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Deep sequencing reveals high bacterial diversity and phylogenetic novelty in pit mud from Luzhou Laojiao cellars for Chinese strong-flavor Baijiu

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ABSTRACT

The pit mud (PM) in fermentation cellar is a complex ecosystem that hosts diverse microbial communities that contribute to the production of Chinese strong-flavor Baijiu (CSFB). However, the microbial ecology of PM, particularly the extent of their phylogenetic novelty remains poorly understood. Here we conducted Illumina MiSeq sequencing to explore the diversity and novelty patterns of PM bacterial communities from Luzhou Laojiao cellars in use for 40 and 400 years. High diversity indices were found in the PM with 16 phyla and 105 genera. Interestingly, the compositions of dominant genera of the PM were significantly different than that reported previously for PM sampled from other geographic sites, suggesting greater microbial diversity of PM. The dominant genus of *Caproiciproducens*, a caproic acid-producing bacterium, is the first reported for Chinese Baijiu production. Our results demonstrate that the PM hosts a large number of novel taxa, with 26% of the total OTUs (operational taxonomic units) distant to cultured counterparts. The class Clostridia within Firmicutes presented the highest proportion of novel OTUs. Most novel OTUs were initially isolated from diverse environments, the most abundant of which came from Chinese Baijiu brewing ecosystems, highlighting the huge culturing gap within the PM, but at the same time suggesting the importance of these OTUs in CSFB production. The data presented in this study significantly increases the number of bacteria known to be associated with CSFB production and should help guide the future exploration of microbial resources for biotechnological applications.

1. Introduction

Baijiu, Chinese liquor, is one of the oldest distillates in the world with an annual production of 13 million metric tons, and $ 8.8 billion in sales (Jin, Zhu, & Xu, 2017; Song, 2016; Zheng & Han, 2016). Chinese Baijiu can be classified into 12 categories based on their flavor characteristics (Zheng & Han, 2016). Chinese strong-flavor Baijiu (CSFB), also called "Luzhou-flavor Baijiu", dominates the market, accounting for > 70% of total Baijiu consumption (Liu, Tang, Zhao, Gu, et al., 2017). CSFB is produced by distillation of mixed, fermented grains (e.g., sorghum, wheat, and rice), and the fermentation procedure is a recycling process that occurs in cellars lined with pit mud (PM) (Zheng et al., 2013; Zheng & Han, 2016). PM, an essential material for CSFB fermentation, is a specific fermented clay that contains bacteria, archaea, and fungi, that are involved in various biological processes, contributing to the production of aromatic compounds in CSFB (Liu, Tang, Zhao, Liu, et al., 2017; Tao et al., 2014). Thus, the composition of PM microbial communities largely determines the flavor and quality of CSFB. To clarify the effect of microbial mechanisms on CSFB production, extensive studies were performed to study the PM microbial communities (Hu, Du, Ren, & Xu, 2016; Huang, Wang, Wei, Liu, & Li, 2015; Tao et al., 2014; Zheng et al., 2013; Zheng et al., 2015). These studies have shown that PM microbial communities are considerably complex and community composition varies with cellar age (Tao et al., 2014; Zheng et al., 2013) and geographical location (Huang et al., 2015; Liang, Luo, Zhang, Wu, & Zhang, 2016; Wang & Zhang, 2014).

Luzhou Laojiao, a world-famous Baijiu company located in Luzhou, Sichuan province of China, is a top representative of CSFB manufacturers (Yao et al., 2015; Zheng & Han, 2016). The production of Luzhou Laojiao has a long history dating back from the Ming dynasty (1573 CE) (Zhao, Zhang, & Zhou, 2009). According to previous studies (Li, Lin, Liu, Wang, & Luo, 2016; Sun et al., 2016), the composition of the microbial communities for Baijiu production was significantly shaped by environmental variables. After hundreds of years of

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Table 1
The physicochemical properties and NGS data for investigated PM samples in the cellars in use for 40 and 400 years.

		PM samples							
		A1	A2	A3	A4	B1	B2	B3	B4
Physicochemical properties	pH	5.25	5.74	6.49	6.08	6.26	6.69	5.97	6.45
	Moisture (%)	45.25	39.68	40.29	44.14	40.96	41.87	38.12	42.88
	Humic matters (%)	16.0	13.7	9.9	10.4	16.6	13.9	9.9	15.6
	NH_4^+-N (g/kg)	2.27	2.31	2.14	2.86	2.05	2.48	1.88	2.65
	AP (g/kg)	1.01	0.61	0.79	1.11	1.10	0.53	0.66	1.28
	AK (g/kg)	3.19	2.43	3.78	3.52	3.65	3.08	4.18	3.51
	Caproic acid (g/kg)	6.25	6.08	6.07	6.97	6.10	7.96	6.76	7.91
	Acetic acid (g/kg)	2.07	2.76	2.11	1.46	2.46	2.54	2.42	2.51
	Butyric acid (g/kg)	1.61	1.70	1.36	1.01	1.03	2.28	1.97	3.38
16S rRNA gene NGS analysis	Qualified sequences	32,033	59,041	62,984	75,230	77,707	76,137	83,267	83,413
	Sequences length (bp)	424.86	424.81	421.27	429.24	426.93	425.99	425.94	426.75
	OTUs (97% identity)[a]	255	276	258	256	273	231	338	235
	Coverage (%)[a]	99.9	99.9	99.9	99.8	99.9	99.9	99.9	99.9
	Chao1[a]	276.40	289.50	280.33	310.62	346.33	259.80	368.11	254.18
	Shannon[a]	5.92	6.14	5.91	4.16	5.79	5.65	5.95	4.90

[a] The indices were calculated based on a cutoff of 97% similarity of 16S rRNA gene sequences and 29,269 sequences per sample.

domestication, microbes involved in Luzhou Laojiao production formed a specific community structure based on the geographical climate in Luzhou and the unique manufacturing practices employed (Wang & Zhang, 2014; Xu et al., 2017). To date, some 400-year old cellars are still employed for CSFB production by the Luzhou Laojiao company. In the Chinese Baijiu industry, a cellar that has been in usage for a longer time is considered able to produce higher quality CSFB. Due to their excellent production performance and important history, the ancient cellars of Luzhou Laojiao, in continuous use for centuries, are under the protection of the National Cultural Heritage Conservation Board in China and are also recommended for inclusion on the Tentative List of World Cultural Heritage sites (Teachcn, 2011; Zhao et al., 2009). Overall, the Luzhou Laojiao cellars provide an excellent model to study the microbial effect on CSFB production. Understanding the PM microbial composition within these systems will be helpful for the development of Chinese Baijiu industry.

Previous studies focused on the PM microbial communities of the Luzhou laojiao cellars largely relied on low resolution community fingerprinting approaches (Ding, Wu, Huang, & Zhou, 2015; Liu, Tang et al., 2015; Liu, Zhao et al., 2015; Xiong et al., 2013; Zheng et al., 2013; Zheng et al., 2015). Although those molecular techniques provided a first description of abundant populations, the extent of microbial diversity remains unexplored. A study using next generation sequencing (NGS) strategies determined bacterial sequences from 2 individual PM samples from cellars of different age (Zheng et al., 2015). However, due to the limited number of samples, only limited conclusions can be drawn about bacterial diversity in the PM. In addition, previous studies carried out in PM largely focused on the diversity of the abundant taxa and their impact on CSFB production, but little is known about the genetic novelty of microbial communities despite the fact that many novel species have been isolated form PM (Chen et al., 2015; Liu et al., 2014; Ma et al., 2016; Yin et al., 2016). The discovery of multiple novel species suggests PM might be a promising source of new taxa, but it is unclear how many different microbes contribute to these systems. This lack of information limits our understanding of the brewing microecosystem and hinders the further exploration of new microbial resources for biotechnological applications.

The aim of this study was to determine the diversity and novelty patterns of PM bacterial communities from Luzhou Laojiao cellars in use for 40 and 400 years with MiSeq sequencing (NGS from Illumina). Our results demonstrate the microbial communities are deeply diverse and include many novel taxa, suggesting the need for more detailed ecophysiological studies. The results will help us define the overall structure of microbial communities responsible for Chinese Baijiu production and identify beneficial bacteria for further application.

2. Materials and methods

2.1. Materials

Samples of PM were removed from fermentation cellars that are affiliated with Luzhou Laojiao in Luzhou, Sichuan province of China. Luzhou is located in a humid subtropical climate zone (105°27′13.80″ E, 28°53′6.90″ N). The main geographical climate conditions (1951–2013) are as follows: altitude 335 m, annual average temperature 17.8 °C, annual temperature range 19.3 °C, annual average rainfall 1115 mm, annual average relative humidity 83%, and annual sunshine time 1300 h (Yang, Tang, Yang, Liu, & Zhao, 2014).

We selected cellars used for approximately 40 and 400 years for sampling, and 4 cellars were selected for each cellar age. PM samples (50 g) were collected from all corners and the center of each cellar wall and then combined. The PM samples taken from the cellars used for approximately 40 and 400 years are hereafter referred to as A1, A2, A3, and A4 for the 40-year cellar samples, and B1, B2, B3, and B4 for the 400-year cellar samples. The 40- and 400-years cellars are located in 2 different workshops at spatial scales of 10 km. The raw materials and the brewing processes used for CSFB production in the 2 different workshops are the same.

2.2. Physicochemical properties

The moisture of PM was measured by a dry/wet weight measurement method after drying at 105 °C for 3 h. The pH was measured at a 1:5 (wt/vol) ratio in ultrapure water with a pH meter (PB10, Sartorius, Gottingen, Germany). The concentration of humic matter was determined as described by Mehlich (1984). The concentration of ammonium nitrogen (NH_4^+-N), available phosphorous (AP), and available potassium (AK) were detected according to previous literature (Shen, 2007). The organic acids were extracted as described previously (Yuan, Zhang, Zeng, & Zhang, 2017) and the concentration of caproic, acetic, and butyric acid were quantified using a gas chromatograph (Clarus 500, PerkinElmer, Waltham, MA, USA) equipped with a flame ionization detectors (FID) and a CP-Wax 57 CB column (50 m × 0.25 mm × 0.2 μm Acidic). Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. The initial column temperature was 250 °C. The injection volume was 1 μL. The column temperature was programmed as follows: 35 °C for 8 min, increased at 3 °C/min to 85 °C, increased at 5 °C/min to 155 °C, increased at 10 °C/min to 210 °C, and then maintained at 210 °C for 15 min.

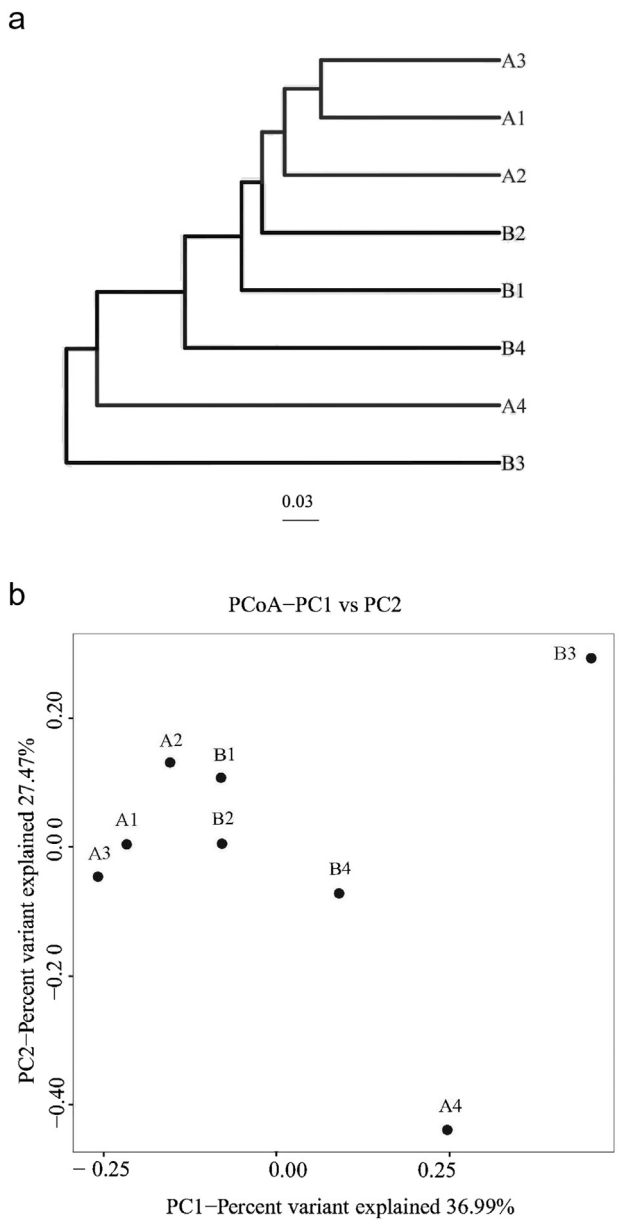

Fig. 1. UniFrac cluster analysis of bacterial communities of PM samples from cellars in use for 40 and 400 years. The UPGMA tree (a) where the *scale bar* indicates the distance between clusters in UniFrac units, and the score plot of PCoA (b) are shown. The scatter plot is of principal coordinate 1 (PC1) vs. principal coordinate 2 (PC2). The percentages are the percentage of variation explained by the components.

2.3. DNA extraction and Illumina MiSeq sequencing

DNA was extracted from the PM using the EZNA™ soil DNA kit (Omega Bio-Tek, Norcross, GA, USA) in accordance with the manufacturer's instructions. A set of primers designed by GENEWIZ Inc. (Suzhou, Jiangsu Province, China) was used to amplify the V3, V4, and V5 hypervariable regions of the 16S rRNA. The V3–V4 regions were amplified using forward primers containing the sequence "CCT ACG GRR BGC ASC AGK VRV GAA T" and reverse primers containing the sequence "GGA CTA CNV GGG TWT CTA ATC C". The V4–V5 regions were amplified using forward primers containing the sequence "GTG YCA GCM GCC GCG GTA A" and reverse primers containing the sequence "CTT GTG CGG KCC CCC GYC AAT TC". The PCR mixtures (20 μL) contained 1 μL of DNA template, and 2 μL of 10 × PCR buffer (50 mM KCl, 10 mM Tris-HCl and 1.5 mM $MgCl_2$), 200 μM of dNTPs, 0.1 μM of each primer, and 1 U of Taq DNA polymerase (Promega, Madison, WI, USA). The amplification was as follows: 3 min at 94 °C; 24 cycles of 5 s at 94 °C, 90 s at 57 °C, and 10 s at 72 °C; a final step of 72 °C for 5 min, and then cooling at 10 °C.

DNA libraries were validated by Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA), and quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 300 paired-end (PE) configuration. Image analysis and base calling were performed using the MiSeq Control Software (MCS) of the MiSeq instrument. The sequences were processed and analyzed by GENEWIZ Inc.

2.4. Data analysis

The sequencing data were processed using the QIIME platform (Version 1.9.1; http://qiime.org/tutorials/tutorial.html). The forward and reverse reads were joined and assigned to samples based on the barcode sequences and then truncated by removing the barcode and primer sequences. Quality filtering of the joined sequences was performed and sequences that did not fulfill the following criteria were discarded: sequence length < 200 bp, no ambiguous bases, mean quality score ≥ 20. Qualified sequences were grouped into operational taxonomic units (OTUs) at a 97% sequence identity using the clustering program VSEARCH (Version 1.9.6) against the Silva 119 database (https://www.arb-silva.de/) (Quast et al., 2012). The Ribosomal Database Program (RDP) classifier was used to classify OTUs into taxonomic categories at a confidence threshold of 80% (Wang, Garrity, Tiedje, & Cole, 2007). The Shannon index was determined as an indicator of the species diversity as described previously (Schloss et al., 2009). Bacterial community comparative analysis was performed based on the UniFrac analysis (Lozupone, Hamady, & Knight, 2006), followed by principal coordinate analysis (PCoA) and unweighted pair grouping method with arithmetic mean clustering using QIIME. We explored the 16S rRNA gene novelty of the data set by BLAST identity search against GenBank sequences (searched April 2017). The identity of each single sequence was related both to the closest environmental match (CEM) and to the closest cultured match (CCM) available in GenBank. A cut-off for sequence identity at the species level is generally considered to be 97% (Stackebrandt & Goebel, 1994). Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) for the Windows operating system.

2.5. Accession number

The MiSeq sequencing data were submitted to the Sequence Read Archive (SRA) of the NCBI database as the BioProject ID PRJNA 389631 (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Physicochemical characterization of the PM

The surveyed PM samples covered a wide range of variability in physicochemical concentrations (Table 1). Ranges for pH, moisture, and humic matter were 5.25–6.69, 38.12–45.25%, and 9.9–16.6%, respectively. The concentrations of NH_4^+-N, AP, and AK were 1.88–2.86 g/kg, 0.53–1.28 g/kg, and 2.43–4.18 g/kg, respectively. For organic acids, all samples had a high concentration of caproic acid (6.07–7.96 g/kg), followed by acetic acid (1.46–2.76 g/kg), and butyric acid (1.01–3.38 g/kg). There were not statistically different levels of these physicochemical elements in the different groups ($p < 0.05$, *t*-test), but organic acids were more abundant in the 400-year PM samples compared to the 40-year PM samples (7.18 ± 0.91 g/kg (mean ± SD) vs. 6.34 ± 0.43 g/kg for caproic acid; 2.48 ± 0.05 g/kg vs. 2.10 ± 0.53 g/kg for acetic acid; and 2.17 ± 0.97 g/kg vs.

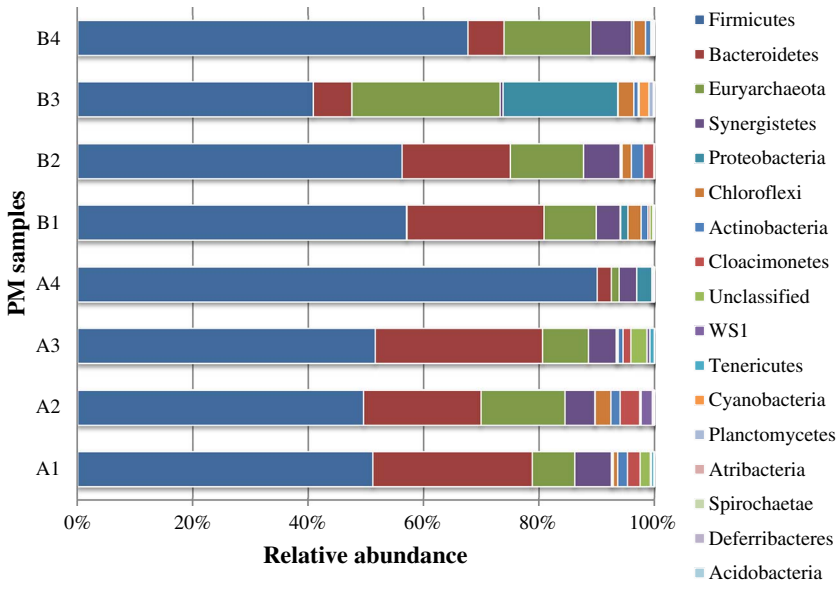

Fig. 2. Taxonomic classifications of bacterial communities at the phylum level in PM samples from cellars in use for 40 and 400 years. The class "Unclassified" refers to the group that could not be precisely assigned to any known taxonomic group at the phylum level.

1.42 ± 0.31 g/kg for butyric acid, respectively).

3.2. The diversity of bacterial community in PM

Sequencing of the PM resulted in 32,033–83,413 qualified sequences for each sample with an average sequence length of 425.72 bp (Table 1). In total, 430 different OTUs (range 231–338 OTUs) with a sampling coverage of ≥ 99.8% were obtained based on 97% sequence identity and 29,269 sequences per sample. The level of coverage indicated that the sequences identified represent a near full of bacterial sequences present in the PM samples. The Shannon indices, providing an estimate of bacterial alpha diversity in each sample, ranged from 4.16–6.14, and were fairly similar in the 2 different groups, with 5.53 ± 0.92 in the 40-year PM samples, and 5.57 ± 0.47 in the 400-year PM samples, suggesting similar overall species diversity. The similarity among the bacterial communities in the PM samples was evaluated using UPGMA cluster analysis (Fig. 1a). This analysis revealed that the bacterial communities could be clustered into 3 groups. Group I included samples A1, A2, A3, B1, B2, and B4. Groups II and III contained only one sample, A4 and B3, respectively. This clustering profile was further supported by PCoA analysis (Fig. 1b). However, there was no clear clustering of PM samples either by UPGMA or PCoA analysis within the groups, indicating significant inter-individual variations in the bacterial communities.

3.3. Phylogenetic structure of the bacterial community in PM

The phylogenetic analysis indicated that all identified bacterial sequences fell within 16 phyla (Fig. 2). The majority of sequences belonged to 8 major phyla (average relative abundance > 1%): Firmicutes, Bacteroidetes, Euryarchaeota, Synergistetes, Proteobacteria, Chloroflexi, Actinobacteria, and Cloacimonetes. Based on the average relative abundance, the most abundant phylum was Firmicutes (58.04%), followed by Bacteroidetes (16.88%), and Euryarchaeota (11.70%). At the genus level, sequences from the PM samples represented 105 different groups (Table S1); the most abundant 30 genera are shown in Fig. 3. We defined dominant genera as those shared by all PM samples and with average relative abundances > 1%. These 11 genera (*Methanobrevibacter*, *Caproiciproducens*, *Petrimonas*, *Lactobacillus*, *Sedimentibacter*, *Proteiniphilum*, *Syntrophomonas*, *Aminobacterium*, *Christensenellaceae* R-7, *Caldicoprobacter*, and *Olsenella*) constituted 45.57% to 58.95% of the total abundance of each PM sample (Fig. 3). Compared with the proportions of the dominant genera in the 40-year PM samples, the proportions of most groups decreased slightly in the 400-year PM samples. However, the top 2, *Methanobrevibacter* and *Caproiciproducens*, increased their average relative abundance by 117.07% (from 6.61% to 14.34%) and 51.42% (from 8.28% to 12.54%), respectively.

3.4. Genetic novelty of the bacterial community in PM

BLAST analysis revealed a total of 112 OTUs that were distantly related to any previously reported cultured counterparts (CCM < 97%) in the GenBank database, suggesting that the PM samples might contain substantial genetic novelty (Fig. 4, Table S2). The dispersion plot of both CCM and CEM values showed that 66% of the novel OTUs were located in the section with CCM < 97% and CEM > 97%, indicating a widespread distribution of similar OTUs that were far from cultured organisms, revealing a significant culturing gap. We selected the 20 most abundant OTUs in this cultured gap section and tracked their ecological distribution using data deposited into GenBank. Interestingly, the closest relatives for the OTUs were recovered from diverse environments, but the most abundant matches were from Chinese Baijiu brewing samples (strong aromatic liquor pit, PM, and yellow water) (Table 2). A group of 38 OTUs located on the section with both CEM and CCM < 97% were considered phylotypes with the highest novelty, which represent species that were either not cultured previously or were not been previously detected in nature. Notably, some OTUs (7) were < 93% similar to all sequences in GenBank, indicating the presence of high-rank novel phylogenetic taxa in the PM samples. Regardless of the abundance, the rare and novel OTUs accounted for 26% of the total (430 OTUs).

Using the RDP classifier, the great majority of the novel OTUs could be assigned to established taxonomic categories at the phylum/class-level (mostly within Firmicutes) (Table 3). At the class level, the most abundant group was Clostridia, with 67 OTUs, followed by Bacteroidia with 11, and Actinobacteria with 5. The novelty pattern plots at the class level were separately analyzed for the 40- and 400-years PM samples, and a highly similar pattern was observed (Fig. 5). The major exceptions were OTU227 and OTU41, specifically detected in the 40-year group, and OTU313, related to Clostridia, was exclusively identified in the 400-year PM samples (Table S2).

4. Discussions

In this study, we conducted MiSeq sequencing to explore the diversity and novelty patterns of PM bacterial communities from Luzhou

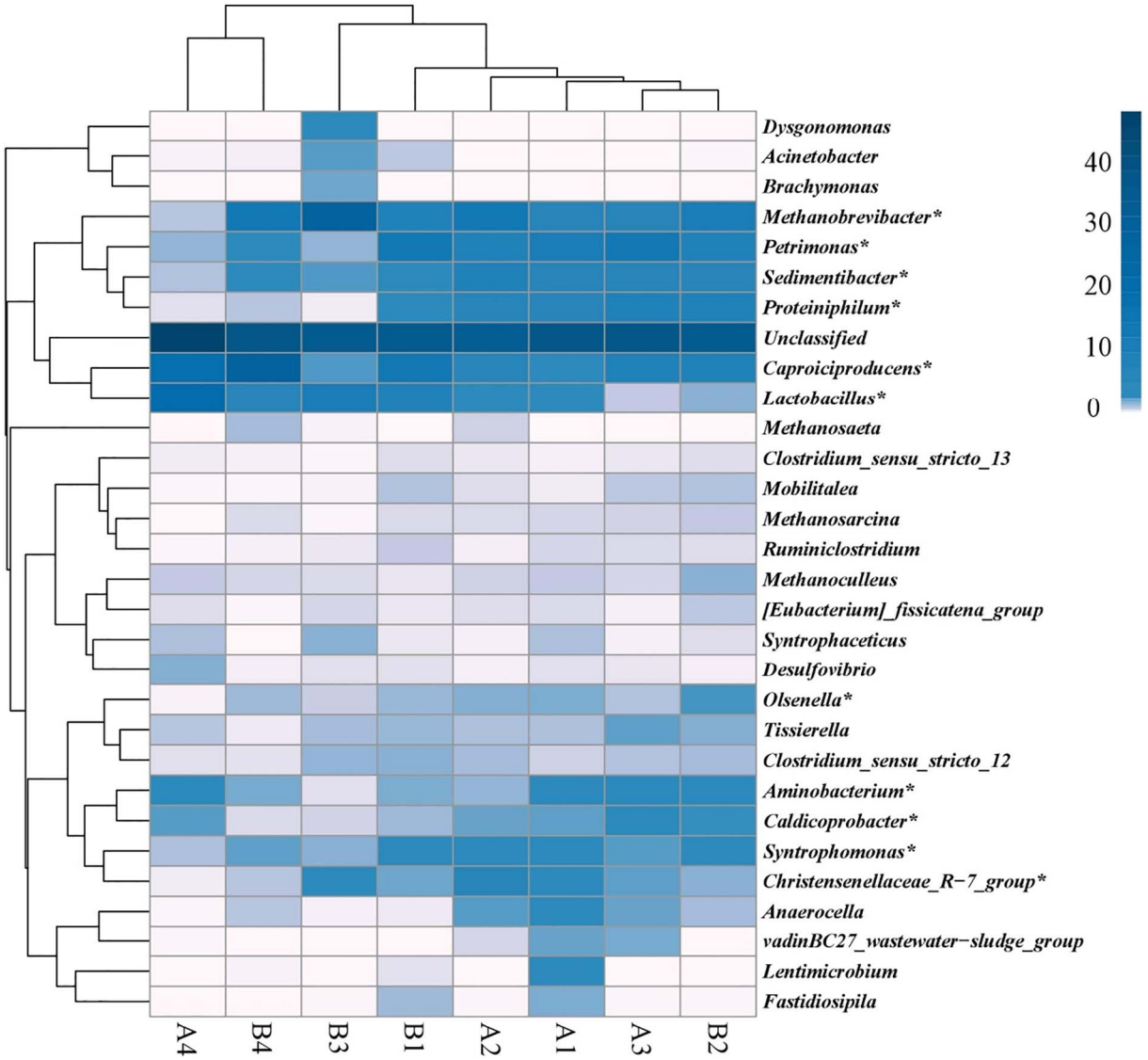

Fig. 3. Relative proportion of the bacterial community at the genus level in PM samples from cellars used for 40 and 400 years. The heat map illustrates the abundance of the top 30 genera, and the *scale bar* shows the variation range of the normalized abundance (%) of the bacterial community. The symbol "*" is used to indicate genera shared by all PM samples and with average relative abundances > 1%.

Laojiao cellars in use for 40 and 400 years. A previous study reported bacterial sequences from 2 individual PM samples of different ages from Luzhou Laojiao cellars, and found a significantly higher diversity index in the sample from the older cellar (Zheng et al., 2015). However, our study used multiple samples (4 cellars for each cellar age) and our analysis did not reveal significant differences in Shannon index values due to significant inter-individual variations in each group. These conflicting results suggest that a more complex PM microbial structures of the cellars than previously thought (Zheng et al., 2015). Nevertheless, it is worth mentioning that the results presented in this study comprise, to our knowledge, the first assessment of the genetic novelty of microbial communities in PM. Thus, it is clear that this study represents a more comprehensive investigation of PM bacterial diversity in the cellars used in the production of CSFB.

The most abundant phylum in the PM was Firmicutes, followed by Bacteroidetes and Euryarchaeota. The high rank diversity observed in this study is in agreement with that estimated from the previously studied PM (Ding, Wu, Zhang, Zheng, & Zhou, 2014; Hu et al., 2016; Huang, Xiong, Hu, Liang, & Zhao, 2017; Tao et al., 2014). However, compared with other PM datasets that determined previously by NGS analysis (Huang et al., 2017; Tao et al., 2014), we found that the compositions of dominant genera changed markedly between geographic sites. For example, a recent study identified *Sphingobacterium*, *Acetobacter*, *Alcaligenes*, and *Acinetobacter* as prevalent genera in PM sampled from Baiyunbian (a famous Baijiu manufacturer in Hubei province of China) (Huang et al., 2017), significantly different from those observed in the PM sampled from a Baijiu manufacturer located in Mianzhu, Sichuan province (Tao et al., 2014). Although sequences from *Sphingobacterium*, *Acetobacter*, *Alcaligenes*, and *Acinetobacter* were retrieved in our survey, together they only represented 0.46% of the total bacterial abundance. More interestingly, of the dominant genera identified herein, only *Lactobacillus* was highly expressed in both previous studies (Huang et al., 2017; Tao et al., 2014). The differential dominance of the PM bacterial groups in different studies could be explained by a combination of methodological and environmental factors. However, previous studies showed that the 16S rRNA fingerprinting of PM bacterial communities was significantly correlated with geographic sites (Huang et al., 2015; Liang et al., 2016), indicating geographical restriction of microbial distribution. Indeed, CSFB is produced in an open environment (Jin et al., 2017), and the microbial community of the starting inoculum (*Daqu* starter) can be greatly affected by environmental factors (Li et al., 2016). Microbes in this

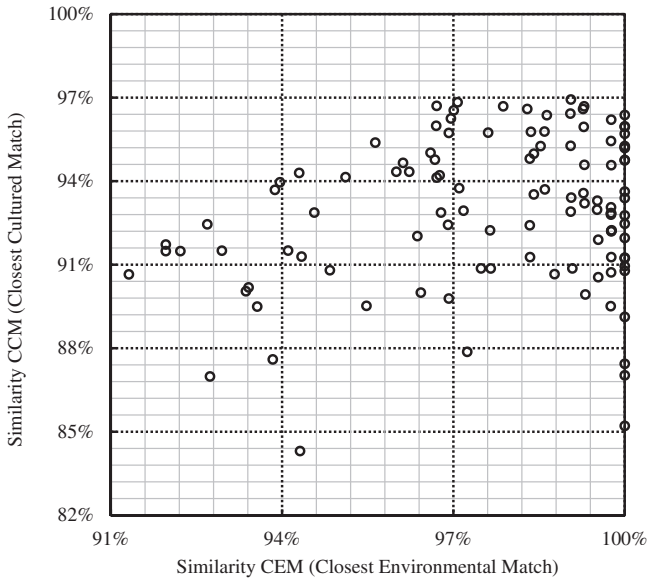

Fig. 4. Dispersion plot of the CEM and CCM similarities for the novel OTUs retrieved from PM samples in the cellars in use for 40 and 400 years.

Table 3
Phylum/class-level novelty distribution for the novel OTUs found in investigated PM samples, following their abundance.

Taxonomic group	Number of sequences	Number of OTUs	Abundances (%)
Unclassified	1632	8	0.697
Firmicutes			
Unclassified	9976	8	4.26
Clostridia	5422	67	2.316
Erysipelotrichia	61	2	0.026
Bacteroidetes			
Unclassified	307	2	0.131
Bacteroidia	2176	11	0.929
Actinobacteria			
Actinobacteria	207	5	0.088
Synergistetes			
Synergistia	1343	3	0.574
Chloroflexi			
Anaerolineae	277	2	0.118
Proteobacteria			
Alphaproteobacteria	70	2	0.03
Cyanobacteria			
Cyanobacteria	478	1	0.204
Planctomycetes			
Planctomycetacia	15	1	0.006

starting inoculum for grain fermentation can migrate into the PM, so different geographic sites may provide unique climate environments to select specifically adapted microbes for CSFB fermentation. Further studies are required to better understand the relationship between the PM microbial community and environmental conditions, but the data presented here suggest that the microbial diversity of PM is far broader than our current database collection suggests.

The known metabolic properties of the dominant bacteria identified in our study provide insights into roles in CSFB production. In general, the genera of *Caproiciproducens*, *Petrimonas*, *Lactobacillus*, *Proteiniphilum*, *Christensenellaceae*, *Caldicoprobacter*, and *Olsenella* produce organic acids (e.g., caproic, lactic, acetic, and butyric acid), ethanol, CO_2, and H_2 as the major end products of carbohydrate fermentation (Bouananedarenfed et al., 2011; Grabowski, Tindall, Bardin, Blanchet, & Jeanthon, 2005; Hahnke, Langer, Koeck, & Klocke, 2016; Kim et al., 2015; Li, Jensen, Højberg, Canibe, & Jensen, 2015; Morotomi, Nagai, & Watanabe, 2012; Stiles & Holzapfel, 1997). *Sedimentibacter* and *Aminobacterium* ferment amino acids to acetic and butyric acid (Hamdi et al., 2015; Imachi et al., 2016). *Syntrophomonas* can degrade long-chain fatty acids into acetic acid and H_2 (Crable et al., 2016). *Methanobrevibacter* is known as a hydrogenotrophic methanogen, and can produce CH_4 from H_2/CO_2 (Rea, Bowman, Popovski, Pimm, & Wright, 2007). The representative aromatic compounds of CSFB are predominantly ethyl caproate balanced with ethyl lactate, ethyl acetate, and ethyl butyrate (Zheng & Han, 2016). The prevalence of these dominant bacteria in the PM highlights their dominant roles in organic matter degradation and supplying soluble substrates for aromatic ester formation and the methanogenesis that occurs during CSFB production.

The genera of *Methanobrevibacter* and *Caproiciproducens* are of great interest because they are most highly represented in the PM samples, and apparently, they increased as a proportion of the total microbes with cellar age. Interestingly, previous studies have shown that methanogens, together with fermentative bacteria, can enhance organic

Table 2
The 20 most abundant OTUs in the cultured gap section retrieved from the investigated PM samples, with their abundance (%), occurrence, taxonomy, and similarity (%-S) with the CCM and the CEM.

OTU ID	Number of sequences	Abundance	Occurrence	Taxonomy	CCM	%-S	CEM	%-S	Isolation source
9	7578	3.24	8	Firmicutes	NR108634	92.77	JX576081	100.00	PM
28	1213	0.52	7	Bacteroidetes	DQ278861	91.23	AB818640	100.00	PM
29	1198	0.51	8	Synergistetes	NR113331	95.23	JX576077	100.00	PM
41	1045	0.45	4	Bacteria	KC854351	91.27	KJ734842	99.76	Yellow water[a]
84	975	0.42	6	Firmicutes	NR115800	90.78	KU656398	100.00	Anaerobic reactor
70	722	0.31	8	Firmicutes	EU481702	93.06	AB818592	99.76	PM
75	656	0.28	8	Firmicutes	NR115693	92.20	JX575838	99.76	PM
76	648	0.28	7	Firmicutes	DQ882650	96.21	JX575871	99.76	PM
59	565	0.24	7	Bacteroidetes	KP233808	95.95	KM250996	100.00	Biochemical reactor
102	434	0.19	8	Firmicutes	AF550610	90.73	JX575979	99.76	PM
91	388	0.17	7	Firmicutes	NR118109	95.70	JX575915	100.00	PM
67	381	0.16	8	Firmicutes	FJ808605	96.69	JX575953	99.29	PM
104	355	0.15	8	Firmicutes	NR074915	89.13	AB699890	100.00	Strong aromatic liquors pit
61	346	0.15	8	Bacteria	LN907772	92.22	KU667179	99.76	Anaerobic digestion reactor
93	277	0.12	8	Firmicutes	KC331170	92.80	HQ224818	99.76	Anaerobic baffled reactor
163	260	0.11	8	Firmicutes	NR115693	91.96	JX575967	100.00	PM
121	199	0.08	8	Firmicutes	KC331180	92.98	KC110472	99.52	Rhizosphere of constructed wetland
213	198	0.08	8	Firmicutes	AB910750	95.97	KU656397	100.00	Anaerobic reactors
95	197	0.08	8	Bacteroidetes	KT149222	87.03	KT167048	100.00	Food waste digestion
218	189	0.08	7	Firmicutes	JN874874	95.26	JX575945	100.00	Faeces of *Homo sapiens*

[a] Yellow water: a byproduct formed during Baijiu brewing.

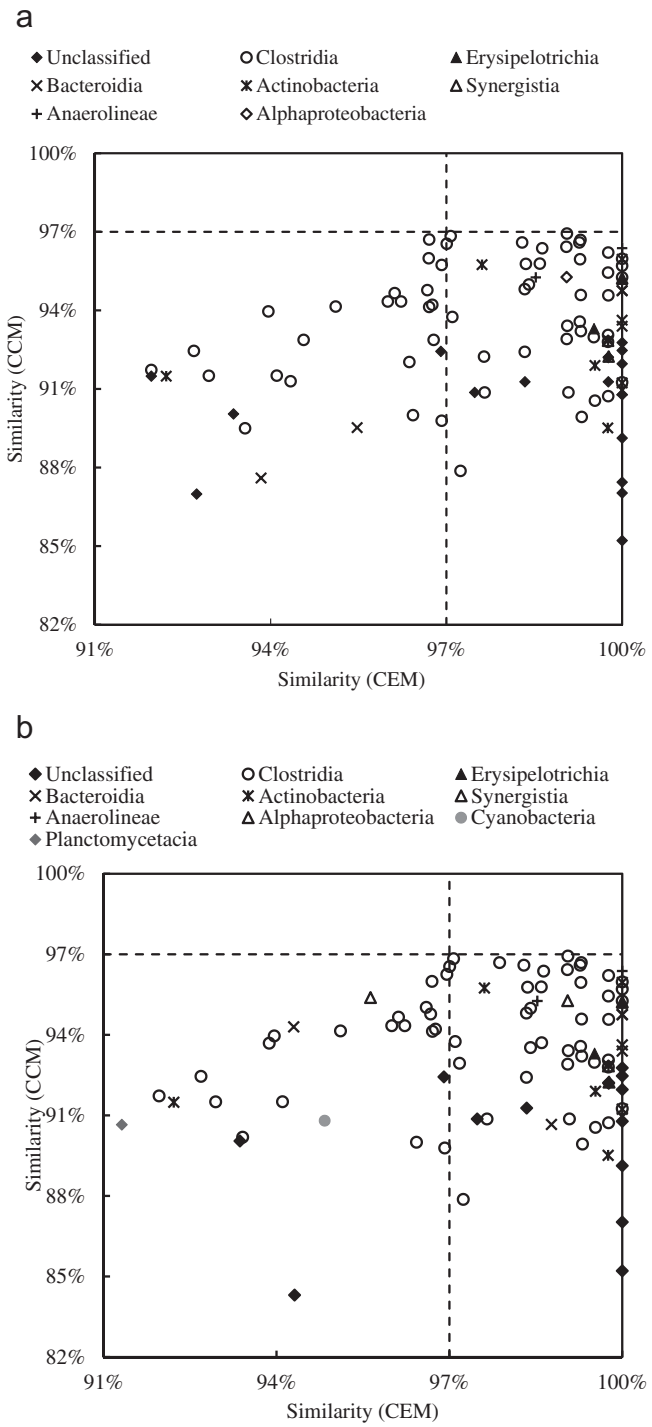

Fig. 5. Novelty pattern plots for the different taxa at the class level found in the PM samples in the cellars in use for 40(a) and 400 (b) years.

acid production through syntrophic interactions (Felchner-Zwirello, Winter, & Gallert, 2013), thus the co-occurrence of high abundances of *Methanobrevibacter* and *Caproiciproducens* may indicate efficient caproic acid production. This finding corresponds to the highest caproic acid level in the PM samples found in this study and an increased caproic acid level in the 400-year PM samples. Ethyl caproate, produced from the esterification of caproic acid and ethanol, is a key component affecting CSFB quality and higher levels of ethyl caproate enhance this quality (Xu, Wang, Fan, Mu, & Chen, 2009). The microbial community structure shift may therefore explain why an older cellar is important for high quality CSFB production (Zheng et al., 2013). More importantly, in the Chinese Baijiu industry, an efficient approach to increase CSFB quality is to increase the content of ethyl caproate by screening and utilizing caproic acid-producing bacteria (Liu, Tang, Zhao, Liu, et al., 2017; Xu et al., 2009). To our knowledge, this identification of *Caproiciproducens* is the first reported for Chinese Baijiu production, and further studies are required to verify the presence of this beneficial bacterium in PM and to determine its contribution to production.

The BLAST analysis revealed that the PM harbors a high degree of genetic novelty in the 16S sequences, and most sequences were identified as Clostridia and Bacteroidia. The presence of Clostridia is of great interest as these are essential microbes active in Chinese Baijiu production, responsible for the formation of various aromatic compounds (e.g., organic acids, alcohols, and phenols). Many Clostridia species have been previously isolated in pure culture from PM (Hu, Du, & Xu, 2015; Liu, Du, Wang, & Xu, 2017; Liu et al., 2014; Xue & Xue, 2016; Yin et al., 2016). However, our data suggests that the system apparently contains both abundant and new taxa that are substantially different from any other pure culture previously characterized. This case highlights the need for full classification of the microbial species in PM. Furthermore, we found that most of the novel OTUs placed in the cultured gap section were previously identified in Chinese Baijiu brewing ecosystems. According to Lynch and Neufeld (2015), the presence of rare taxa in an ecosystem can reflect environmental selection and rare taxa may serve as reservoirs of functional diversity. Therefore, the recurrent presence of these rare and novel OTUs in our dataset may suggest their importance in CSFB production. The discrepancy between the microbes retrieved from environment by cultivation and molecular methods is frequently attributed to the insufficiency of suitable conditions to recover microbes from natural settings. However, some low abundance microbes can become dominant under a favourable environment. For example, a recent study using a combination of NGS and cultivation methods explored members of rare groups from a deep-sea coral reef community, and all of the isolates obtained were rare taxa, based on the corresponding NGS data (Jensen, Lynch, Ray, Neufeld, & Hovland, 2014). In the past decades, new approaches and methodological improvements allowed the identification of thousands of 16S RNA sequences now deposited in GenBank. However, 38 OTUs in this study had a very low similarity to both CCM and CEM, and thus the system may represent a unique community for the identification of novel taxa associated with CSFB production. The challenge now is to isolate these microbes in pure culture for detailed ecophysiological studies.

5. Conclusions

This study conducted MiSeq sequencing to explore the diversity and novelty patterns of PM bacterial communities from Luzhou Laojiao cellars in use for 40 and 400 years. The results indicated that bacterial communities of the PM are highly diverse and 11 dominant genera were identified with species that may play important roles in CSFB production. As far as we know, the high proportion in the PM of *Caproiciproducens*, a caproic acid-producing bacterium, was not reported previously. The relative abundance of *Caproiciproducens* also increased significantly in the 400-year PM sample. This community structure shift may explain why an older cellar is important for high quality CSFB production. Also, we observed a large number of novel taxa in the PM samples, showing that a large fraction of the bacterial diversity must still be fully characterized. Although the function of these microbes remains unclear, our survey of the ecological distribution of 20 abundant OTUs suggested that many may be ecologically important for CSFB production. Therefore, further investigation will be required to develop cultivation methods to identify new unseen diversity and to learn more about the ecophysiology of these microbes and their roles in CSFB fermentation.

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Appendix A. Supplementary data

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References

Bouananedarenfed, A., Fardeau, M. L., Grégoire, P., Joseph, M., Kebbouchegana, S., Benayad, T., ... Ollivier, B. (2011). *Caldicoprobacter algeriensis* sp. nov. a new thermophilic anaerobic, xylanolytic bacterium isolated from an Algerian hot spring. *Current Microbiology, 62*(3), 826–832.

Chen, X. R., Shao, C. B., Wang, Y. W., He, M. X., Ma, K. D., Wang, H. M., ... Ruan, Z. Y. (2015). *Paenibacillus vini* sp. nov., isolated from alcohol fermentation pit mud in Sichuan Province, China. *Antonie Van Leeuwenhoek, 107*(6), 1429–1436.

Crable, B. R., Sieber, J. R., Mao, X. W., Alvarez-Cohen, L., Gunsalus, R., Loo, R. R. O., ... Mcinerney, M. J. (2016). Membrane complexes of *Syntrophomonas wolfei* involved in syntrophic butyrate degradation and hydrogen formation. *Frontiers in Microbiology, 7*, 1795.

Ding, X. F., Wu, C. D., Huang, J., & Zhou, R. Q. (2015). Interphase microbial community characteristics in the fermentation cellar of Chinese Luzhou-flavor liquor determined by PLFA and DGGE profiles. *Food Research International, 72*, 16–24.

Ding, X. F., Wu, C. D., Zhang, L. Q., Zheng, J., & Zhou, R. Q. (2014). Characterization of eubacterial and archaeal community diversity in the pit mud of Chinese Luzhou-flavor liquor by nested PCR-DGGE. *World Journal of Microbiology and Biotechnology, 30*(2), 605–612.

Felchner-Zwirello, M., Winter, J., & Gallert, C. (2013). Interspecies distances between propionic acid degraders and methanogens in syntrophic consortia for optimal hydrogen transfer. *Applied Microbiology and Biotechnology, 97*(20), 9193–9205.

Grabowski, A., Tindall, B. J., Bardin, V., Blanchet, D., & Jeanthon, C. (2005). *Petrimonas sulfuriphila* gen. nov., sp. nov., a mesophilic fermentative bacterium isolated from a biodegraded oil reservoir. *International Journal of Systematic and Evolutionary Microbiology, 55*(3), 1113–1121.

Hahnke, S., Langer, T., Koeck, D. E., & Klocke, M. (2016). Description of *Proteiniphilum saccharofermentans* sp. nov., *Petrimonas mucosa* sp. nov. and *Fermentimonas caenicola* gen. nov., sp. nov. isolated from mesophilic lab-scale biogas reactors and emended description of the genus *Proteiniphilum*. *International Journal of Systematic and Evolutionary Microbiology, 66*(3), 1466–1475.

Hamdi, O., Ben, H. W., Postec, A., Bouallagui, H., Hamdi, M., Bonin, P., ... Fardeau, M. L. (2015). *Aminobacterium thunnarium* sp. nov., a mesophilic, amino acid-degrading bacterium isolated from an anaerobic sludge digester, pertaining to the phylum *Synergistetes*. *International Journal of Systematic and Evolutionary Microbiology, 65*(2), 609–614.

Hu, X. L., Du, H., Ren, C., & Xu, Y. (2016). Illuminating anaerobic microbial community and cooccurrence patterns across a quality gradient in Chinese liquor fermentation pit muds. *Applied and Environmental Microbiology, 82*(8), 2506–2515.

Hu, X. L., Du, H., & Xu, Y. (2015). Identification and quantification of the caproic acid-producing bacterium *Clostridium kluyveri* in the fermentation of pit mud used for Chinese strong-aroma type liquor production. *International Journal of Food Microbiology, 214*, 116–122.

Huang, Y. N., Xiong, X. M., Hu, Y. L., Liang, Y. X., & Zhao, S. M. (2017). Bacterial community and diversity in pit mud of Baiyunbian liquor analyzed by PCR-DGGE and high-throughput sequencing. *Microbiology China, 44*(2), 375–383.

Huang, Z. G., Wang, Y. L., Wei, C. H., Liu, Y. M., & Li, M. (2015). Diversity of bacteria community structure in pit mud from different regions and analysis of its correlations with physicochemical factors. *Liquor-Making Science and Technology, 11*, 18–25.

Imachi, H., Sakai, S., Kubota, T., Miyazaki, M., Saito, Y., & Takai, K. (2016). *Sedimentibacter acidaminivorans* sp. nov., an anaerobic, amino acids-utilizing bacterium isolated from marine subsurface sediment. *International Journal of Systematic and Evolutionary Microbiology, 66*(3), 1293–1300.

Jensen, S., Lynch, M. D., Ray, J. L., Neufeld, J. D., & Hovland, M. (2014). Norwegian deep-water coral reefs: Cultivation and molecular analysis of planktonic microbial communities. *Environmental Microbiology, 17*(10), 3597–3609.

Jin, G. Y., Zhu, Y., & Xu, Y. (2017). Mystery behind Chinese liquor fermentation. *Trends in Food Science and Technology, 63*, 18–28.

Kim, B. C., Jeon, B. S., Kim, S. I., Kim, H., Um, Y., & Sang, B. I. (2015). *Caproiciproducens galactitolivorans* gen. nov. sp. nov., a bacterium capable of producing caproic acid from galactitol, isolated from a wastewater treatment plant. *International Journal of Systematic and Evolutionary Microbiology, 65*(12), 4902–4908.

Li, P., Lin, W. F., Liu, X., Wang, X. W., & Luo, L. X. (2016). Environmental factors affecting microbiota dynamics during traditional solid-state fermentation of Chinese daqu starter. *Frontiers in Microbiology, 7*(253), 1237.

Li, X. Q., Jensen, R. L., Højberg, O., Canibe, N., & Jensen, B. B. (2015). *Olsenella scatoligenes* sp. nov., a 3-methylindole (skatole) and 4-methylphenol (*p*-cresol) producing bacterium isolated from pig faeces. *International Journal of Systematic and Evolutionary Microbiology, 65*(4), 1227–1233.

Liang, H. P., Luo, Q. C., Zhang, A., Wu, Z. Y., & Zhang, W. X. (2016). Comparison of bacterial community in matured and degenerated pit mud from Chinese Luzhou-flavour liquor distillery in different regions. *Journal of the Institute of Brewing, 122*(1), 48–54.

Liu, B., Du, H., Wang, X. S., & Xu, Y. (2017). Detecting source of *p*-cresol in strong flavor Chinese liquor by high throughput sequencing. *Microbiology China, 44*(1), 108–117.

Liu, C. L., Huang, D., Liu, L. Y., Zhang, J., Deng, Y., Chen, L., ... Dai, L. R. (2014). *Clostridium swellfunianum* sp. nov., a novel anaerobic bacterium isolated from the pit mud of Chinese Luzhou-flavor liquor production. *Antonie Van Leeuwenhoek, 106*(4), 817–825.

Liu, M. K., Tang, Y. M., Zhao, K., Gu, Y. F., Ren, D. Q., Yao, W. C., ... Zhang, X. P. (2017). Recent advances in research on the community, isolation, and application of microbes in the pit mud used in manufacture of Chinese Strong-flavor Baijiu. *Microbiology China, 44*(5), 1222–1229.

Liu, M. K., Tang, Y. M., Zhao, K., Liu, Y., Guo, X. J., Ren, D. Q., ... Zhang, X. P. (2017). Determination of the fungal community of pit mud in fermentation cellars for Chinese strong-flavor liquor, using DGGE and Illumina MiSeq sequencing. *Food Research International, 91*, 80–87.

Liu, M. K., Tang, Y. M., Zhao, K., Ren, D. Q., Yao, W. C., & Tian, X. H. (2015). Analysis of actinobacteria community and diversity in the pit mud of Chinese luzhou-flavour liquor. *Acta Ecologica Sinica, 35*(3), 858–864.

Liu, M. K., Zhao, K., Tang, Y. M., Ren, D. Q., Yao, W. C., Tian, X. H., ... Deng, B. (2015). Analysis of *Clostridium* cluster I community diversity in pit mud used in manufacture of Chinese Luzhou-flavor liquor. *Food Science and Biotechnology, 24*(3), 995–1000.

Lozupone, C., Hamady, M., & Knight, R. (2006). UniFrac—an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics, 7*(1), 371.

Lynch, M. D. J., & Neufeld, J. D. (2015). Ecology and exploration of the rare biosphere. *Nature Reviews Microbiology, 13*(4), 217–229.

Ma, K. D., Chen, X. R., Guo, X., Wang, Y. W., Wang, H. M., Zhou, S., ... Ruan, Z. Y. (2016). *Bacillus vini* sp. nov. isolated from alcohol fermentation pit mud. *Archives of Microbiology, 198*(6), 559–564.

Mehlich, A. (1984). Photometric determination of humic matter in soils, a proposed method. *Communications in Soil Science and Plant Analysis, 15*(12), 1417–1422.

Morotomi, M., Nagai, F., & Watanabe, Y. (2012). Description of *Christensenella minuta* gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order *Clostridiales*, and proposal of *Christensenellaceae* fam. nov. *International Journal of Systematic and Evolutionary Microbiology, 62*(1), 144–149.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... Glockner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*(D1), D590–D596.

Rea, S., Bowman, J. P., Popovski, S., Pimm, C., & Wright, A. D. (2007). *Methanobrevibacter millerae* sp. nov. and *Methanobrevibacter olleyae* sp. nov., methanogens from the ovine and bovine rumen that can utilize formate for growth. *International Journal of Systematic and Evolutionary Microbiology, 57*(3), 450–456.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., ... Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology, 75*(23), 7537–7541.

Shen, Y. F. (2007). *Handbook of Chinese liquor making technology* (1st ed). Beijing: China Light Industry Press.

Song, S. Y. (2016). 2015 Annual working report of Baijiu branch, market committee, famous Baijiu collection committee, and design and equipment committee of China alcoholic drinks association. *Liquor-Making Science and Technology, 6*, 17–28.

Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in Bacteriology. *International Journal of Systematic and Evolutionary Microbiology, 44*(4), 846–849.

Stiles, M. E., & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology, 36*(1), 1–29.

Sun, W. N., Xiao, H. Z., Peng, Q., Zhang, Q. G., Li, X. X., & Han, Y. (2016). Analysis of bacterial diversity of Chinese Luzhou-flavor liquor brewed in different seasons by Illumina Miseq sequencing. *Annals of Microbiology, 66*(3), 1293–1301.

Tao, Y., Li, J. B., Rui, J. P., Xu, Z. C., Zhou, Y., Hu, X. H., ... Li, X. Z. (2014). Prokaryotic communities in pit mud from different-aged cellars used for the production of Chinese strong-flavored liquor. *Applied and Environmental Microbiology, 80*(7), 2254–2260.

Teachcn. Top ten brands of wine in China. (2011). http://www.teachcn.com/Content.asp?id=733 (Accessed 17.06.19).

Wang, M. Y., & Zhang, W. X. (2014). Analysis of microbial community structure in pit mud from two Chinese *Luzhou-flavor* liquor producing areas. *Microbiology China, 41*(8), 1498–1506.

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology, 73*(16), 5261–5267.

Xiong, Y., Cheng, Q., Tang, Y. M., Wu, Y. Y., Ren, D. Q., & Chen, C. P. (2013). PCR-DGGE analysis of Lactobacillus diversity in pit muds of different cellar-aged Luzhou Laojiao liquor. *Chinese Journal of Applied & Environmental Biology, 19*(6), 1020–1024.

Xu, Y., Wang, D., Fan, W. L., Mu, X. Q., & Chen, J. (2009). Traditional Chinese biotechnology. In G. T. Tsao, P. Ouyang, & J. Chen (Eds.). *Biotechnology in China II: Chemicals, energy and environment* (pp. 189–233). Berlin, Heidelberg: Springer Berlin Heidelberg.

Xu, Y. Q., Sun, B. G., Fan, G. S., Teng, C., Xiong, K., Zhu, Y. P., ... Li, X. T. (2017). The brewing process and microbial diversity of strong flavour Chinese spirits: A review. *Journal of the Institute of Brewing, 123*(1), 5–12.

Xue, Z. K., & Xue, Y. (2016). Polyphasic identification of a hexanoic acid bacteria strain from the pit mud and study on its acid producing ability. *Liquor-Making Science and Technology, 6*, 65–71.

Yang, J. Z., Tang, Y., Yang, Q. S., Liu, B. H., & Zhao, X. L. (2014). Climate environment characteristics for producing high quality Luzhou-flavor liquor in Weihai of Shandong province. *Food and Fermentation Technology, 20*(2), 67–71.

Yao, F., Yi, B., Shen, C. H., Tao, F., Liu, Y. M., Lin, Z. X., & Xu, P. (2015). Chemical analysis of the Chinese liquor Luzhoulaojiao by comprehensive two-dimensional gas chromatography/time-of- flight mass spectrometry. *Scientific Reports, 5*, 9553.

Yin, Q., Tao, Y., Zhu, X. Y., Zhou, Y., He, X. H., Cheng, L., ... Li, D. P. (2016). *Clostridium liquoris* sp. nov., isolated from a fermentation pit used for the production of Chinese Strong-flavored liquor. *International Journal of Systematic and Evolutionary Microbiology, 66*(2), 749–754.

Yuan, Y. J., Zhang, Q. Y., Zeng, L. Y., & Zhang, W. X. (2017). Analysis of bacterial community and acid, ester contents in pit mud with different characteristics. *Food and Fermentation Industries, 43*(1), 44–48.

Zhao, J. S., Zhang, S. Y., & Zhou, Z. H. (2009). The historic and cultural traceability of the traditional brewing technology of Luzhoulaojiao. *Food and Fermentation Technology, 45*(4), 6–8.

Zheng, J., Liang, R., Zhang, L. Q., Wu, C. D., Zhou, R. Q., & Liao, X. P. (2013). Characterization of microbial communities in strong aromatic liquor fermentation pit muds of different ages assessed by combined DGGE and PLFA analyses. *Food Research International, 54*(1), 660–666.

Zheng, Q., Lin, B. R., Wang, Y. B., Zhang, Q. P., He, X. X., Yang, P., ... Huang, X. H. (2015). Proteomic and high-throughput analysis of protein expression and microbial diversity of microbes from 30-and 300-year pit muds of Chinese Luzhou-flavor liquor. *Food Research International, 75*, 305–314.

Zheng, X. F., & Han, B. Z. (2016). Baijiu (白酒), Chinese liquor: History, classification and manufacture. *Journal of Ethnic Foods, 3*(1), 19–25.