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ORIGINAL ARTICLE

Monitoring the microbial community succession and diversity of Liangzhou fumigated vinegar during solid-state fermentation with next-generation sequencing

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Abstract

This study reveals the microbial community succession and diversity during the whole solid-fermentation processes of naturally fermented Liangzhou fumigated vinegar (LZFV). Dynamics and diversity of microbial community succession in "Daqu" starter and other fermentation stages (starch saccharification, alcoholic fermentation, and acetic acid fermentation) were monitored using a metagenomic approach involving high-throughput sequencing. Meanwhile, dynamic changes of characteristic flavor compounds of vinegar were determined by gas chromatograph (GC) analysis. The result showed that the microbiota composition exhibited rich diversity. Twenty-five bacterial and 18 fungal genera were found in the whole fermentation process where *Lactobacillus*, *Acetobacter*, *Aspergillus*, *Saccharomyces*, and *Alternaria* were the predominant microorganisms. Alpha diversity metrics showed that bacterial diversity in Daqu was greater than that in AF and AAF. By contrast, fungal diversity increased from Daqu to AF and decreased in the initial stage (5–8 days) of AAF then remained relatively steady. Hence, these results could help understand dynamics of microbial community succession in continuous fermentation of traditional Chinese vinegars. The LZFV fermentation is a continuous process with spontaneous growth that affects the dynamics of microbial communities. Continuous changes of micro-environment conditions in substrate affect the diversity and structure of microbiota. Microbial growth and metabolism were closely related to the changes in the physicochemical characteristics of the cultures. The microbial flora composition showed rich diversity, and with the increase in brewing time and the change in micro-ecological environmental conditions; the microbial community showed a complex dynamic changes.

Keywords Microbial community succession · Diversity · Solid-state fermentation · Liangzhou fumigated vinegar · High-throughput sequencing

Introduction

As a flavoring agent and an important feedstock, vinegar has a long history of over 3000 years in China (Xu et al. 2011). Liangzhou fumigated vinegar (LZFV) is one of Chinese traditional vinegar with local characteristic, which is naturally fermented by a mixture of various microorganisms (i.e., yeasts, molds, and bacteria) in the solid state (Fig. 1). This process differs from similar products in Western countries that use only one or a few microorganisms to ferment (Zhao and Li 2005). It generally involves preparation of the starter "Daqu" (made from barley and pea by spontaneous growth of the microorganisms on/in it), starch saccharification (SS), alcoholic fermentation (AF), and acetic acid fermentation (AAF) (Chen et al. 2009; Wu et al. 2013). The special fermentation technology is also reflected in simultaneous SS and AF after inoculating Daqu starter and yeast—in general, this process lasts 3 days in a closed tank at room temperature—the fermented product is called "Jiupei." This is followed by an open-style process of AAF after Jiupei is mixed with some aged vinegar mother "Cupei"—the acetic acid fermentation product) in a fermentation vat, and this continues for about 10 days (Zhao and Yun 2016). This fermentation, with its coexistence of various microorganisms, provides numerous enzymes for synthesis of flavor

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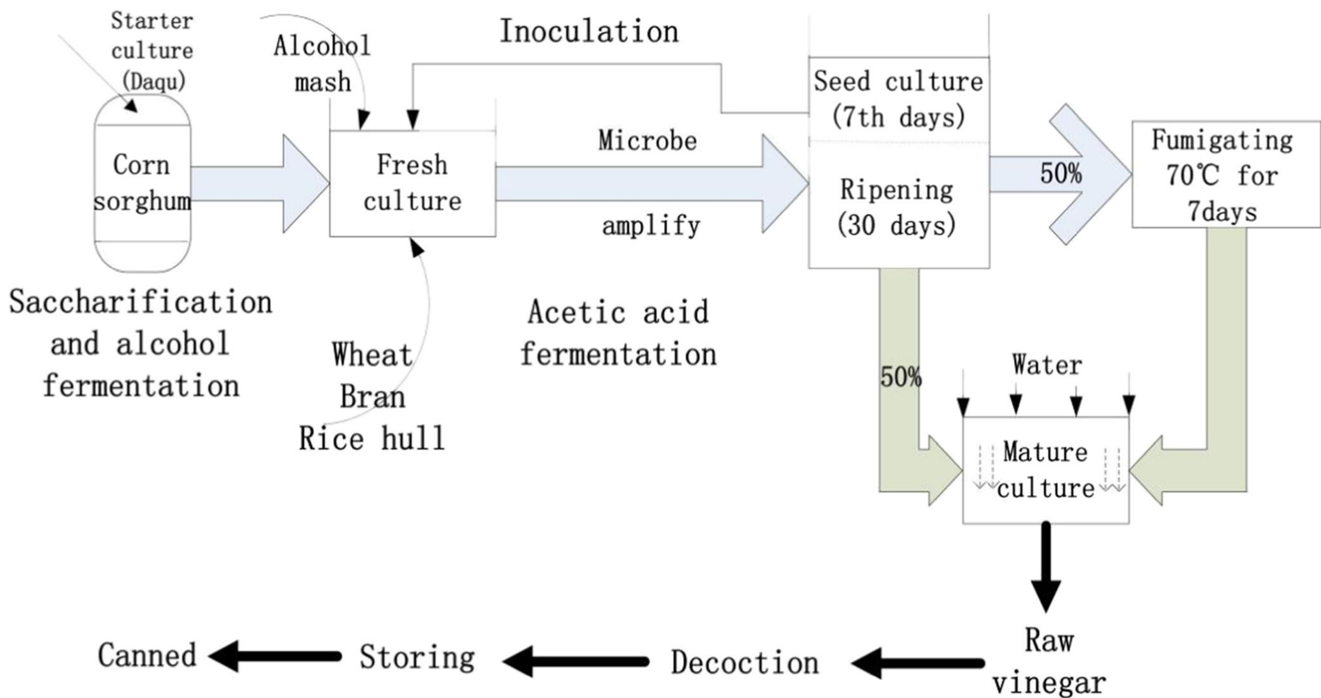

Fig. 1 The traditional brewing processing of the Liangzhou fumigated vinegar

and functional substances, such as organic acids, amino acids, acetoin (ACT), and the volatile component ligustrazine (He et al. 2004; Xu et al. 2007). Because of the uniqueness and complexity of raw materials, technology, and environmental conditions of LZFV fermentation, it is difficult to study the composition and succession of the microbial community during fermentation process. However, the microbial diversity and its dynamic change are significant for the quality and characteristics of the fermentation products (Chen et al. 2013). In order to control the fermentation process well, and ensure vinegar quality, it is necessary to explore the changes in the microbial community during LZFV fermentation.

The culture-independent technique based on a next-generation sequencing system, such as pyrosequencing, has been described as a more complete alternative to capture the complexity of the communities present in different fermented products (Roh et al. 2010; Jung et al. 2011; Illeghems et al. 2012; Ercolini 2013). Such a system was recently applied to some famous vinegars produced using solid-state fermentation technology (Nie et al. 2013; Lu et al. 2016; Nie et al. 2017). However, it has never been used to study the microbiota present in LZFV fermentation. Due to the lack of understanding of the function microbial community succession in the traditional fermentation process, vinegar flavor control is still mainly by virtue of experience. So, in the present work, we researched the composition, function, and succession of microorganisms in different stages of LZFV fermentation process in order to provide references for related enterprises to improve process control and vinegar quality.

Materials and methods

Sampling

Daqu and the original cultures (the solid fermentative substrate of LZFV) used in this study were collected from Yimin Food Industry Company, located in Wuwei, Gansu, China. To monitor the microbial succession and diversity during solid-state fermentation, we periodically collected six culture samples (1, 3, 5, 8, 10, and 13 days) from the same location. All these culture samples were collected at a depth of approximately 25 cm from the upper surface in the fermentation vat, and were numbered T1, T3, T5, T8, T10, and T13, respectively. Approximately 200 g per sample was collected and placed into sterile blue-cap bottles and immediately stored in an ice-box. The samples were stored at −80 °C until used for genomic DNA extraction and physicochemical analysis.

Physicochemical analysis

An approximately 10-g sample was homogenized with 30 ml of de-ionized water, and the pH was measured using a pH meter (Mettler Toledo, USA). The total acidity was analyzed by titration using standardized solution (0.1 M sodium hydroxide) with phenolphthalein as the indicator (Nie et al. 2013). Residual sugar amount was estimated using the 3,5-dinitrosalicylate method as described by Miller (1959), and the amino nitrogen was analyzed according to Thomas and Ingledew (1990). The ethanol concentration in samples was determined by high-performance liquid chromatography according to Wu et al. (2013). Gas chromatograph-flame

ionization detector (Agilent Technologies, USA) analysis after headspace solid-phase micro extraction (Supelco, USA) was used to determine ACT, tetramethylpyrazine (TTMP), and ethylacetate (EA) according to Richter et al. 2013 and Zhao and Yun 2016.

Microbial viable cell count of the main functional communities

The five functional microbial communities (including acetic acid bacteria, lactic acid bacteria, heat-resistant bacteria, yeasts, and molds) viable cell count were carried out according to the method of Zhao (2016).

DNA extraction and PCR amplification

Samples were homogenized using liquid nitrogen (flash-frozen in liquid nitrogen and then rapidly thawed in a water-bath at 65 °C for 2 min, and repeated three times), and approximately 500 mg of sample was then added to the extraction buffer [100 mM Na_3PO_4, 100 mM Trise HCl, 100 mM ethylene diaminetetraacetic acid, 1.5 M NaCl, 1% cetyltrimethyl ammoniumbromide (CTAB, *w*/*v*), 2% polyvinylpolypyrrolidone (PVPP, *w*/*v*), pH 8.0]. CTAB was used to remove polysaccharides from vinegar samples, and PVPP was used for polyphenolic component adsorption. Total DNA was extracted according to previously described methods (Mamlouk et al. 2011; Nie et al. 2013). All DNA concentrations were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at OD260/OD280 ratios of 1.7–2.0. DNA integrity was assessed using agarose gel electrophoresis.

Two pairs of universal primers were respectively used to amplify the bacterial V4 region of 16S rDNA and the fungal internal transcribed spacer (ITS) region: primers 520F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′) were used to amplify bacteria 16S rDNA, and ITS1F (5′-CTTG GTCATTTAGAGGAAGTAA-3′) and 2043R (5′-GCTG CGTTCTTCATCGTGC-3′) to amplify the fungal ITS region. The PCR mixture (25 μl) contained 5 × Q5 reaction buffer, 5 × Q5 GC high enhancer, 2.5 mM dNTP, 5 ng of extracted total DNA, 10 μM of each primer and 1.25 U of Q5 polymerase. The sizes of PCR products were assessed by electrophoresis on 1% (*w*/*v*) agarose gel.

Pyrosequencing

The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purified products were quantified using a spectrophotometer (NanoDrop Technologies). Next, equal amounts (100 ng) of amplicons from different samples were pooled and purified prior to pyrosequencing using the ethanol precipitation method. The amplicon mixture was applied to a Genome Sequencer FLX454 Titanium System (454 Life Sciences, Branford, Connecticut, USA) (Liu et al. 2015; Polka et al. 2015).

Multivariate statistical analyses

The community-supported software package v1.11.0 (Schloss et al. 2009) was used to describe and compare microbial communities and systematic layout planning methodology was used to analyze the number of sequences assigned to each cluster (regarded as Operational Taxonomic Units, OTUs). To describe microbial diversity (including dominance, richness, and equitability), based on OTU richness, we calculated biodiversity indices: Chao1, ACE, Simpson index (Simpson 1949) and Shannon–Weaver index (Haruta et al. 2006). Principal component analysis was then performed to group microorganisms using Canoco for Windows v4.5 software (Wageningen UR, Netherlands). Venn diagrams were drawn using Venny 2.0.2, and hierarchical clustering analysis was performed to draw heatmaps using Hemi 1.0.3.7-Heatmap Illustrator.

Results

Dynamic changes in physicochemical characteristics during the whole fermentation

Dynamic changes in physicochemical properties during the whole fermentation are shown in Fig. 2. During LZFV fermentation, the temperature changed significantly. In the first 3 days (the AF), temperature increased slowly from 22.6 °C to 28.3 °C, followed by the AAF when temperature rose rapidly from 28.3 °C to 45.2 °C; after 8 days, it gradually fell to 32.9 °C. Concentration of residual sugar gradually increased in the first 3 days; ethanol content in samples also constantly rose and reached a maximum (6.9%, *v*/*v*) at 3 days. The content of total acidity increased rapidly from 0.45% (*w*/*w*) in original culture to 4.58% at 13 days. The free amino nitrogen also increased throughout the whole fermentation but pH gradually decreased from 4.66 to 3.18. AAF is a critical stage for production of organic acids, which are the dominant compounds of vinegar. Temperature, pH, ethanol, total acidity, and residual sugar were the key physicochemical metrics to monitor AAF (Fig. 2). The characteristic flavor compounds of LZFV (such as ACT, TTMP, and EA) had significant differences at the different stages of fermentation—among them, ACT, as a TTMP precursor, reached a peak of 1.46 g/100 g at 3 days of fermentation, and then gradually decreased. However, TTMP and EA continuously accumulated during the whole fermentation process.

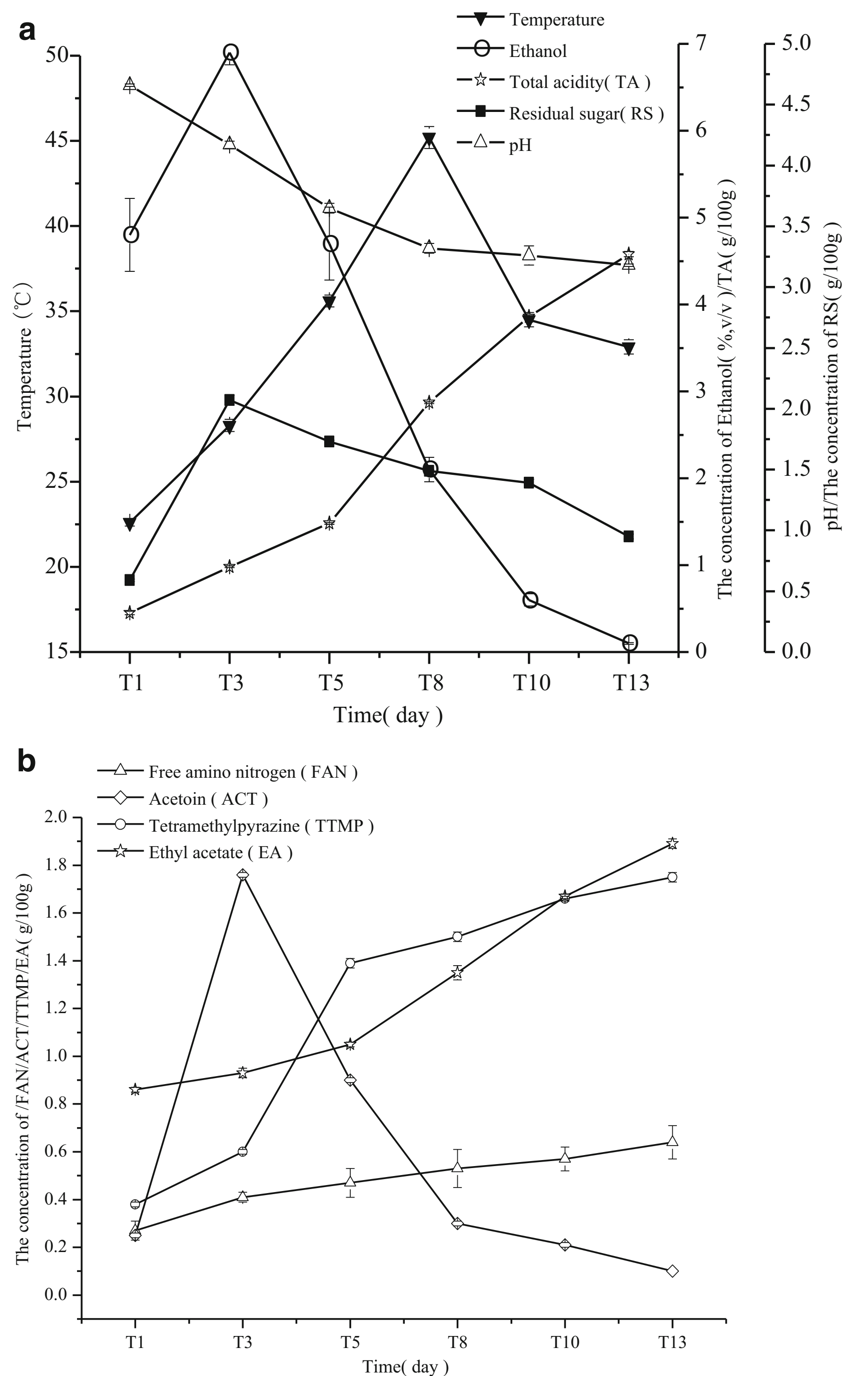

Fig. 2 Dynamic changes in physicochemical characteristics during LZFV fermentation. **a** described the dynamic changes in the key physicochemical metrics, including temperature, pH, total acidity, residual sugar, and ethanol concentration during LZFV fermentation. **b** described the characteristic flavor compounds (free amino nitrogen, acetoin, tetramethylpyrazine, and ethylacetate) of vinegar during LZFV fermentation

Dynamic changes of main functional microbial communities cell counts in Liangzhou vinegar brewing process

In order to understand the influence of major functional microbial communities on quality of vinegar well, the five functional microbial communities (including acetic acid bacteria, lactic acid bacteria, heat-resistant bacteria, yeasts, and molds) viable cell count were carried out (in Fig. 3). The results showed that the number of molds was the highest on the first day of fermentation, and gradually decreased as the fermentation progressed. During the first 5 days of fermentation, the yeast had a small increase with the prolongation of fermentation time. It reached the maximum on the fifth day, and then decreased. The presence of yeast was almost undetectable after the tenth day. However, the number of bacteria reached the maximum on the eighth day. Among them, acetic acid bacteria, lactic acid bacteria, and heat-resistant bacteria

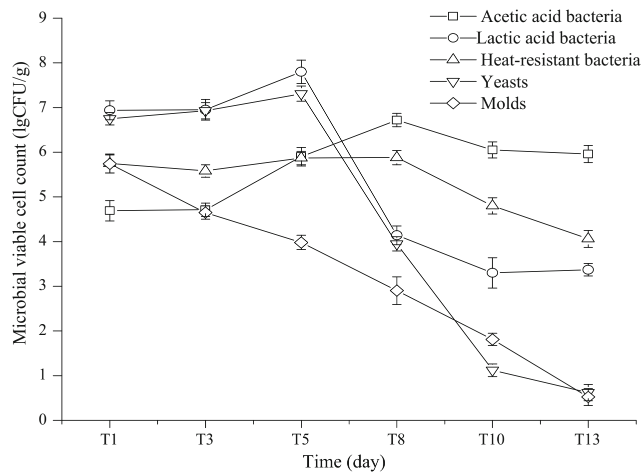

Fig. 3 Succession of major functional microbial communities during the fermentation process

gradually increased in the late stage of fermentation, and they were becoming the dominant communities. These were related to the accumulation of metabolites and changes in pH.

Changes of microbial diversity in the whole fermentation

The diversities of bacterial and fungal communities during the whole LZFV fermentation were systematically investigated (Tables 1 and 2). The alpha diversity metrics (Tables 1 and 2) showed the richness and evenness of microbiota in vinegar samples. The four alpha diversity metrics were computed to ensure that all metrics closely matched the true diversity of LZFV. The Chao1 analysis indicated that the sample in the early fermentation period (1 day) consisted of more phylotypes than other phases, and the samples in the transition period (3–5 days) had the least microbial population among all samples. Shannon, Simpson and ACE metrics also indicated the same trend in bacterial diversity. This showed that most bacteria could adapt to the culture environment on the first day of fermentation. During the transition period, as ethanol gradually accumulated, bacteria that could not tolerate the high ethanol concentration died. In the AAF process, the acidogenic bacteria and extreme microorganisms grew rapidly and organic acids quickly accumulated, and so bacteria with a low tolerance of acetic acid disappeared with longer times of fermentation—causing a decline in microbial diversity.

However, fungal diversity showed a different trend—the sample with the most phylotypes was the transition period (5 days), but the initial (1 day) and final fermentation period (13 days) samples had small populations. Shannon, Simpson, and ACE metrics also indicated the same trend. Thus, the transition period showed this environment was suitable for growth and metabolism of most fungi participating in fermentation.

Dynamics and succession of microbial community during whole fermentation

To evaluate the contribution of OTUs among the different cultures of LZFV fermentation, Venn diagrams were constructed (Figs. 4 and 5).

The dynamic changes in the microbial community during the whole fermentation of LZFV were investigated periodically. It showed that the microbial community composition dynamically changed—every sample had some specific OTUs and also had some common OTUs to others.

Bacterial components were rich (413 and 388 OTUs) in Daqu and the sample for the initial period of fermentation (T1); however, for samples during the AAF process (T5, T8, and T10), OTUs gradually declined (111–143 OTUs) and then remained relatively steady. Toward the end of fermentation, the number of OTUs also gradually increased. Specific bacterial genera had higher relative abundance (> 50%) in Daqu and the T1 sample, and common genera represented < 10%. In contrast, common

Table 1 Bacterial community diversity metrics (distance < 0.03) during LZFV fermentation

Sample ID	Reads	0.97					
		OTU	ACE	Chao1	Shannon	Simpson	Coverage
DQ	24128	413	297	275	2.44	0.1942	0.9976
T1	25516	388	323	308	3.04	0.1093	0.9977
T3	12812	141	147	137	0.46	0.8717	0.9969
T5	18153	116	133	112	0.83	0.6214	0.9981
T8	15809	111	222	137	1.11	0.4499	0.9973
T10	19991	143	214	164	1.24	0.4162	0.9976
T13	17615	239	298	226	1.38	0.3733	0.9962

Values are the means of three replicates

Table 2 Fungal community diversity metrics (distance < 0.03) during LZFV fermentation

Sample ID	Reads	0.97					
		OTU	ACE	Chao1	Shannon	Simpson	Coverage
DQ	28380	80	97	96	1.92	0.2288	0.9993
T1	25350	107	124	126	1.65	0.4196	0.9991
T3	30482	121	124	122	2.08	0.2951	0.9998
T5	34777	164	195	195	1.45	0.5614	0.9990
T8	29358	154	154	153	1.97	0.3590	0.9997
T10	30447	136	136	136	2.50	0.2415	0.9998
T13	21182	102	103	102	2.49	0.2215	0.9999

Values are the means of three replicates

fungal genera had relative abundance > 45% and specific genera < 10% in Daqu, and the relative abundance of specific genera in each sample was ≤ 15%. This phenomenon indicated that composition of the bacterial community changed more quickly than for the fungal community during the whole LZFV fermentation, and also implied that Daqu starter mainly provided the fungus source for LZFV fermentation. Bacteria in Daqu also participated in the fermentation, but were not the dominant microorganisms—the dominant bacteria in the fermentation mainly came from the “vinegar mother” and the external environment.

Analysis of microbial composition and community structure in the whole fermentation

The microbial composition at different levels is shown in Table 3. The relative taxonomic abundance of microbial community at the genus level, and the relative abundance of all genera of > 0.1% are shown in Fig. 4.

The microbial community structure analysis using high-throughput sequencing showed a more diverse bacterial community compared with the fungal community in the LZFV process. The bacteria changed more quickly than fungi (Table 3 and Fig. 6). The microbial community of Daqu was

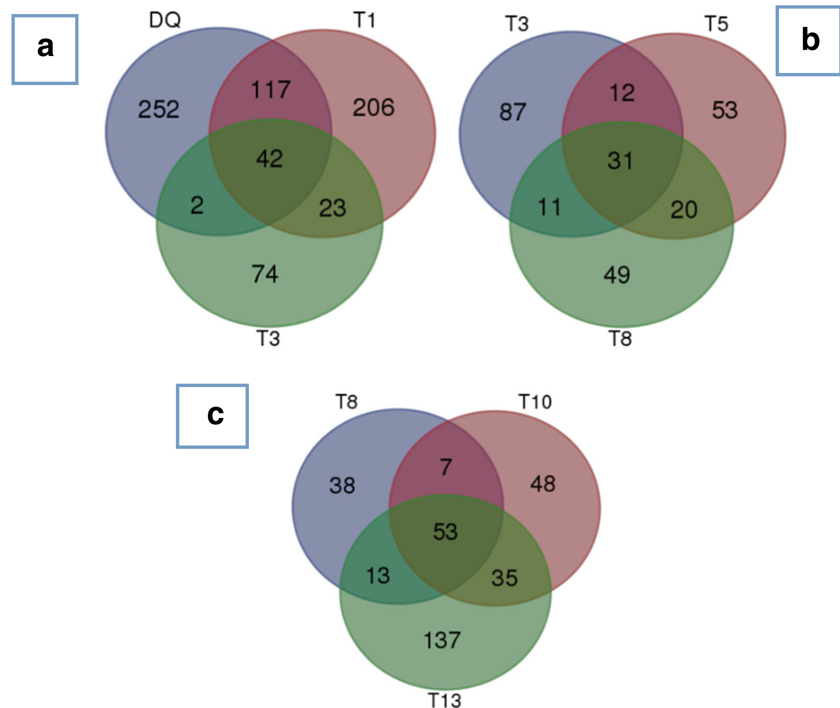

Fig. 4 Venn diagrams showing specific and common OTUs of bacterial communities (**a**–**c**) during the LZFV fermentation. Samples were classified at the genus level. **a** Represents the contribution of OTUs (bacterial genus level) in Daqu starter (DQ) and the initial fermentation stage of LZFV fermentation process (T1, the 1st day culture sample; T3, the 3rd day culture sample). **b** Represents the contribution of OTUs (bacterial genus level) in the intermediate stage of fermentation during LZFV fermentation process (T3, the third day culture sample; T5, the fifth day culture sample; T8, the eighth day culture sample). **c** Represents the contribution of OTUs (bacterial genus level) in the last stage of fermentation during LZFV fermentation process (T8, the eighth day culture sample; T10, the tenth day culture sample; T13, the 13th day culture sample)

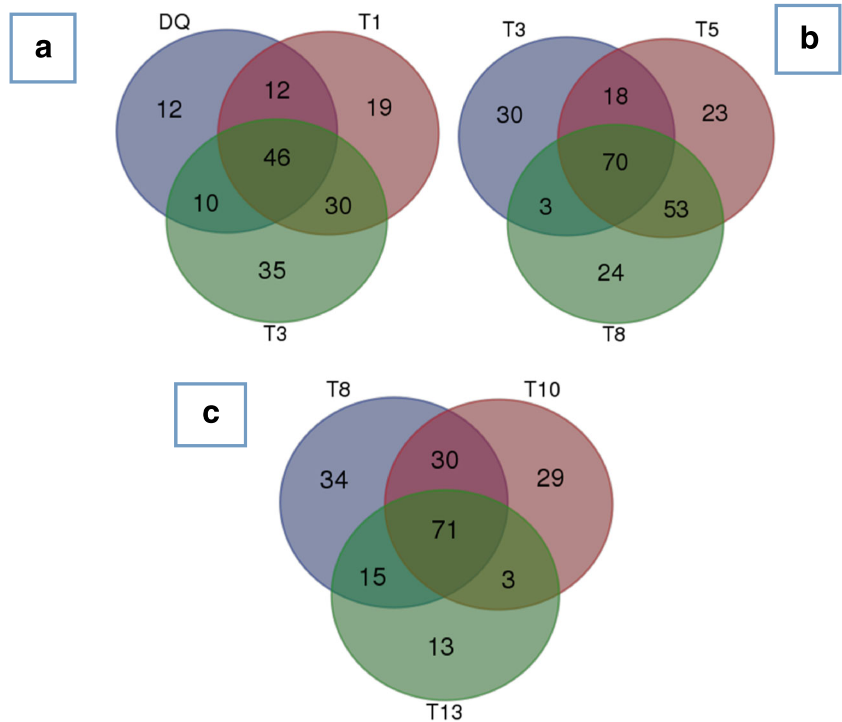

Fig. 5 Venn diagrams showing specific and common OTUs of fungal communities (**a**–**c**) during LZFV fermentation. Samples were classified at the genus level. **a** Represents the contribution of OTUs (fungal genus level) in Daqu starter (DQ) and the initial fermentation stage of LZFV fermentation process (T1, the first day culture sample; T3, the third day culture sample). **b** Represents the contribution of OTUs (fungal genus level) in the intermediate stage of fermentation during LZFV fermentation process (T3, the third day culture sample; T5, the fifth day culture sample; T8, the eighth day culture sample). **c** Represents the contribution of OTUs (fungal genus level) in the last stage of fermentation during LZFV fermentation process (T8, the eighth day culture sample; T10, the tenth day culture sample; T13, the 13th day culture sample)

composed of 24 genera—*Streptomyces*, *Bacillus*, *Thermoactinomyces*, *Lactobacillus*, and *Erwinia* were the dominant bacterial genera and had relative abundance > 5%. The T1 sample also had multitudinous bacteria—it contained 25 genera, except *Geobacillus*, its bacterial composition was the same as Daqu, and there was no clearly dominant bacterial genus among them. *Lactobacillus* was the dominant bacterial genus with relative abundance highest in T3, indicating that *Lactobacillus* were the major functional microorganisms in the AF process. With increasing time, the relative abundance of *Lactobacillus* was gradually reduced and that of *Acetobacter* gradually increased. During the AAF, total abundance of *Lactobacillus* and *Acetobacter* was > 90%—the relative abundance of *Acetobacter* increased as AAF progressed, indicating that they were the predominant functional microorganisms for AAF of vinegar fermentation.

The 18S-26S rDNA ITS region analysis showed that the dominant members of the fungal community in Daqu were *Aspergillus* (35.67%), *Thermoascus* (40.96%), and *Monascus* (12.86%). The period of 1–3 days was when SS and AF were carried out simultaneously, so *Aspergillus* and *Saccharomyces* were the dominant fungi and the total relative abundance of the two genera exceeded 50%: T1 was 52.89% and T3 was 70.78%. In addition, *Alternaria*, *Monascus*,

Table 3 The microbial composition of samples at different taxonomic levels

Sample	Bacteria					Fungi				
	Phylum	Class	Order	Family	Genus	Phylum	Class	Order	Family	Genus
DQ	6	12	20	42	55	4	11	21	28	43
T1	12	20	31	57	84	4	15	29	39	56
T3	5	7	13	22	27	4	18	32	46	64
T5	5	8	14	22	27	4	18	34	45	69
T8	6	8	13	21	28	4	14	28	43	66
T10	6	8	16	28	37	4	15	29	41	64
T13	6	11	21	43	56	4	16	27	36	54

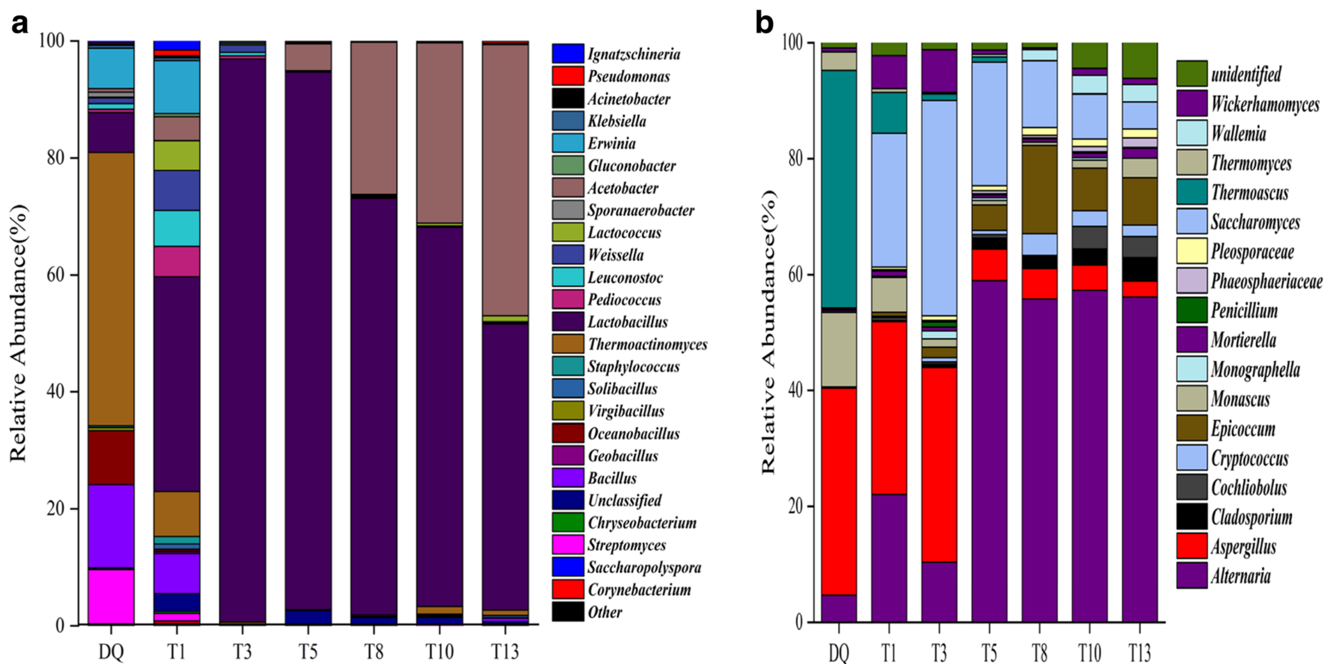

Fig. 6 Phylogenetic taxonomy for bacterial (**a**) and fungal (**b**) dynamic changes at the genus level during LZFV fermentation. All genera are shown in Fig. 6, which the relative taxonomic abundance are more than 0.1%

Thermoascus, and *Wickerhamomyces* also had higher contents and their relative abundances were > 1%. The relative abundance of *Aspergillus*, *Saccharomyces*, and *Wickerhamomyces* sharply decreased after 3 days of fermentation indicating that these three genera were the predominant functional microorganisms for the SS and AF processes. During the AAF, the relative abundance of *Alternaria* was basically stable (approximately 55%) and *Epicoccum*, *Mortierella*, *Cladosporium*, *Cochliobolus*, *Monascus*, and *Wallemia* appeared or their relative abundance increased—thus, we conjectured that they were the predominant functional microorganisms in the AAF process.

Hierarchical cluster analysis of microorganisms in the whole fermentation

Hierarchical cluster analysis was used to explore the changes in different microorganisms during the whole fermentation, and heatmaps of bacterial and fungal genera were produced (Fig. 7a and b, respectively). The bacterial community composition of Daqu and T1 had some similarity, those for T3 and T5 were identical, and those among T8, T10, and T13 were very similar. The samples of the AF process (T1 and T3) had similar fungal community composition, and the samples of AAF (T5, T8, T10, and T13) had identical fungal community composition.

There were three stages during the LZFV fermentation. The first stage was 1–3 days, with the major functional microorganisms being *Aspergillus* and *Saccharomyces*. The second stage was 3–8 days, and this was a preparatory phase of AAF. In this stage, microbial composition changed quickly, many functional microorganisms of AF weakened and those of AAF appeared or grew rapidly—so samples of this period contained not only ethanol but also acetic acid (Fig. 2). The last stage was during 8–13 days, when the microbial community was almost stable, although the acetic acid content consistently increased (Fig. 2).

Discussion

Many microbiological studies have been performed to examine the process of traditional Chinese vinegar fermentation by culture-independent techniques, especially PCR-denaturing gradient gel electrophoresis (DGGE) (Haruta et al. 2006; Xu et al. 2011; Nie et al. 2013, 2017). Xu monitored the microbial community during solid-state AAF of Zhengjiang aromatic vinegar using PCR-DGGE, and showed that there were 12 genera of bacteria and four genera of fungi during the fermentation process (Xu et al. 2011). Nie researched the microbial succession and diversity during solid-state fermentation of Tanjin Duliu mature vinegar using PCR-DGGE, and identified 18 genera of bacteria and eight genera of fungi (Nie et al. 2013). Nie detected 22 bacterial and six fungal genera in a study of the microbial community succession during the fermentation of Shanxi aged vinegar (Nie et al. 2017).

Our research explored the microbial succession and diversity during solid-state fermentation of LZFV by high-throughput sequencing, a revolutionary technology characterized by low cost and high-throughput of data (Zhu et al. 2014). Results showed that every sample contained more than 27 bacterial and 43 fungal genera, indicating that more microorganisms were detected by high-throughput sequencing than by PCR-DGGE. Therefore, obtaining microbial diversity

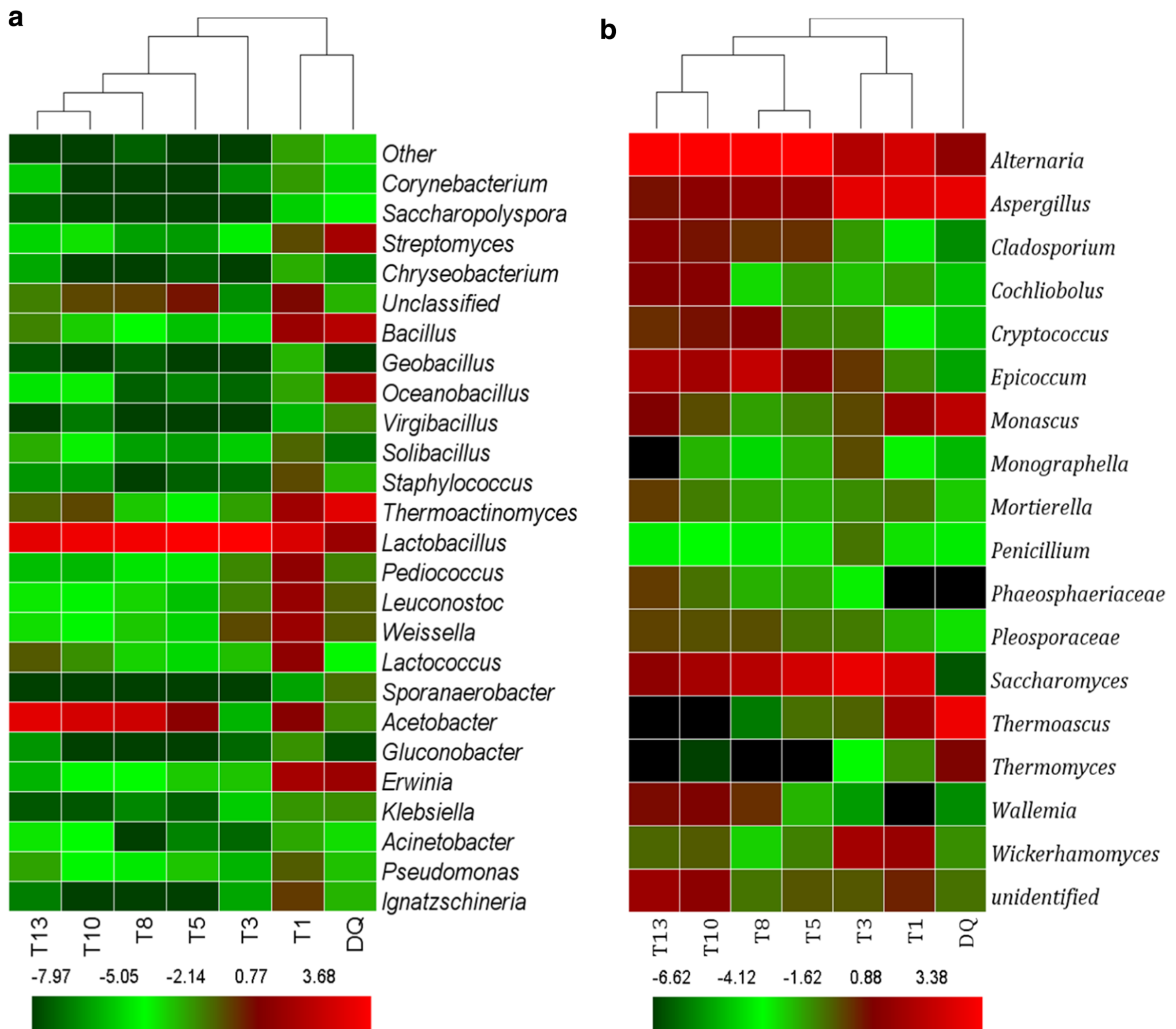

Fig. 7 Hierarchical cluster analysis of microorganisms in whole fermentation. Red represents positive and black represents negative; the gradient from blue to red represents the microbial relative abundance gradually rising

information in Daqu will help us to understand its impact on the quality of vinegar products. Like many other Daqu types, Liangzhou-Daqu is made in a hot environment (35–60 °C). We found that the dominant bacterial genera in Daqu were *Streptomyces*, *Bacillus*, *Thermoactinomyces*, and *Erwinia*—most species in these genera are resistant to high temperature. Many *Streptomyces* strains can be used for isolating enzymes such as α-galactosidases which can hydrolyze raffinose family oligosaccharides—mainly raffinose and stachyose, anti-nutritional factors in legumes of soybean—to increase nutritional value (Cao et al. 2010). *Thermoactinomyces* have been detected in many high-temperature Daqu samples (Zhu et al. 2014; Yao et al. 2014, 2015) that exhibit high enzyme production, such as of cutinolytic esterase and alkaline protease (Fett et al. 2000; Divakar et al. 2006). *Bacillus* was reported to be continuously present in Daqu, and they contribute to the evolution of flavor and activities of enzymes such as amylases and proteinases (Zheng et al. 2011) in vinegars and liquors. Our previous study showed that TTMP was the characteristic flavor compound of LZFV, and that flavor of the vinegar product was enhanced significantly with increasing of TTMP (Han 2013). *Bacillus* is the main genus involved in TTMP production (Larroche et al. 1999; Xiao et al. 2009; Zhu et al. 2010).

The fungi needed for LZFV fermentation almost all came from Daqu—only one unclassified genus, belonging to *Phaeosphaeriaceae*, appeared in the fermentation period and showed a rising trend. *Aspergillus* (35.67%), *Thermoascus* (40.96%), and *Monascus* (12.86%) were regnant in the LZFV Daqu fungal community. This was very different for Shanxi-Daqu (Wu et al. 2004; Chen

et al. 2009) and Duliu-Daqu (Nie et al. 2013) with *Saccharomyces* more abundant, but *Thermoascus* less abundant. The different microbial flora might be the main reason why the flavor characteristics of the vinegars differ. For LZFV fermentation, we found that *Aspergillus*, *Saccharomyces*, *Wickerhamomyces*, and *Lactobacillus* played important roles in the SS and AF process, *Acetobacter* were predominant functional microorganisms in the AAF process, and *Lactobacillus* had the highest relative abundance in the early fermentation period and declined in the AAF stage.

As used for Shanxi-aged vinegar, AF was carried out in a static and anoxic environment suitable for the survival of *Saccharomyces* and *Lactobacillus*. Alcohol gradually increased as *Saccharomyces* grew. Many microorganisms that could not tolerate high alcohol died and competition abated, so that *Lactobacillus* could grow quickly. However, after alcohol accumulated, Cupei was added to the vat with abundant other microorganisms and manually stirred once daily—this process would introduce oxygen. The increased oxygen limited the growth of *Lactobacillus*, so aerobic *Acetobacter* grew rapidly, and acetic acid accumulated most at the end of fermentation. This explains why the relative abundance of *Acetobacter* was low at 3 days and increased after 5 days. These results are consistent with the LZFV fermentation process (An 2011).

Lactobacillus populations played a strong role in improving the taste of vinegar. They provide substrates for esterification by yeasts and affect both the technological properties and microbial stability of the final products through production of lactic acid (Yousif et al. 2010). Our research also showed that abundance of *Bacillus*, *Solibacillus*, and *Lactococcus* increased at the end of the fermentation stage, and these are related to flavor-forming. *Bacillus* is involved in the synthesis of ACT and TTMP. *Solibacillus* might contribute to increases in the precursor of esters in fermentation (Li 2014), and were the flavor-forming bacteria during the AAF process. In addition, *Alternaria* was also found during the entire process and its relative abundance was stable during the AAF process, but its function remains unclear. It was also detected during the whole fermentation of Shanxi-aged vinegar (Nie et al. 2017).

Conclusions

The present work revealed that a complex microbial community was involved in spontaneously fermented LZFV. Not only naturally selected species gradually grew and reproduced in the fermentation process, but also promoters of vinegar fermentation. The LZFV fermentation is a continuous process with spontaneous growth that affects the dynamics of microbial communities. Continuous changes of micro-environment conditions in substrate affect the diversity and structure of microbiota. These results will be quite useful for understanding the relationship between microorganisms and the characters or quality of vinegar. Thus, we can conclude that the traditional solid-state fermentation process of LZFV is extremely complex. Its essence is the microbial community succession and a series of biochemical reactions caused by them, to which vinegar flavor formation is closely related.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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