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Prokaryotic Communities in Pit Mud from Different-Aged Cellars Used for the Production of Chinese Strong-Flavored Liquor

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Chinese strong-flavored liquor (CSFL) accounts for more than 70% of all Chinese liquor production. Microbes in pit mud play key roles in the fermentation cellar for the CSFL production. However, microbial diversity, community structure, and cellar-age-related changes in pit mud are poorly understood. Here, we investigated the prokaryotic community structure and diversity in pit-mud samples with different cellar ages (1, 10, 25, and 50 years) using the pyrosequencing technique. Results indicated that prokaryotic diversity increased with cellar age until the age reached 25 years and that prokaryotic community structure changed significantly between three cellar ages (1, 10, and 25 years). Significant correlations between prokaryotic communities and environmental variables (pH, NH_4^+, lactic acid, butyric acid, and caproic acid) were observed. Overall, our study results suggested that the long-term brewing operation shapes unique prokaryotic community structure and diversity as well as pit-mud chemistry. We have proposed a three-phase model to characterize the changes of pit-mud prokaryotic communities. (i) Phase I is an initial domestication period. Pit mud is characterized by abundant *Lactobacillus* and high lactic acid and low pH levels. (ii) Phase II is a transition period. While *Lactobacillus* abundance decreases dramatically, that of *Bacteroidetes* and methanogens increases. (iii) Phase III is a relative mature period. The prokaryotic community shows the highest diversity and capability to produce more caproic acid as a precursor for synthesis of ethyl caproate, the main flavor component in CSFL. This research provides scientific evidence to support the practical experience that old fermentation cellars produce high-quality liquor.

Chinese strong-flavored liquor (CSFL), also called "Luzhou flavor liquor," accounts for more than 70% of Chinese liquor production (1). It is produced by the unique and traditional Chinese solid-state fermentation technique, which has a history of several thousand years. In brief, a cellar is constructed by digging a rectangular soil pit in which the entire inner wall is covered with precultured pit mud. The precultured pit mud is usually prepared by mixing aged pit mud (as an inoculum), fresh common soil, and water and incubating the mixture for about a year in an anaerobic cellar before use. The raw materials for the fermentation, including wheat, sorghum, and corn, are mixed, crushed, and distilled by steaming. The steamed raw material is supplied with 2% to 3% (wt/wt) Daqu-starter, which mainly includes mold and yeast, and placed into the cellar. The cellar is sealed with common mud, and fermentation is allowed to proceed for 60 days. Fermented material is then taken out of the cellar and distilled to make Chinese liquor. The process described above is periodically repeated after new fermentation materials are supplied.

Microbes in the pit mud produce various flavor components such as butyric acid, caproic acid, and ethyl caproate. In particular, ethyl caproate is recognized as a key component affecting the CSFL flavor and quality. In general, CSFL quality improves with increasing cellar age. High-quality liquor is produced only in old cellars, which are maintained at least for 20 years by continuous use (2, 3). In particular, some long-aged cellars have been used for several hundred years without interruption, and well-known CSFLs such as Wuliangye, Jiannanchun, and Luzhoulaojiao are brewed in such long-aged cellars (1, 4). High CSFL quality is attributed to the maturing process of pit mud, which results in a well-balanced microbial community structure and diversity in the pit mud to produce distinctive flavors.

The pit mud acts as a source of inoculum and a habitat of microbes in the brewing fermentation cellar for the CSFL production. Previously, the pit-mud microbiota had been studied using cultivation-dependent and -independent approaches. Members of *Clostridium*, *Bacillus*, and *Sporolactobacillus* genera were isolated and identified from the pit-mud samples (5, 6). Members of six bacterial phyla, including *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, unclassified *Bacteria*, and *Proteobacteria*, and of one archaeal phylum, *Euryarchaeota*, were identified from the pit-mud samples using denaturing gradient gel electrophoresis (DGGE) and clone library analysis of the 16S rRNA gene (7, 8). However, big discrepancies in microbial composition existed among previous investigations. This may be attributable to differences in sampling locations in a pit, cellar ages, and laboratory techniques employed to characterize the community structure. In addition, most of the previous studies on the pit-mud microbiota using traditional cultural and molecular methods cannot provide details of the phylogenetic composition and cellar-age-related changes of the pit-mud microbial community. Without such in-

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TABLE 1 Chemical properties in the pit mud

Variable	Values for cellar of age (yr)[a]: 1	10	25	50
pH	3.57 ± 0.06[a]	5.00 ± 0.78[ac]	5.51 ± 0.64[bc]	5.81 ± 0.33[bc]
NH_4^+ (g kg^{-1})	1.86 ± 0.06[a]	2.70 ± 0.47[ac]	4.21 ± 0.67[b]	3.55 ± 0.68[bc]
Moisture (%)	43.3 ± 1.9[a]	44.5 ± 2.9[a]	45.1 ± 2.5[a]	44.1 ± 5.0[a]
Humic matter (%)	30.1 ± 3.6[a]	26.4 ± 1.2[a]	24.9 ± 7.3[a]	21.4 ± 5.6[b]
TN (%)	16.0 ± 0.6[a]	16.8 ± 2.0[a]	18.1 ± 2.1[a]	16.2 ± 1.7[a]
Acetic acid (g kg^{-1})	4.13 ± 0.63[a]	2.94 ± 0.70[a]	4.37 ± 0.98[a]	3.98 ± 0.86[a]
Butyric acid (g kg^{-1})	2.89 ± 0.20[a]	1.77 ± 0.1[b]	2.02 ± 0.63[b]	1.63 ± 0.24[b]
Caproic acid (g kg^{-1})	5.79 ± 0.72[a]	6.51 ± 3.35[a]	20.0 ± 8.66[b]	15.2 ± 7.7[b]
Lactic acid (g kg^{-1})	166.5 ± 12.5[a]	59.1 ± 21.3[b]	26.2 ± 10.3[b]	29.8 ± 4.5[b]

[a] All data are presented as means ± standard deviations ($n = 5$). Values with different letters in a row mean significant differences at $P < 0.05$ as determined by ANOVA.

formation, the specific microbes controlling the CSFL quality cannot be identified.

In this study, we investigated prokaryotic communities in the pit mud from different-aged cellars used for CSFL production with the 16S rRNA gene pyrosequencing technique. Pit-mud samples were collected from a famous CSFL manufacturer located in Sichuan province, China. The aims were to (i) characterize prokaryotic community structure and diversity and their cellar-age-related changes in the pit mud, (ii) identify important prokaryotic populations in the maturing process of pit mud and their possible roles in the CSFL quality, and (iii) unveil the relationships between chemical properties of pit mud and prokaryotic community structure and diversity.

MATERIALS AND METHODS

Sample collection. Pit mud samples were collected from a famous brewing manufacturer located in Mianzhu city, Sichuan province, China. We selected cellars used for 1, 10, 25, and 50 years for sampling. Five cellars were selected for each cellar age. Triplicate samples were collected in each cellar. Thus, 60 samples were obtained from 20 cellars representing four ages. After sequencing, we found that prokaryotic communities were similar among triplicate samples from the same cellar. Thus, sequencing data of triplicate samples from the same cellar were merged and used for the downstream analysis. Samples were transferred to the laboratory on ice and kept at −80°C.

Chemical property analysis. Pit-mud moisture was determined with a gravimetric method by drying soils at 60°C for 48 h immediately after the sampling. The pH was measured by a pH meter in the slurry, with a 1:5 ratio of pit mud to deionized water. Total nitrogen (TN) was determined with the Kjeldahl method (9). NH_4^+ concentration was determined using the sodium salicylate method (10). Humic acid content was determined according to the method described by Mehlich (11). Major organic acids (lactic acid, acetic acid, butyric acid, and caproic acid) were quantified using an ion chromatograph (Metrohm 761 Compact IC) equipped with a conductivity detector and an ion exclusion column (Metrosep Organic Acids 6.1005.200) following the method described by Rozendal et al. (12).

DNA extraction, PCR amplification, and pyrosequencing. Genomic DNA was extracted using a PowerSoil DNA isolation kit (MoBio Laboratories). For pyrosequencing, the V4-V5 hypervariable region of 16S rRNA genes was amplified with universal primers 515F (5′-GTGYCAGCMGCCGCGGTA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′) (13). The detailed PCR conditions were as described by Li et al. (14). The bar-coded amplicons were pooled with equimolar concentrations of the samples and sequenced using a GS FLX+ pyrosequencing system (454 Life Sciences).

Pyrosequencing data processing. The raw sequences were sorted based on unique sample tags, trimmed for sequence quality, and denoised using QIIME pipeline (15). Chimera sequences were removed using the Uchime algorithm (16). The sequences were clustered by the complete-linkage clustering method incorporated in the QIIME pipeline. Operational taxonomic units (OTUs) were classified using 97% of 16S rRNA gene sequence similarity as a cutoff. Only the OTUs containing at least 5 reads were considered to be valid OTUs in this study. Shannon index and Chao1 estimator values were calculated in RDP at 97% sequence similarity (http://pyro.cme.msu.edu/). The phylogenetic affiliation of each sequence was analyzed by RDP Classifier at a confidence level of 80%. The original pyrosequencing data are available at the European Nucleotide Archive (see below).

Statistical analysis. Overall structural changes of prokaryotic communities were evaluated by principal coordinates analysis (PCoA) in Fast UniFrac (http://bmf.colorado.edu/fastunifrac/). The cluster analysis (CA) was conducted with the unweighted-pair group method using average linkages (UPGMA) based on the Bray-Curtis distance in PAST (17). The statistical significance among data sets was assessed by PerMANOVA using the weighted PCoA scores in PAST (http://folk.uio.no/ohammer/past/). The Mantel test was applied to evaluate the correlations between prokaryotic communities and environmental variables using PASSaGE (18). Environmental variables providing the highest Pearson's correlation coefficients with prokaryotic communities were selected using the BioEnv procedure, and variance partitioning analysis (VPA) was performed to quantify the relative contributions of environmental variables using the varpart procedure in the R package Vegan (http://cran.r-project.org/web/packages/vegan/index.html). Redundancy analysis (RDA) was performed using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY). The statistical significance of the difference between the means of samples was tested by one-way analysis of variance (ANOVA) with the Tukey *post hoc* test.

Nucleotide sequence accession number. The original pyrosequencing data are available at the European Nucleotide Archive (accession no. PRJEB4986).

RESULTS

Pit-mud chemical properties. Levels of pit-mud moisture, humic content, TN, and acetic acid from different cellar samples did not significantly differ. However, the pH, NH_4^+, humic matter, caproic acid, butyric acid, and lactic acid contents changed significantly with increasing cellar age (Table 1). 1-year samples had the highest concentrations of lactic acid and butyric acid but the lowest pH and concentrations of NH_4^+ and caproic acid. While pH and NH_4^+ were significantly increased ($P < 0.05$) in the 10-year samples, the lactic acid content declined by 65% in the 10-year samples compared to that in the 1-year samples ($P < 0.01$) and continued to decline in the 25-year and 50-year samples. Caproic acid content increased significantly after 25 years. Moreover, significant differences between the 25-year and 50-year samples were not observed in most measured variables.

Overall prokaryotic community structure and diversity. In total, 494,293 qualified reads with an average length of 397 bp

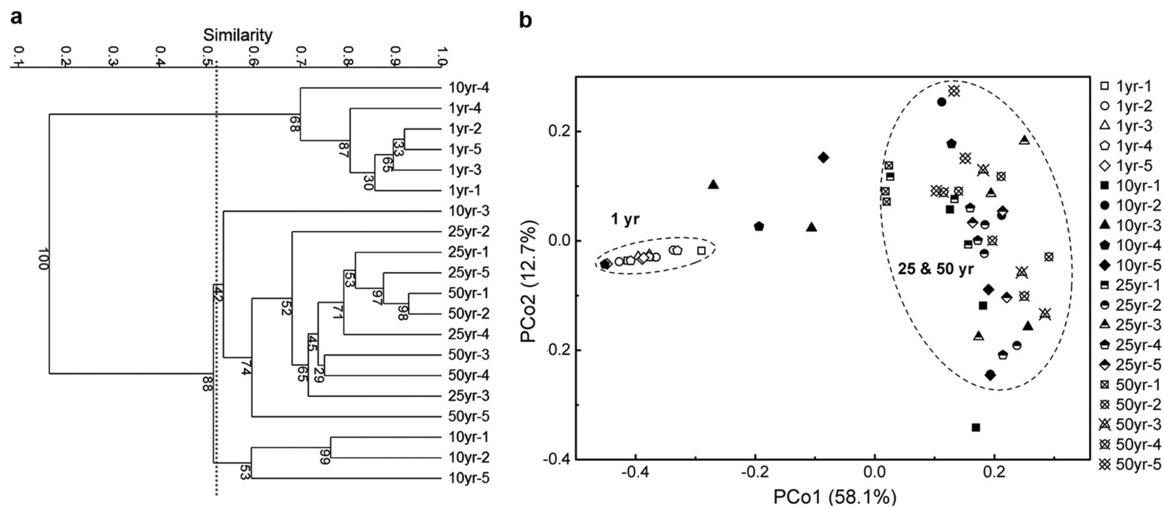

FIG 1 Data represent cluster analysis of pit-mud prokaryotic communities based on Bray-Curtis distances (a) and principal coordinate analysis using weighted UniFrac (b). Five replicate samples with same cellar age are shown as "#yr-1" to "#yr-5"; e.g., "10yr-1" represents the first replicate sample from 10-year-old cellars.

were obtained from all pit-mud samples and each sample contained 6,568 to 10,992 reads (see Table S1 in the supplemental material). A total of 796 OTUs were obtained based on 3% dissimilarity in 16S rRNA sequences, considering those OTUs with ≥5 sequences to be valid ones. Rarefaction analysis indicated that all prokaryotic communities were well represented since the rarefaction curves were approaching the saturation plateau (see Fig. S1 in the supplemental material).

Based on the relative abundances of OTUs, prokaryotic communities in 20 cellars formed three clusters as follows: (i) group I contained all the 1-year samples and a 10-year sample, (ii) group II contained three 10-year samples, and (iii) group III contained all the 25- and 50-year samples and a 10-year sample (Fig. 1a). The grouping patterns determined on the basis of all the taxonomic ranks from the order to the OTU level were similar (data not shown).

PCoA analysis was conducted to evaluate similarities in different communities using Unifrac approaches. Although there was a slight difference between the cluster analysis and PCoA results, similar clustering patterns were observed. One-year samples tended to form clusters, and 25- and 50-year samples formed clusters as well. The 10-year samples were either distributed close to the 1-year cluster or dispersed within the 25- and 50-year cluster, suggesting that they were in a transitional state. The differences among 25-year and 50-year samples were larger than those among 1-year samples. Cellar age was the main factor in the first principal coordinate axis (PCo1) and contributed 58.1% of the total variation (Fig. 1b).

The lowest diversity was observed in the 1-year samples. Shannon's diversity index increased significantly with cellar age ($P < 0.05$) from 1-year-old to 25-year-old pit mud, and it became constant in the 25-year-old to 50-year-old pit mud. Richness levels (OTUs) were similar in the 1-year and 10-year samples and significantly increased in the 25-year and 50-year samples ($P < 0.05$). In general, no significant differences between the 25-year and 50-year samples in the diversity and richness indices were observed (Table 2).

General phylogenetic composition of pit-mud community. In total, 91% of total reads were affiliated with bacterial phyla and 9.0% of total reads were assigned to archaeal phyla. The dominant bacterial phyla (>5% of total reads) were *Firmicutes* (64.5%) and *Bacteroidetes* (16.6%) (see Table S2 in the supplemental material). Archaea were dominated by *Euryarchaeota* (9.0%). *Crenarchaeota* reads (0.007%) were rare. The relative abundance of archaeal reads increased in the 10-year and older samples.

Prokaryotic compositions in the 10- to 50-year samples were not significantly changed. We defined core prokaryotic genera as those detected in all pit-mud samples and with relative abundance higher than 1.0% in the 10- to 50-year samples. These dominant genera included 10 bacterial genera, *Lactobacillus*, *Petrimonas*, *Clostridium* IV, *Sedimentibacter*, *Syntrophomonas*, *Spirochaetes* SHA-4, unclassified *Porphyromonadaceae*, *Anaerobrancaceae*, *Clostridiaceae* 1, and *Ruminococcaceae*, and 4 archaeal genera, *Methanoculleus*, *Methanosarcina*, *Methanobacterium*, and *Methanobrevibacter* (Table 3). In general, these genera constituted 66% to 68% of the total abundance and they significantly changed in abundance with cellar age.

Among 796 OTUs detected in all the pit-mud samples, the relative abundances of 90% OTUs were less than 1%. A total of 232 OTUs (29.1% of total OTU numbers) were shared by all samples. In addition, 91 (11.4%) unique phylotypes were observed in only one of the samples (see Fig. S2 in the supplemental material). These data indicated that the cellar-age-related changes occurred at the OTU level.

Cellar-age-related changes of prokaryotic community struc-

TABLE 2 Prokaryotic diversity indices calculated based on a cutoff of 97% similarity of 16S rRNA gene sequences and 5,652 reads per sample[a]

Cellar age (yr)	Chao1	Observed OTU	Shannon index
1	403.2 ± 72.1[ac]	247.2 ± 18.0[ac]	3.26 ± 0.55[a]
10	335.1 ± 33.1[bc]	221.2 ± 17.3[a]	4.70 ± 0.74[b]
25	457.1 ± 62.0[a]	287.5 ± 19.2[b]	5.61 ± 0.16[c]
50	413.3 ± 69.5[ac]	275.0 ± 33.8[bc]	5.50 ± 0.30[c]

[a] All data are presented as means ± standard deviations ($n = 5$). Values with different letters in a column mean significant differences at $P < 0.05$ as determined by ANOVA.

TABLE 3 Relative abundances of core prokaryotic communities in the pit mud from cellars of different ages

Phylum and genus	% relative abundance in cellars of age (yr)[a]: 1	10	25	50
Bacteroidetes	1.37 ± 0.63[a]	20.83 ± 9.22[b]	23.32 ± 4.45[b]	18.62 ± 6.01[b]
Petrimonas	0.94 ± 0.45[a]	11.19 ± 5.31[b]	16.69 ± 2.73[b]	12.02 ± 4.95[b]
Unclassified *Porphyromonadaceae*	0.28 ± 0.14[a]	6.45 ± 4.04[b]	3.71 ± 1.13[b]	4.39 ± 1.51[b]
Euryarchaeota	0.58 ± 0.29[a]	10.36 ± 4.29[b]	11.44 ± 1.77[b]	12.61 ± 3.32[b]
Methanobacterium	0.15 ± 0.11[a]	1.85 ± 1.30[ac]	2.85 ± 2.42[bc]	4.57 ± 1.74[b]
Methanobrevibacter	0.12 ± 0.06[a]	1.42 ± 0.76[b]	1.94 ± 0.37[b]	2.05 ± 0.68[b]
Methanoculleus	0.16 ± 0.13[a]	5.43 ± 4.73[bc]	1.56 ± 0.35[a]	2.05 ± 1.93[ac]
Methanosarcina	0.12 ± 0.05[a]	1.00 ± 1.04[a]	4.52 ± 1.11[b]	3.05 ± 1.94[b]
Firmicutes	95.68 ± 0.95[a]	63.12 ± 9.06[b]	53.99 ± 5.71[b]	54.42 ± 9.95[b]
Unclassified *Anaerobrancaceae*	0.11 ± 0.06[a]	1.21 ± 0.64[b]	2.62 ± 0.92[c]	2.46 ± 0.75[c]
Unclassified *Clostridiaceae* 1	0.16 ± 0.06[a]	0.96 ± 0.42[a]	4.97 ± 1.10[b]	7.27 ± 2.93[b]
Sedimentibacter	1.57 ± 0.20[a]	4.05 ± 1.58[b]	4.78 ± 0.84[b]	3.34 ± 1.11[b]
Lactobacillus	62.28 ± 7.88[a]	16.07 ± 16.85[b]	3.28 ± 2.25[b]	4.23 ± 4.60[b]
Clostridium IV	1.12 ± 0.23[a]	8.79 ± 6.27[b]	9.57 ± 2.91[b]	12.71 ± 7.38[b]
Unclassified *Ruminococcaceae*	0.50 ± 0.14[a]	1.39 ± 0.43[ac]	3.43 ± 1.29[b]	1.96 ± 0.85[c]
Syntrophomonas	0.45 ± 0.09[a]	4.74 ± 1.47[b]	4.53 ± 1.32[b]	3.48 ± 1.68[b]
Spirochaetes	0.12 ± 0.05[a]	1.58 ± 1.84[a]	3.88 ± 1.78[b]	3.35 ± 2.13[b]
Spirochaetes SHA-4	0.12 ± 0.04[a]	1.58 ± 1.84[a]	3.60 ± 1.78[b]	3.16 ± 2.10[b]

[a] All data are presented as means ± standard deviations ($n = 5$). Values with different letters in a row mean significant differences at $P < 0.05$ as determined by ANOVA.

ture. At the phylum level, *Firmicutes* dominated in the 1-year samples (95.7% of total reads) and dramatically decreased in abundance in the 10-year and older samples. However, *Bacteroidetes*, *Euryarchaeota*, and *Spirochaetes* increased significantly in the 10-year samples compared to the 1-year samples (Fig. 2 and Table 3). The decrease in *Firmicutes* was mainly driven by the decrease in the abundance of genus *Lactobacillus*, corresponding to the decrease in lactic acid concentration (Table 1). However, some *Firmicutes* groups, such as unclassified *Anaerobrancaceae*, unclassified *Clostridiaceae* 1, *Sedimentibacter*, *Clostridium* IV, unclassified *Ruminococcaceae*, and *Syntrophomonas*, increased their relative abundances in this process; those increases corresponded to the increase in methanogens and caproic acid concentration. These prokaryotic groups included recognized syntrophs, e.g., *Syntrophomonas*. Few *Euryarchaeota* sequences were detected in the 1-year samples. However, *Euryarchaeota* abundance significantly increased in the 10-year and older samples. Methanogens were mainly composed of hydrogenotrophic *Methanobacterium*, *Methanobrevibacter*, and *Methanoculleus* and acetoclastic *Methanosarcina* that utilize both hydrogen/CO_2 and acetate. Pure acetoclastic methanogens, such as *Methanosaeta*, were not detected in pit-mud samples.

In the 1-year samples, bacteria were dominated by OTU1574 (62.3% ± 7.9%), affiliated with *Lactobacillus*, followed by other

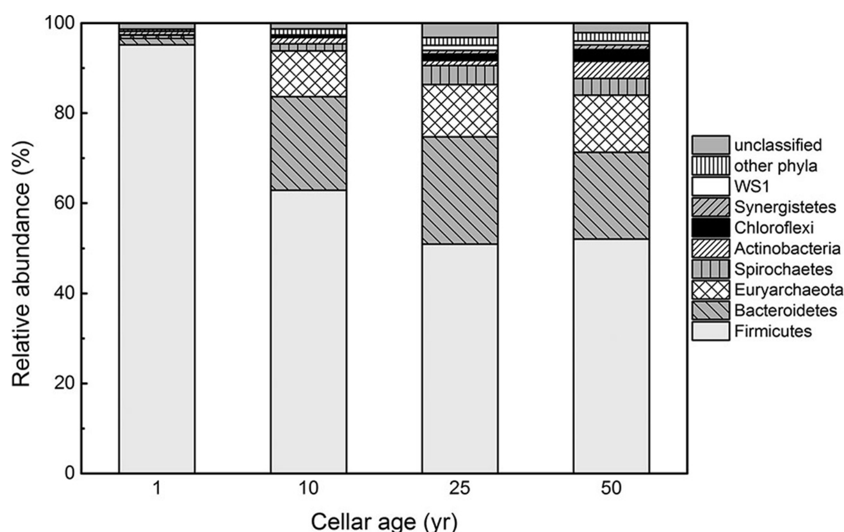

FIG 2 Taxonomic classification of prokaryotic reads retrieved from different pit-mud samples at the phylum level using the RDP classifier with a confidence threshold of 80%.

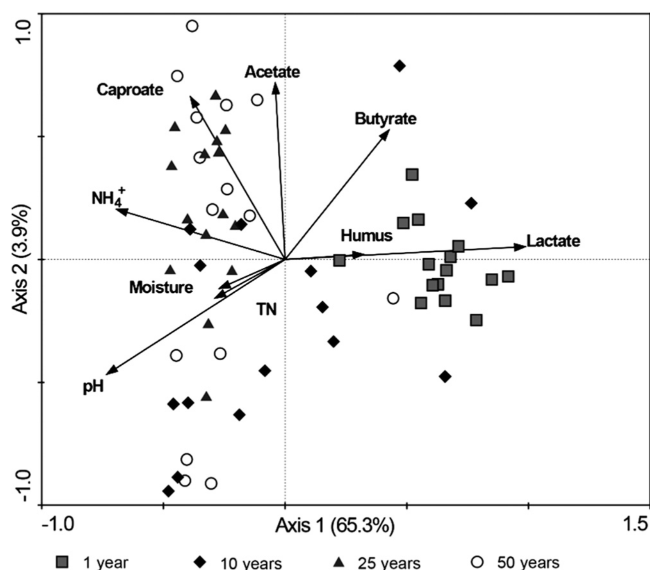

FIG 3 Redundancy analysis of prokaryotic community structure and measurable variables in the pit-mud samples. Arrows indicate the direction and magnitude of measurable variables associated with prokaryotic community structures.

abundant (>1%) OTUs belonging to OTU3924 (*Bacillus*), OTU811 (*Garciella*), OTU2976 (*Clostridium* XlVa), and OTU1593 (*Desmospora*). In the 10-year and older samples, the relative abundance of *Lactobacillus* OTU1574 decreased to up to 2.6% to 15.8% of total reads, and other dominant OTUs in the 1-year samples affiliated with *Bacillus*, *Garciella*, and *Desmospora* decreased to up to <0.1% of total reads. In contrast, the most abundant OTUs in the 10-year and older samples were mainly affiliated with *Petrimonas* (OTU1171, 2564), *Clostridium* IV (OTU2212), *Sedimentibacter* (OTU3437), unclassified *Clostridia* (OTU3893), *Methanoculleus* (OTU1262), *Methanosarcina* (OTU1750), *Methanobacterium* (OTU1234), and *Methanobrevibacter* (OTU1474) (see Fig. S3 in the supplemental material).

Relationships between prokaryotic communities and environmental variables. Redundancy analysis (RDA) was performed to discern the possible relationship between prokaryotic community structure and environmental parameters (Fig. 3). Overall, the two axes explained 69.2% of the variation in microbial community differentiation, suggesting the remarkable correlation between prokaryotic community structure and environmental factors. Lactic acid levels mainly correlated positively with prokaryotic communities in the 1-year samples. However, caproic acid levels mainly correlated with those in the 25- and 50-year samples. TN, humic acid, and moisture content levels were not significantly correlated with the changes of prokaryotic community.

The Mantel test further demonstrated that lactic acid, pH, NH_4^+, and butyric acid levels in the pit mud were significantly correlated with prokaryotic community structure at the OTU level ($P < 0.01$). Pearson's correlation analysis showed that pH and NH_4^+ content were positively correlated with the relative abundances of *Bacteroidetes* and *Euryarchaeota* but negatively with that of *Firmicutes* (see Table S3 in the supplemental material); however, lactic acid and butyric acid concentration levels showed contrasting correlations. The pH and NH_4^+ content increased with cellar age, corresponding to the increase in community diversity and caproic acid levels but to the decline in lactic acid levels and the relative abundance of *Lactobacillus* (Table 1 and 3).

Variance partitioning analyses (VPA) indicated that 64.7% of the variance in prokaryotic community structure could be explained by three major variables, lactic acid, pH, and NH_4^+, which could independently explain 62.9%, 34.8%, and 30.4% of the total variation. Moreover, significant interactive effects among three major variables, such as interaction of lactic acid with pH (33.7%), lactic acid with NH_4^+ (29.5%), and pH with NH_4^+ (17%), were observed (see Fig. S4 in the supplemental material).

DISCUSSION

The pit-mud microbiota is recognized to play important roles in the quality of Chinese liquor. For the first time, we unveiled prokaryotic community structure and diversity and their cellar-age-related changes in pit mud from CSFL fermentation cellars by the high-throughput pyrosequencing technique. Our results provided mechanistic explanations to link prokaryotic community structure and cellar-age-related changes with CSFL quality.

Cellar-age-related changes in prokaryotic community structure. PCoA analysis indicated that prokaryotic communities in the 1-year samples were different from those in the 25-year and 50-year samples. The diversity of prokaryotes increased with cellar age and kept constant in the 25-year and 50-year samples (Fig. 1 and Table 2), while those in the 10-year pit-mud samples were in the transition state. On the basis of the observations reported above, we can separate the changes of pit-mud prokaryotic communities into three distinct phases. Phase I is the initial domestication period. During this phase, the starting microbial community is selected and niche assembled under specific environmental conditions (e.g., low pH and high lactic acid) created by the fermentation process in the cellar. The number of species (200 to 300 OTUs) was significantly lower than in most soil, generally harboring more than 1,000 OTUs (19). The loss in diversity and species richness had likely resulted from niche selection and the filtering out of species that could not tolerate the pit-mud environment. Although we did not investigate the prokaryotic community composition of parental soil used for the preparation of pit mud in this study, many studies have revealed that the bacterial community in aerobic soil is mainly composed of *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (19, 20). However, the pit-mud prokaryotic community was dominated by phylum *Firmicutes* (95.7%) after 1-year of domestication. This suggested that *Firmicutes* were selected by pit-mud conditions as the most acclimated prokaryotes.

Phase II is a transitional period during which the community structure changes dramatically. At this stage, we observed a significant increase in prokaryotic diversity. While the abundance of *Firmicutes* decreased, the abundances of *Bacteroidetes*, *Spirochaetes*, and archaea (mainly methanogens) increased (Table 3). It is likely that niche-selected populations from soil, the fermentation inoculum (Daqu-starter), or the surrounding air environment have gradually adapted to the biogeochemical environment of pit mud. In addition, long-term and periodic brewing fermentation lead to the production of organic acids and H_2 and CO_2 in pit mud, facilitating the growth of methanogens.

Phase III is the relative mature period of the pit-mud prokaryotic community. Prokaryotic diversity and richness are stable during this period and are markedly higher than in the young pit mud. The specific microbiota in the pit mud is selected by periodic fermen-

tation and enrichment for more than 25 years without interruption. As a result, mutual collaborations and interactions among different prokaryotic species lead to a well-balanced prokaryotic community in the pit mud, which produces more caproic acid than in phases I and II; this also results in CSFL quality improvement.

Influence of environmental variables on the prokaryotic community. This study revealed that lactic acid and pH were the most important factors influencing prokaryotic community structure in the pit mud. The pH was very low in the 1-year pit mud and increased in the older cellars. This corresponded to the high concentration of lactic acid in the 1-year pit mud and the high abundance of *Lactobacillus* bacteria, which produce lactic acid as the major end product in carbohydrate fermentation. The accumulation of lactic acid was likely to lower the pH in the 1-year pit-mud samples. This is consistent with a report that lactic acid accumulation led to the decline of ruminal pH (21). Belenguer et al. (22) also found that lactate and butyrate levels were largely correlated with pH. A decrease in soil pH tends to reduce overall diversity and to change the composition of prokaryotic communities (23, 24). Interestingly, a strong correlation of the prokaryotic community with the NH_4^+ level was also observed. The balance of NH_4^+ and fatty acid content may influence pH directly and, indirectly, the prokaryotic community structure. Niche-driven changes in the pit-mud prokaryotic community supported that environmental factors, not historical factors, determine the shift in the structure of a prokaryotic community (20, 25) in the pit mud.

Core prokaryotic communities and their relationships to CSFL quality. The core microbiota (also called the core genera or populations) consists of dominant generalists. The genera *Petrimonas*, unclassified *Clostridiaceae*, *Methanoculleus*, *Methanosarcina*, and *Methanobacterium* were identified from pit-mud samples by DGGE or clone library methods in previous studies (7, 8, 26, 27). Here, we identified 14 genera, including 5 previously identified genera and *Lactobacillus*, *Clostridium* IV, *Sedimentibacter*, *Syntrophomonas*, *Spirochaetes* SHA-4, *Methanobrevibacter*, and unclassified *Porphyromonadaceae*, *Anaerobrancaceae*, and *Ruminococcaceae*, as the core microbiota in the pit mud using the pyrosequencing technique. The core microbiota contains fermentation bacteria, syntrophs, and methanogens, which play important roles in the maturing process of pit mud. The *Lactobacillus* species produce lactic acid as the major end product in carbohydrate fermentation (28). *Petrimonas* is reported to produce acetic acid, hydrogen, and CO_2 during glucose fermentation (29). *Sedimentibacter* ferments amino acids and glycine to ethanol or to acetic acid and butyric acid (30, 31). *Syntrophomonas* can syntrophically degrade long-chain fatty acids into acetic acid and H_2 in a coculture with methanogens (32, 33). The roles of some core genera in the pit mud, such as those of *Clostridium* IV, *Spirochaetes* SHA-4, and unclassified *Porphyromonadaceae*, *Anaerobrancaceae*, and *Ruminococcaceae*, were not well understood. *Methanoculleus* and *Methanobacterium* are hydrogenotrophic methanogens, while *Methanosarcina* utilize both H_2 and acetic acid (34). The *Methanosaeta*, characterized by exclusive use of acetate, were not detected, suggesting that the hydrogenotrophic pathway predominated the CH_4 production in the pit mud.

It is recognized that the quality of CSFL is highly correlated with ethyl caproic acid production through esterification of ethanol and caproic acid (35, 36). RDA indicated that caproic acid was mainly correlated with those prokaryotic communities in the 25- and 50-year samples (Fig. 3), indicating the importance of prokaryotes in the production of caproic acid. Caproic acid is a side product in the acidogenic fermentation of a complex biomass or sugars (37, 38). It is generally produced by anaerobic bacteria such as *Clostridium kluyveri* (39) and *Eubacterium pyruvativorans* (40) or by cocultures of *C. kluyveri* with ruminal cellulolytic bacteria using cellulose as a substrate (41). In this study, we observed significant correlations between caproic acid content and the relative abundances of four core prokaryotic populations (*Lactobacillus*, *Clostridium* IV [OTU2212], unclassified *Clostridiaceae* 1 [OTU3893], and unclassified *Anaerobrancaceae* [OTU3251]) ($P < 0.01$). Among them, *Lactobacillus* abundance was negatively correlated with caproic acid formation. It has been previously reported that the lactic acid producers restrain the production of caproic acid (42). In contrast, the relative abundances of three other core genera showed positive correlations to caproic acid production, and they were more abundant in old pit-mud samples. To our knowledge, no isolates closely related to these core genera or OTUs have been reported. We speculate that these core bacterial populations are involved in the formation of caproic acid or other flavoring components.

The abundances of methanogens were significantly higher in the 10-year to 50-year pit mud than in the 1-year pit mud. Although methanogens do not directly produce caproic acid, they can enhance caproic acid production through syntrophic cooperation between caproic-acid-producing bacteria and methanogens (5). Caproic acid formation and many other fermentation reactions are hydrogenogenic (38) under anaerobic conditions. The interspecies hydrogen transfer between caproic acid-producing or fermenting bacteria and methanogens controls hydrogen partial pressure under a certain threshold, which makes caproic acid formation and fermentation reactions thermodynamically more favorable.

In summary, this study revealed prokaryotic community structure and diversity in the pit mud from CSFL cellars of different ages using the pyrosequencing technique and identified the main factors influencing community structure. Fourteen core prokaryotic genera were identified whose species might play important roles in the maturing process of pit mud. Prokaryotic diversity and community structure became stable after 25 years or longer, which corresponded to the accumulation of caproic acid. Cellar-age-related changes of the prokaryotic community and diversity in the pit mud provided scientific evidence to support the practical experience that old fermentation cellars produce high-quality CSFL. Further research will be aimed at elucidating metabolic pathways and multiple syntrophic networks in the pit mud using metagenomic and metatranscriptomic approaches and at isolating representative key species for characterization of their biochemical pathways related to CSFL production.

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