P5.1, and P10.1 was 62.7, 52.0, and 66.1%, respectively, and dominated during the whole AAF process. The relative abundance of *Rhodospirillales* in three batches was lower in the day 1 vinegar *Pei* at the beginning of AAF fermentation (<3% of total day 1 vinegar *Pei* sequences), but increased and prevailed during the later stages of AAF. *Lactobacillales* and *Rhodospirillales* were the most prevalent bacterial communities during the AAF process. *Lactobacillales* members were characterized by an increased tolerance to acidity (low pH range), and were the most abundant bacteria during AAF. Members of this group are anaerobes, and are major contributors of lactic acid, which promote a soft taste by moderating the irritating sour smell (Wu et al. 2012). In addition, *Rhodospirillales*, especially some *Acetobacter* genus, not only produce acetic acid but also act as acetoin biosynthetic bacteria in various fermentation processes (Chen et al. 2010). Thus, *Lactobacillales* and *Rhodospirillales* greatly influence the flavor and taste of vinegar during AAF. The relative abundance of *Rickettsiales* was higher in the initial fermentation process and accounted for

21.9, 26.4, and 22.6% in P3.1, P5.1, and P10.1, respectively; however, it decreased rapidly (<0.5% of total vinegar *Pei* sequences) by the end of AAF. It could be that raw materials initially had a relatively high amount of *Rickettsiales*, but could not accommodate the increasing acidity and decreasing pH of the AAF environment. The special fermentation technique of brewing Qishan vinegar ensures survival of local multispecies and prevents vinegar from contamination. Smaller groups including *Bacillales* (<5.4% of total vinegar *Pei* sequences), *Pseudomonadales* (<1.7% of total vinegar *Pei* sequences), *Rhizobiales* (<2.6% of total vinegar *Pei* sequences) and *Xantchomonadales* (<1.1% of total vinegar *Pei* sequences) varied in different batches during AAF. *Clostridiales* also showed relatively low abundance in all samples (<2.5% of total vinegar *Pei* sequences) expect for P.5.19, where none was detected.

Bacterial communities at genus level in vinegar *Pei* were also determined (Fig. 3b). Generally, the prevailing bacteria during AAF were *Lactobacillus*, *Acetobacter*, *Weissella*, *Leuconostoc* and *Bacillus*. For Tianjin Duliu aged vinegar, only the upper half layer vinegar *Pei* is stirred during AAF, and the dominant bacteria detected during this process were *Lactobacillus*, *Nostoc*, *Acetobacter*, and *Gluconacetobacter* (Nie et al. 2013). Meanwhile, *Nostoc* and *Gluconacetobacter* were absent in Qishan vinegar. As for Zhenjiang aromatic vinegar, using the day 7 vinegar *Pei* as a starter to initiate the AAF process (Wang et al. 2015), *Lactobacillus* was observed to be the predominant genus during the early stages of AAF while *Acetobacter*, *Lactococcus, Gluconacetobacter, Enterococcus* and *Bacillus* dominated at later stages (Wang et al. 2016). This was not the case in Qishan vinegar. Therefore, *Lactobacillus* and *Acetobacter* are the common dominant bacteria genera in the AAF of Chinese cereal vinegars. The other dominant microbial communities likely contribute to the main differences among these vinegars. Unlike in submerged vinegar production, *Gluconacetobacter* was the predominant species in wine vinegar production (Trcek et al. 2016). Compared to Qishan vinegar fermentation, wine vinegar is dominated by relatively homogeneous bacterial community (Trcek et al. 2016). The individual vinegar brewing techniques therefore have a great effect on the bacterial composition, which, in turn, reflects the

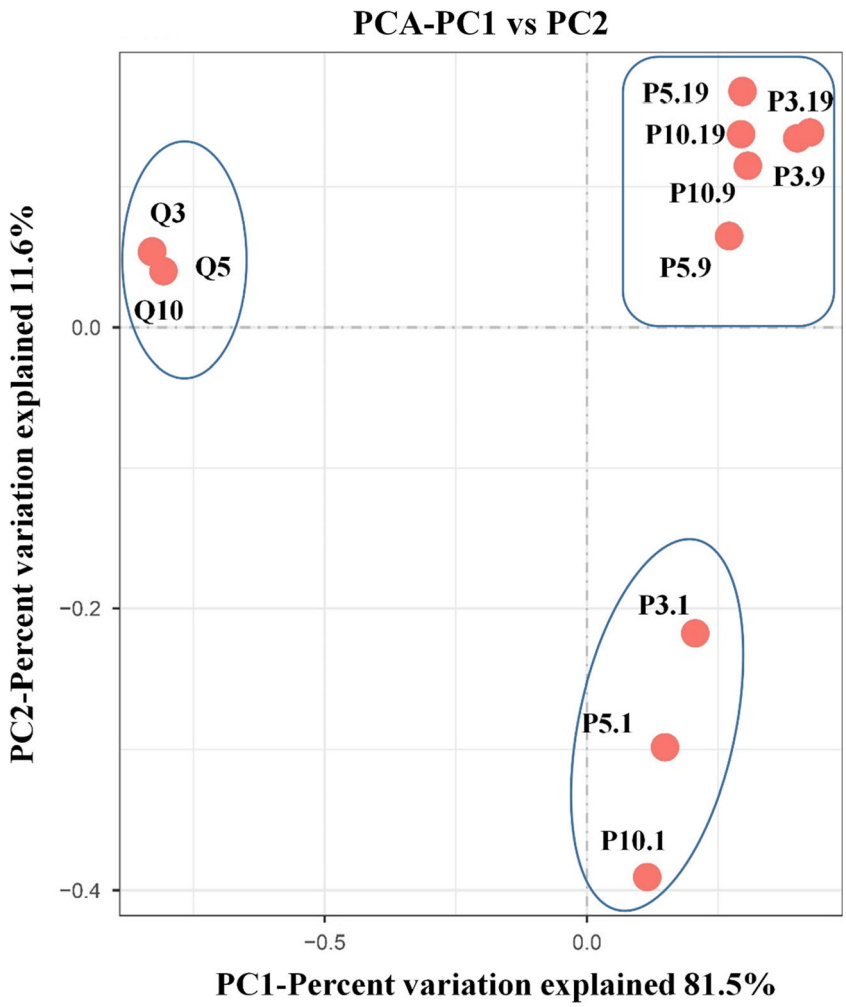

Fig. 4 Principal component analysis (PCA) analysis of Daqu and vinegar *Pei* samples based on Illumina HiSeq Sequencing

difference in the final vinegar products. In summary, the three batches of samples had largely similar dominant bacteria including *Lactobacillus*, *Acetobacter*, *Weissella*, *Leuconostoc* and *Bacillus* during AAF of Qishan vinegar.

Statistical analysis of bacterial diversity and its relationships with physiochemical factors

Principal component analysis (PCA) was applied to compare the differences and similarities in bacterial communities at different fermentation stages. The first principal component accounted for 81.5% of total variability, and the second principal component accounted for 11.6% of total variability. The cumulative score therefore, of the first two principal components was 93.1%, which showed a good representation of the results (Fig. 4). The samples collected from Q3, Q5, and Q10 gathered into one group, the samples of day 1 vinegar *Pei* (initial AAF process) (P3.1, P5.1 and P10.1) tended to form a cluster while the vinegar *Pei* samples of day 9 (mid AAF process) (P3.9, P5.9, and P10.9) and day 19 (later AAF process) (P3.19, P5.19 and P10.19) were clustered together (Fig. 4). This depicted the obvious difference between Daqu and vinegar *Pei*. It also portrayed the differences between day 1 vinegar *Pei* and mid- and later stages vinegar *Pei* during AAF. Furthermore, the clustering of day 9 and day 19 vinegar *Pei* samples showed the close similarity of the bacterial community during the mid- and later stages of AAF. This results can be interpreted that the acid environment might have influenced the microbial ecology in the mid- and later stages of AAF by favoring an acidophilic bacterial community.

Physiochemical factors generally have significant effects on microbial communities (Williams and Crawford 1983). In this study, RDA was applied to examine the correlation between physiochemical parameters and bacterial composition. As shown in Fig. 5, the first canonical axes accounted for 85.7% of the variance, and the second canonical axes accounted for 6.6% of the variance, resulting in the cumulative score of 92.3%, suggesting a notable correlation between bacterial structure and physiochemical parameters. The RDA result showed the high and positive association between *Enterbacteriales* and Daqu samples. Furthermore, *Richkettsiales* and *Xanthomonadales* were positively correlated with P3.1, P5.1 and P10.1. *Lactobacillales* on the other hand, correlated positively with P3.9, P5.9 and P10.9. *Rhodospirillales* was highly and positively correlated with mid- and later stages of vinegar *Pei*. Meanwhile, *Rhodospirillales* and *Lactobacillales* were more associated with acidity and humidity, respectively, which indicated that acidity and humidity significantly influence the phylotypes of *Rhodospirillales* and *Lactobacillales*, respectively. The RDA result also indicated that *Rhodospirillales* could adapt to the stressful conditions during the later stages of AAF fermentation; this results corresponds with those of previous reports (Li et al. 2016a). Moreover, as the results indicated, the acidic environment allowed the acidophilic bacterial community to thrive and restricted undesired microbes.

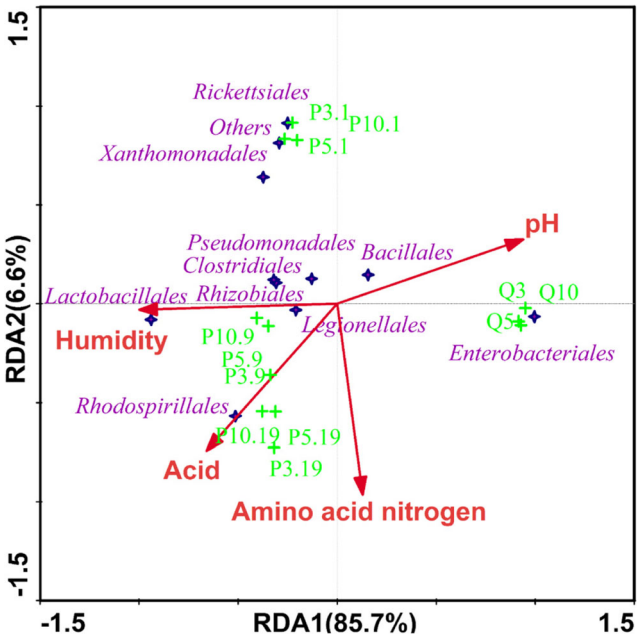

Fig. 5 Redundancy analysis (RDA) correlation triplet depicting the abundance of bacterial community obtained by Illumina HiSeq sequencing in relation to physiochemical characteristics of Daqu and vinegar *Pei* samples

In short, physiochemical parameters influence different bacterial communities at different stages of the fermentation process. More so, the different fermentation stages, e.g., Daqu fermentation and AAF, form clusters, respectively, showed the uniformity and stability of the Qishan vinegar brewing process.

Group analysis of bacteria in three batches of entire SSF

All samples were divided into three batches based on fermentation stage: T3 (Q3, P3.1, P3.9, and P3.19), T5 (Q5, P5.1, P5.9, and cP5.19), and T10 (Q10, P10.1, P10.9 and P10.19).

Table 3 Analysis of similarities (ANOSIM) and multi-response permutation procedures (MRPP) analysis to test for differences in bacterial communities among different batches[a]

Group	ANOSIM		MRPP	
	R-value	*P*-value	*R*-value	P-value
T3-T5	−0.198	0.728	−0.089	0.691
T3-T10	−0.031	0.523	−0.034	0.705
T5-T10	−0.188	0.755	−0.094	0.790

[a] R < 0 means non-significant differences between the two batches, P > 0.05 means statistically non-significant

Analysis of similarities (ANOSIM) and multi-response permutation procedures (MRPP) analysis were conducted to compare the similarities and differences between any two of these batches. Both ANOSIM and MRPP tests demonstrated that any two batches had similar bacterial communities during vinegar fermentation ($R < 0$, $P > 0.05$) (Table 3). These results provided further evidence for the constancy of bacterial dynamics during the brewing process of Qishan vinegar. The consistency of the vinegar brewing process was due to the fact that there were no obvious differences in enzyme activity, physiochemical parameters, and bacterial composition of the Daqu samples. The successive bacterial community dynamics of vinegar *Pei* during AAF contributed to the stable quality of the final vinegar product. In addition, natural selection of the desired bacterial community under prolonged environmental pressure contributed to the stability of different batches during the entire SSF.

Conclusion

In summary, Illumina HiSeq sequencing revealed variations in bacterial communities at different fermentation stages of Qishan vinegar. The results demonstrated that *Enterobacteriales* members were the most dominant order in Daqu, whereas *Lactobacillales* and *Rhodospirillales* phylotypes prevailed in vinegar *Pei* during the AAF stage. The dominant and characteristic bacteria genera of Qishan vinegar during the AAF process were *Lactobacillus*, *Acetobacter*, *Weissella*, *Leuconostoc* and *Bacillus*. Quantitative PCR also revealed that the abundance of the 16S rRNA gene in AAF process corresponded mainly to LAB and AAB. Combined with PCA and RDA analysis, our results confirmed that bacterial community structure could be affected by physiochemical factors, which, in turn, influences the unique bacterial composition at different fermentation stages. ANOSIM and MRPP analyses results provided further evidence for the stability of bacterial dynamics during the brewing process of traditional Chinese Qishan vinegar. The final quality of the Qishan vinegar is determined by the formation of flavor compounds, including organic acids, amino acids and volatile compounds. Microbes are the key factor to flavor formation, and contribute the main metabolites in vinegar. This knowledge about the microbial composition could provide basic information to improve the composition of starter cultures. In addition, the relationship between flavor formation and the microbial community at different stages of the Qishan vinegar brewing process could be investigated in subsequent studies to further improve the quality and taste of the final vinegar product.

Acknowledgments This project was supported by the National Natural Foundation of China (No. 31271924).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Bokulich NA, Subramanian S, Faith JJ et al (2013) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods 10:57–59

Bokulich NA, Amiranashvili L, Chitchyan K et al (2015) Microbial biogeography of the transnational fermented milk matsoni. Food Microbiol 50:12–19

Carranza P, Grunau A, Schneider T et al (2010) A gel-free quantitative proteomics approach to investigate temperature adaptation of the food-borne pathogen Cronobacter turicensis 3032. Proteomics 10: 3248–3261

Chen JC, Chen QH, Qin G, Sue R, Hui R, He GQ, Gu Q (2010) Simultaneous determination of acetoin and tetramethylpyrazine in traditional vinegars by HPLC method. Food Chem 122:1247–1252

Chen Q, Liu B, Wang J, Che J, Liu G, Guan X (2017) Diversity and dynamics of the bacterial community involved in pig manure biodegradation in a microbial fermentation bed system. Ann Microbiol 67:1–10

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10:996–998

Fraqueza MJ (2015) Antibiotic resistance of lactic acid bacteria isolated from dry-fermented sausages. Int J Food Microbiol 212:76–88

Gao C, Wang A, Wu W, Yin Y, Zhao Y (2014) Enrichment of anodic biofilm inoculated with anaerobic or aerobic sludge in single chambered air-cathode microbial fuel cells. Bioresour Technol 167:124–132

González-Arenzana L, López R, Santamaría P, López-Alfaro I (2013) Dynamics of lactic acid bacteria populations in Rioja wines by PCR-DGGE, comparison with culture-dependent methods. Appl Microbiol Biotechnol 97:6931–6941

Gou M, Wang H, Yuan H, Zhang W, Tang Y, Kida K (2015) Characterization of the microbial community in three types of fermentation starters used for Chinese liquor production. J Inst Brew 121:620–627

Ivone VM, Conceição E, Nunes OC, Manaia CM (2013) Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods. FEMS Microbiol Ecol 83:361–374

Kim SH, Cho HK, Shin HS (2012) Physicochemical properties and antioxidant activities of commercial vinegar drinks in Korea. Food Sci Biotechnol 21:1729–1734

Kittelmann M, Stamm WW, Follmann H, Trüper HG (1989) Isolation and classification of acetic acid bacteria from high percentage vinegar fermentation. Appl Microbiol Biotechnol 30:47–52

Li P, Li S, Cheng L, Luo L (2014) Analyzing the relation between the microbial diversity of DaQu and the turbidity spoilage of traditional Chinese vinegar. Appl Microbiol Biotechnol 98:6073–6084

Li P, Aflakpui FWK, Yu H, Luo L, Lin W (2015a) Characterization of activity and microbial diversity of typical types of Daqu for traditional Chinese vinegar. Ann Microbiol 65:2019–2027

Li P, Liang H, Lin W, Feng F, Luo L (2015b) 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

Li S, Li P, Liu X, Luo L, Lin W (2016a) Bacterial dynamics and metabolite changes in solid-state acetic acid fermentation of Shanxi aged vinegar. Appl Microbiol Biotechnol 100:1–17

Li Z, Li H, Bian K (2016b) Microbiological characterization of traditional dough fermentation starter (Jiaozi) for steamed bread making by culture-dependent and culture-independent methods. Int J Food Microbiol 234:9–14

Liang H, Ye D, Li P, Su T, Wu J, Luo L (2016) Evolution of bacterial consortia in an integrated tannery wastewater treatment process. RSC Adv 6:87380–87388

Liljeberg H, Björck I (1998) Delayed gastric emptying rate may explain improved glycaemia in healthy subjects to a starchy meal with added vinegar. Eur J Clin Nutr 52:368–371

Liu D, Zhang R, Wu H et al (2011) Changes in biochemical and microbiological parameters during the period of rapid composting of dairy manure with rice chaff. Bioresour Technol 102:9040–9049

Ministry of Light Industry of China (1993) General methods of determination for industrial enzymes (QB/T 1803-1993). China Light Industry Press, Beijing

Morales ML, Tesfaye W, Garciaparrilla MC, Casas JA, Troncoso AM (2001) Sherry wine vinegar: physicochemical changes during the acetification process. J Sci Food Agric 81:611–619

Moreau MM, Eades SC, Reinemeyer CR, Fugaro MN, Onishi JC (2014) Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. Vet Microbiol 168:436–441

Nie Z, Zheng Y, Wang M, Han Y, Wang Y, Luo J, Niu D (2013) Exploring microbial succession and diversity during solid-state fermentation of Tianjin duliu mature vinegar. Bioresour Technol 148:325–333

Ooijkaas L (2004) Chinese vinegar and its solid-state fermentation process. Food Rev Int 20:407–424

Peiffer JA, Spor A, Koren O et al (2013) Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc Natl Acad Sci USA 110:6548–6553

Peng Q, Yang Y, Guo Y, Han Y (2015) Analysis of bacterial diversity during acetic acid fermentation of Tianjin Duliu aged vinegar by 454 pyrosequencing. Curr Microbiol 71:195–203

Sun W, Xiao H, Peng Q, Zhang Q, Li X, Han Y (2016) Analysis of bacterial diversity of Chinese Luzhou-flavor liquor brewed in different seasons by Illumina Miseq sequencing. Ann Microbiol 66:1293–1301

Tesfaye W, Morales ML, García-Parrilla MC, Troncoso AM (2002) Wine vinegar: technology, authenticity and quality evaluation. Trends Food Sci Technol 13:12–21

Thomas L, Larroche C, Pandey A (2013) Current developments in solid-state fermentation. Biochem Eng J 81:146–161

Trcek J, Mahnic A, Rupnik M (2016) Diversity of the microbiota involved in wine and organic apple cider submerged vinegar production as revealed by DHPLC analysis and next-generation sequencing. Int J Food Microbiol 223:57–62

Unden G, Zaunmüller T (2009) Metabolism of sugars and organic acids by lactic acid bacteria from wine and must. In: König H, Unden G, Fröhlich J (eds)Biology of microorganisms on grapes, in must and in wine. Springer, Berlin, pp 135–147

Visintin S, Alessandria V, Valente A, Dolci P, Cocolin L (2016) Molecular identification and physiological characterization of yeasts, lactic acid bacteria and acetic acid bacteria isolated from heap and box cocoa bean fermentations in West Africa. Int J Food Microbiol 216:69–78

Wang Z, Lu Z, Yu Y, Li G, Shi J, Xu Z (2015) Batch-to-batch uniformity of bacterial community succession and flavor formation in the fermentation of Zhenjiang aromatic vinegar. Food Microbiol 50:64–69

Wang Z, Lu Z, Shi J, Xu Z (2016) Exploring flavour-producing core microbiota in multispecies solid-state fermentation of traditional Chinese vinegar. Sci Rep 6:26818

Wang X, Ban S, Hu B, Qiu S, Zhou H (2017) Bacterial diversity of Moutai-flavour Daqu based on high-throughput sequencing method. J Inst Brew 123:138–143

Williams RT, Crawford RL (1983) Effects of various physiochemical factors on microbial activity in pea. Can J Microbiol 29:1430–1437

Wu J, Ma Y, Zhang F, Chen F (2012) 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

Wu L, Lu Z, Zhang X, Wang Z, Yu Y, Shi J, Xu Z (2016) Metagenomics reveals flavour metabolic network of cereal vinegar microbiota. Food Microbiol 62:23–31

Xu W, Xu Q, Chen J, Lu Z (2011a) Ligustrazine formation in Zhenjiang aromatic vinegar: changes during fermentation and storing process. J Sci Food Agric 91:1612–1617

Xu W, Huang Z, Zhang X et al (2011b) Monitoring the microbial community during solid-state acetic acid fermentation of Zhenjiang aromatic vinegar. Food Microbiol 28:1175–1181

Yang K, Yue Q, Kong J, Zhao P, Gao Y, Fu K, Gao B (2016) Microbial diversity in combined UAF-UBAF system with novel sludge and coal cinder ceramic fillers for tetracycline wastewater treatment. Chem Eng J 285:319–330

Zheng X, Yan Z, Nout MJR et al (2014) 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

Zhong F, Wu J, Dai Y, Yang L, Zhang Z, Cheng S, Zhang Q (2015) Bacterial community analysis by PCR-DGGE and 454-pyrosequencing of horizontal subsurface flow constructed wetlands with front aeration. Appl Microbiol Biotechnol 99:1499–1512

Zhu W, Nie Y, Xu Y (2017) The incidence and distribution of ochratoxin a in Daqu, a Chinese traditional fermentation starter. Food Control 78:222–229