

Microbial Diversity and Succession in the Chinese Luzhou-Flavor Liquor Fermenting Cover Lees as Evaluated by SSU rRNA Profiles

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Abstract The microbial diversity and the community succession in the fermenting cover lees of Chinese Luzhou-flavor liquor were investigated by small-subunit rRNA (SSU rRNA) culture independent method. All sequences retrieved from the 1, 7 and 60 days fermented cover lees were respectively assigned into the genera of *Streptococcus*, *Acetobacter*, *Arthrobacter*, *Bacillus*, *Staphylococcus*, *Serratia*, *Nocardia*, *Methanoculleus*, *Clostridium*, *Aneurinibacillus*, *Corynebacterium*, *Lactobacillus*, *Microbacterium*, *Trichosporon*, *Saccharomycopsis*, *Sagenomella*, *Talaromyces*, *Eurotium*, *Issatchenkia*, *Zygosaccharomyces*, *Saccharomyces* and TM7 phylum. The fungal *Issatchenkia*, *Saccharomycopsis* and *Talaromyces* and the bacteria *Staphylococcus* and *Lactobacillus* were most abundant in the 1 day fermented cover lees, the fungal *Issatchenkia*, *Saccharomyces* and *Talaromyces* and the bacteria *Bacillus* and *Streptococcus* were dominant in the 7 days cover lees, the archaea *Methanoculleus* and the fungal *Eurotium* and *Talaromyces* were prevalent in the 60 days cover lees. When the microbial community profiles in three samples were compared at species level, the prokaryotic community similarity coefficient was from 0.4042 to 0.5703 and descended to 0.2222, and that of eukaryotic community was from 0.3000 to 0.6000 and followed to 0.5215. These

results suggested that microbial diversity variability and community succession have happened in the cover lees associated with fermentation proceeding and such variability and succession respond for the appearance of some unique flavor of Luzhou-flavor liquor.

Keywords Luzhou-flavor liquor · Microbial diversity · Community succession · Fermenting cover lees

Introduction

Chinese liquor is well known worldwide and very popular in China. In Chinese liquors, there are three main types: sauce fragrance liquor, intense fragrance liquor and light fragrance liquor [1]. Luzhou-flavor liquor is a typical representative of the Chinese intense fragrance liquor, and it is produced in southeast of Sichuan province in China. In 1996 and 2006, the traditional brewing technique of Luzhou-flavor liquor was listed in Chinese intangible cultural heritage list and in world cultural heritage tentative list, respectively [2].

Luzhou-flavor liquor is distilled from the fermented liquor lees in the soil cellar. The liquor lees is the solid medium colonized on by many brewing microorganisms, and it is divided into the cover lees, the foodstuff lees and the bottom lees according to their material composition and position in the cellar (Fig. 1a). The foodstuff lees, located in the middle layer of soil cellar, is the main fermentation region and it consists of the mixture grains such as sorghum, corn, wheat, rice, cavings, the starter Chinese Qu and the distilled foodstuff lees from the last turn. The bottom lees in the bottom of soil cellar, usually named as mother lees, is foodstuff lees that has fermented for three times or more. The cover lees in the top layer contains the

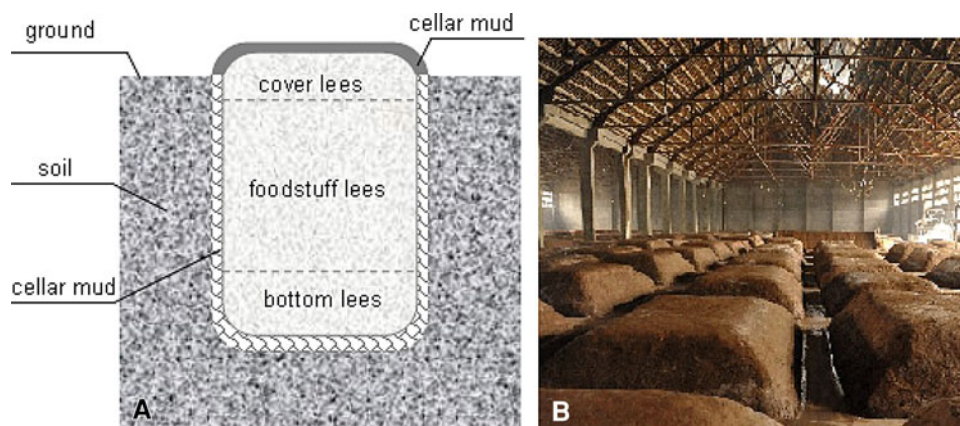
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starter Chinese Qu and the distilled foodstuff lees from the last turn. After these lees are prepared as the rules defined by the process, they are placed into the soil cellar and covered by the cellar mud, then fermented at the natural condition for 60 days or more (Fig. 1b). During the fermenting, the brewing microorganisms, which come from the cellar mud, the starter Chinese Qu, the mother lees and the natural environment surround the soil cellar, are considered to play a key role [2]. And the unique sensorial appearance of Luzhou-flavor liquor is largely dependent on the microbial succession associated with fermenting [2].

The microbial succession is a universal phenomenon in natural fermentation process [3]. The microbial succession in several types of food, such as kimchi, traditional fermented mustard and Vietnamese alcohol starter has been examined by rRNA gene sequence analysis [2]. In recent, some researchers have already initiated the microbial succession analysis in Chinese traditional liquor fermentation by rRNA [1, 2, 4, 5]. The variability of bacteria and fungi present during the traditional fermentation process of Chinese light-fragrance Fen liquor lees was revealed in 2011 [5]. In 2012, Xiang et al. [2] firstly reported the microbial succession in foodstuff lees of traditional Chinese Luzhou-flavor liquor during the fermentation. In most cases, however, these investigations only focused on the microbes in the foodstuff lees but not in the cover lees. The cover lees is an important part of the Chinese traditional liquor cellar micro-ecosystem and it mainly contributes for the lees flavor of Chinese liquor. While, it is not yet known whether the microbial profiles in the cover lees are different from those in the foodstuff lees during the fermentation. In the present study, we have investigated the microbial diversity and their succession in the Luzhou-flavor liquor cover lees during its natural fermentation by 16S rRNA and 18S rRNA gene libraries. The information gathered may be useful to improve our understanding of the composition and succession of the microbes of Luzhou-flavor liquor cover lees during fermenting and to design the effective management of the fermentation process.

Fig. 1 The profile of the Luzhou-flavor liquor soil cellar (a) and the soil cellar filled with the fermenting lees (b)



Materials and Methods

Liquor Lees Sampling

The experiments were carried out on an industrial scale traditional fermenting cover lees at the Luzhou Liquor Co. Ltd (Sichuan, China). The cover liquor lees fermented for 1 day (T1), 7 days (T7) and 60 days (T60) were respectively collected in sterile tubes and then frozen in liquid nitrogen until analysis.

DNA Extraction

The total DNAs were extracted and purified from microbial cells in the cover liquor lees according to the methods described by Xiang et al. [2].

Construction of SSU rRNA Library

Prokaryotic and eukaryotic SSU rRNA in the cover lees were respectively amplified by the 16S and 18S forward and reverse primers (Eu27F: 5'-AGAGTTTGATCCTGGC TCAG-3' and 1490R: 5'-GGTTACCTTGTTACGACTT-3' for bacterial 16S rRNA; Ar28F: 5'-TGGTTGATCCTGCC AGAGG-3' and 1490R: 5'-GGTTACCTTGTTACGACTT-3' for archaeal 16S rRNA; 18SF: 5'-AACCTGGTTGATCC TGCCAGT-3' and 18SR: 5'-TGATCCTTCTGCAGGTTCC ACCTAC-3' for fungal 18S rRNA) [2, 6]. The thermal cycling conditions for 16S and 18S primers were as previously described by Xiang et al. [2]. The amplified fragments were respectively cloned into a plasmid pGEM-T vector according to the manufacturer's protocol. The recombinant plasmids were then transformed into the chemically competent *Escherichia coli* DH 5 α cells and transformants were identified on LB agar plates containing 100 μ g/ml ampicillin, 40 μ g/ml X-gal and 24 μ g/ml IPTG. The white clones were used to construct a library of clones, which corresponded to a single SSU rRNA amplicon.

Sequencing and Phylogenetic Analysis of SSU rRNA

The recombinant plasmids with SSU rRNA were used as templates for sequencing. The 16S rRNA and 18S rRNA sequences without chimeras were submitted to compare with sequences from GenBank and the percent similarity was then determined. The phylogenetic trees based on the 16S rRNA and 18S rRNA were respectively constructed with the MEGA 5.0 by the neighbor-joining method with the Kimura two-parameter model [7].

Statistical Analysis

BioEdit software was employed to carry out the pairwise comparison of all SSU rRNA sequences in this study. The rRNAs with 97 % or higher similarity were clustered into an operation taxonomic unit (OTU), while the others were grouped alone. The rarefaction analysis was performed by the Analytic Rarefaction 1.3 calculator based on the numbers of OTUs versus the numbers of clones [8]. More indices were calculated using Estimates Win 8.20 software for each library. These included S_{obs} , S_{chao1} , S_{ACE} , Shannon diversity (H), Simpson and Coverage. Microbial community relationship of three samples was calculated using Baroni-Urbani and Buser similarity index by DPS 7.5.

Nucleotide Sequence Accession Numbers

The represent SSU rRNA sequences of OTUs in this study were deposited in the GenBank databases at the NCBI. Prokaryotic 16S rRNA sequences GenBank ID: KC337085-KC337102; Eukaryotic 18S rRNA sequences GenBank ID: KC337075-KC337084.

Results

SSU rRNA Libraries of Luzhou Flavor Cover Lees

Each sample was used to produce a library of SSU rRNA clones. A total of 32 archaea clones, 143 bacterial clones and 150 eukaryotic clones were recovered from three fermented cover lees, 27 bacterial clones and 33 eukaryotic clones from T1 sample, 10 archaea clone, 99 bacterial clones and 75 eukaryotic clones from T7 sample, 22 archaea clone, 17 bacterial clones and 42 eukaryotic clones from T60 sample. All clone sequences above without chimeras were sorted into OTUs. It was determined that 6 bacterial OTUs and 6 eukaryotic OTUs were in T1 lees; 1 archaea OTU, 12 bacterial OTUs and 7 eukaryotic were in T7 lees; 2 archaea OTU, 7 bacterial OTUs and 4 eukaryotic OTUs were in T60 lees; and that most OTUs contained

multiple clones, while only few OTUs were unique, represented by only a single clone.

Estimated Microbial Diversity of Cover Lees

The rarefaction curves based on the 16S rRNA and 18S rRNA sequences in three samples were respectively obtained by plotting the number of OTUs observed against the number of clones (Fig. 2). It was calculated that some curves did not reach clear saturation. This result indicates that some microbes might escape from detection. However, the obvious decrease in the rate of OTUs detection on the curve indicated that the major part of the diversity of prokaryotic and eukaryotic microbes in current study had already been detected (Fig. 2). This conclusion was further supported by calculating the coverage of six libraries, which was from 55.7 % to 100 % (Table 1). Biodiversity were calculated for prokaryotic and eukaryotic populations using the Shannon-wiener (H) diversity index and the Simpson dominancy index. The H index and the Simpson index respectively varied from 1.93 to 2.83 and from 6.11 to 13.72 for six gene libraries (prokaryotic libraries: TP1, TP7 and TP60; eukaryotic libraries: TE1, TE7 and TE60) (Table 1). Maximum OTU richness was respectively estimated with S_{Chao1} and S_{ACE} , and it revealed that S_{Chao1} 12 and S_{ACE} 10 for TP1, S_{Chao1} 35.66 and S_{ACE} 32 for TP7, S_{Chao1} 16.33 and S_{ACE} 15.85 for TP60, S_{Chao1} 10 and S_{ACE} 10 for TE1, S_{Chao1} 24 and S_{ACE} 24 for TE7 and S_{Chao1} 23.5 and S_{ACE} 16 for TE60 (Table 1). Based on these estimations, 55.7 %–100 % of the diversities were covered by the applied sampling survey.

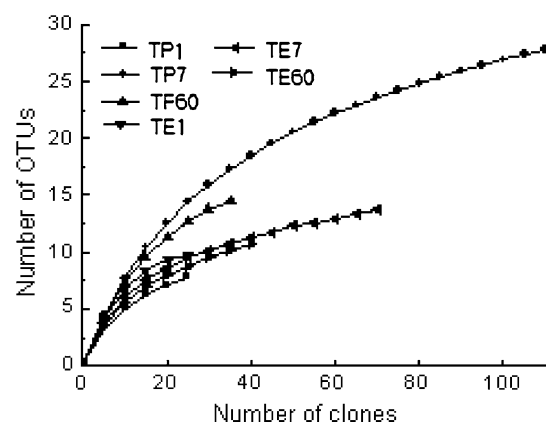


Fig. 2 Rarefaction analysis of fermenting cover lees prokaryotic 16S rRNA and eukaryotic 18S rRNA libraries, displaying the number of OTUs detected versus the number of sequences analyzed. TP1, TP7 and TP60 respectively represented 16S rRNA libraries from 1 day, 7 days and 60 days fermented cover lees. TE1, TE7 and TE60 respectively represented 18S rRNA libraries from 1 day, 7 days and 60 days fermented cover lees

Table 1 Richness and diversity estimations of OTUs derived from 1 day, 7 days and 60 days fermented cover lees prokaryotic 16S rRNA and eukaryotic 18S rRNA gene libraries

Sample	Num of clone	S_{obs}	S_{Chao1}	S_{ACE}	Coverage ^a	H	Simpson
16S rRNA analysis							
TP1	27	8	12	10	72.7	1.93	9.55
TP7	112	25	35.66	32	73.9	2.83	13.72
TP60	39	15	16.33	15.85	93.2	2.49	13
18S rRNA analysis							
TE1	33	10	10	10	100	2.23	11.53
TE7	75	14	24	24	58.3	2.17	7.06
TE60	42	11	23.5	16	55.7	1.96	6.11

^a Percentage of coverage: $S_{obs}/\text{mean}(S_{Chao1}, S_{ACE}) \times 100$

Microbial Distribution and Phylogenesis Based on SSU rRNA

The prokaryotic 16S rRNA without chimeras were rapidly and accurately classified to genus level by program RDP MultiClassifier. The 18S rRNA represent sequences were compared with those in GenBank and were also assigned to a same phylotype. All prokaryotic sequences retrieved in this study respectively fell into the genera of *Lactobacillus*, *Streptococcus*, *Bacillus*, *Staphylococcus*, *Clostridium*, *Acetobacter*, *Serratia*, *Nocardia*, *Aneurinibacillus*, *Corynebacterium*, *Arthrobacter*, *Microbacterium* and TM7 phylum, a lineage of bacteria domain, and achaea domain *Methanoculleus* and *Methanospirillum* genera (Fig. 3a), and those eukaryotic sequences were assigned into *Saccharomyces*, *Zygosaccharomyces*, *Saccharomycopsis*, *Issatchenkia*, *Eurotium*, *Talaromyces* and *Trichosporon*, *Sagenomella* (Fig. 4a). Among these genera, the fungal *Issatchenkia* (8/33), *Saccharomycopsis* (13/33) and *Talaromyces* (8/33) and bacteria *Staphylococcus* (9/27), *Lactobacillus* (13/27) were most abundant in the T1 lees, the fungal *Issatchenkia* (17/75), *Saccharomyces* (17/75) and *Talaromyces* (33/75) and the bacteria *Bacillus* (19/109) and *Streptococcus* (20/109) were dominant in the T7 lees, the archaea *Methanoculleus* (18/39) and the fungal *Eurotium* (13/42) and *Talaromyces* (25/42) were prevalent in the T60 lees (Figs. 3b, 4b).

To further disclose the microbial taxonomic position and relationship in the Luzhou-flavor liquor cover lees, all represent SSU rRNA sequences were submitted to the similarity searches in a public database to infer a possible phylogenetic classification. The BLAST searches revealed that most represent sequences shared more than 99 % similarity with strains in public database at the SSU rRNA level. But the microbes represented by clone TP7-111 showed less than 99 % to the strains available, and it was

assigned into TM7 phylum in bacteria domain. The phylogenetic trees based on 16S rRNA and 18S rRNA of the representative clones and their relational strains in GenBank were respectively constructed and shown in Figs. 3a and 4a. The trees revealed the phylogenetic affiliation of microbes inhabited in fermentation cover lees during the fermentation.

Microbial Community Succession

In general, the microbial succession is a universal phenomenon observed in natural fermentation process and is also a reflection of microbial interaction, competition for intrinsic growth factors [2]. With fermentation proceeding, the prokaryotic community similarity coefficient in three samples was from 0.4042 to 0.5703, and descended to 0.2222 when compared at the species level. And that of eukaryotic community was from 0.3000 to 0.6000 and followed to 0.5215 (Table 2). This result suggested that the microbial community succession has happened in the cover lees associated with fermentation proceeding. Furthermore, the same result was also proclaimed by the variations of H index and simpson index.

Discussion

Compared with microbes in the foodstuff lees, the tendency of microbial diversity in the cover lees was also from low to high and then followed to low during the fermentation process (Table 2). While, microbial distribution in the cover lees is different from those in the foodstuff lees. In the beginning of fermentation process, the microbes inhabited in the cover lees mainly came from the starter. The fungal *Issatchenkia*, *Saccharomycopsis* and *Talaromyces* and bacteria *Staphylococcus* and *Lactobacillus* were most abundant in the T1 sample. In the fermentation industry, some mycelial moulds such as *Aspergillus* and *Rhizopus* are often employed for the degradation and saccharification of grains [9]. While, the strains *Saccharomycopsis fibuligera*, *Issatchenkia orientalis* and *Talaromyces byssochlamydoides* are detected as dominant fungi in T1 sample, but not *Aspergillus* and *Rhizopus*. In previous work, we found that *S. fibuligera*, *I. orientalis* and *T. byssochlamydoides* were also prevalent at the initial fermentation of Luzhou-flavor liquor foodstuff lees [2]. The *S. fibuligera*, *I. orientalis* and *T. byssochlamydoides* have strong amylolytic activity and their roles on hydrolysis of grain starch to sugar have been agreed by many authors [4, 10, 11]. In addition, the strain *S. fibuligera* has a very efficient conversion ability of glucose, starchy biomass and waste starchy substrates into the ethanol [10, 12]. Therefore, it was suggested that the degradation and

Fig. 3 The phylogenetic tree based on the complete 16S rRNA sequences of representative clones from prokaryotic OTUs by using the neighbor-joining method (a) and the representative clone distributions in three samples (b). The scale bar corresponds to 0.02-estimated nucleotide substitution per sequence position. Bootstrap values from 1000 replicates are included

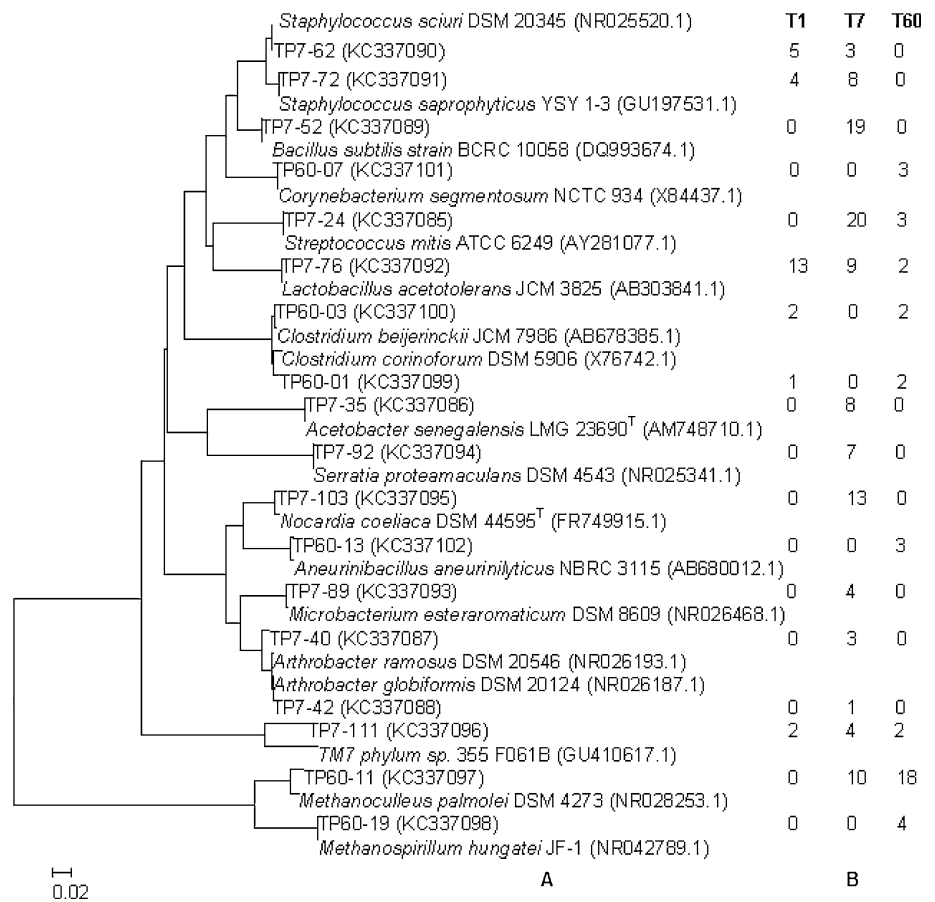


Fig. 4 The phylogenetic tree based on the complete 18S rRNA sequences of representative clones from eukaryotic OTUs by using the neighbor-joining method (a) and the representative clone distributions in three samples (b). The scale bar corresponds to 0.01-estimated nucleotide substitution per sequence position. Bootstrap values from 1000 replicates are included

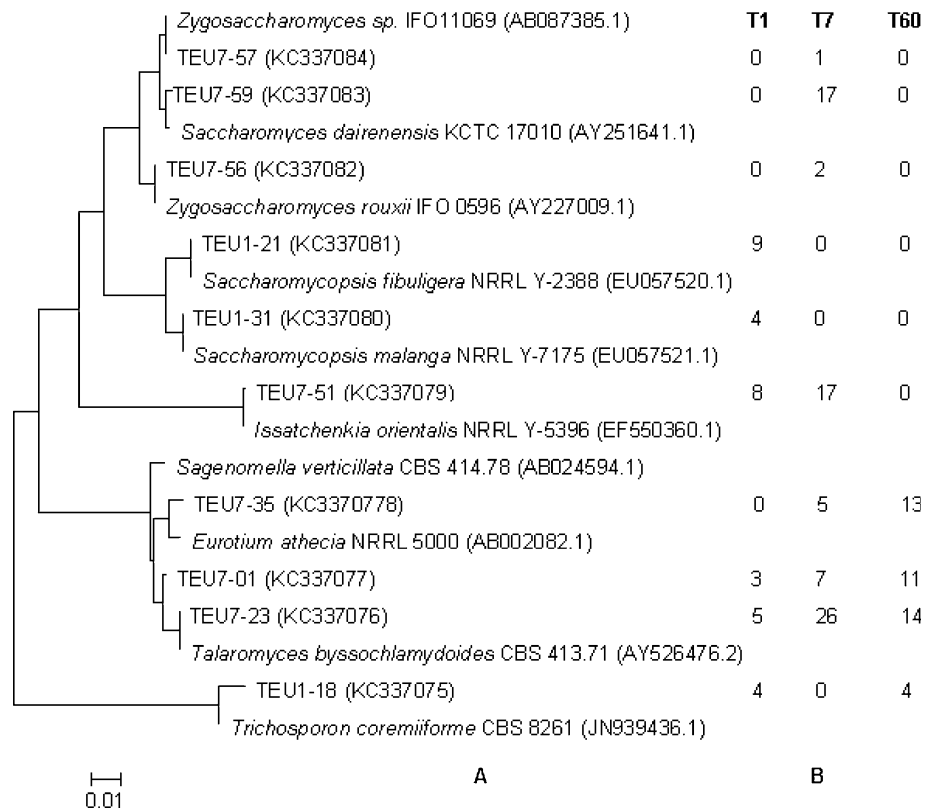


Table 2 The similarity coefficient of Baroni-Urbani and Buser based on prokaryotic and eukaryotic microbial community pairwise comparisons in 1 day, 7 days and 60 days fermented cover lees

Similarity coefficient	T1	T7	T60
Prokaryotic libraries			
T1	1.0000	0.4042	0.5703
T7		1.0000	0.2222
T60			1.0000
Eukaryotic libraries			
T1	1.0000	0.3000	0.6000
T7		1.0000	0.5215
T60			1.0000

saccharification of grains is mediated by *S. fibuligera*, *I. orientalis* and *T. byssochlamydoidea* in the cover lees during the initial fermentation.

The *Saccharomyces cerevisiae* mostly responded for alcoholic fermentation of the Luzhou-flavor liquor foodstuff lees fermented for 7 days [2]. However, *S. cerevisiae* was not detected in the T7 lees although it was predominant in the 7 days foodstuff lees (Fig. 4). And it was replaced by *Saccharomyces dairenensis*. The *S. dairenensis*, an unconventional yeast, can very efficiently selective ferment raw and untouched sugar from many biomass [13]. Furthermore, it is reported that *I. orientalis* has ability to produce ethanol from lignocellulose hydrolysates and cellobiose under different stress conditions [14, 15]. This hinted that the alcoholic fermentation in the T7 lees is mainly employed by *S. dairenensis* and *I. orientalis*. The genus *Eurotium* is an important microbe in the degradation of grains in Chinese Luzhou-flavor liquor lees [4]. And it was abundant in the foodstuff lees when the lees fermented for 7 days [2]. However, in the cover lees, it was not prevalent in the T7 lees. Interestingly, it was the dominant in T60 lees. In the Luzhou-flavor cover lees, the *Eurotium athecium* assigned into the genus *Eurotium* was firstly reported and might also play a primal role in degrading grains. The genera *Methanoculleus* and *Methanospirillum* were dominant in the T60 lees. This result was accordant with many previous studies [1, 2]. Indeed, it is generally accepted that the *Methanoculleus* and *Methanospirillum* can provide material basis for microbial propagating such as caproic acid bacteria, butyric acid bacteria etc. and contribute to produce some characteristic flavor factor of Luzhou-liquor in the process [1].

Usually, only several types of bacteria and fungi are the major microbes contributing to the liquor fermentation process [5, 16]. Our results showed that the predominant microbial community distribution in the cover lees is different from that in the foodstuff lees, and that the collective bio-actions of different dominant archaea, bacteria and fungi living might play important roles in the alcoholic

fermentation and producing flavor factor of Luzhou-flavor liquor. However, whether the inferior other microbes did also tamper with the alcoholic fermentation and the formation of characteristic flavor factor has not yet been known so far. The more detailed information about their activities in the process should receive more attention and further studies.

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