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published in
Canadian Journal of Microbiology
2020

DOI (link to publisher)
10.1139/cjm-2020-0012

document version
Peer reviewed version

Canadian Science Publishing | Canadian Journal of Microbiology

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Journal:	*Canadian Journal of Microbiology*
Manuscript ID	cjm-2020-0012.R1
Manuscript Type:	Article
Date Submitted by the Author:	04-Mar-2020
Complete List of Authors:	Zheng, Yan; Zhengzhou University of Light Industry, School of Food and Biological Engineering Hu, Xiaolong; Zhengzhou University of Light Industry Jia, Zhongjun; Institute of Soil Science Chinese Academy of Sciences Bodelier, Paul; Netherlands Institute of Ecology Guo, Zhiying; Institute of Soil Science Chinese Academy of Sciences Zhang, Yong; Zhengzhou University of Light Industry Li, Fangli; Zhengzhou University of Light Industry He, Peixin; Zhengzhou University of Light Industry
Keyword:	prokaryotic community, different aged pit mud, co-occurrence patterns, 16S rRNA gene, high-throughput sequencing
Is the invited manuscript for consideration in a Special Issue? :	Not applicable (regular submission)

(a) The title:

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Abstract

It is widely believed that the quality and characteristics of Chinese Strong-Flavour Liquor (CSFL) are closely related with the age of the pit mud, and CSFL produced from older pit mud tastes better. This study aims to investigate alteration and interaction of prokaryotic communities across an age gradient in pit mud. Prokaryotic microbes in different aged pit mud (1-, 6- and 10-year) were analyzed using Illumina MiSeq sequencing of 16S rRNA gene. Analysis of 16S rRNA gene indicated that prokaryotic community was significantly altered with ages. Genera *Methanosarcina, Methanobacterium* and *Aminobacterium*, increased significantly with ages, while genus *Lactobacillus* showed a significant decreasing trend with increased ages. Network analysis demonstrated that both synergetic co-occurrence and niche competition were dominated by 68 prokaryotic genera. These genera formed 10 hubs of co-occurrence patterns, mainly under phylum *Firmicutes*, *Euryarchaeota* and *Bacteroidetes*, playing important roles on ecosystem stability of the pit mud. Environmental variables (pH, NH_4^+, available P, available K, and Ca^{2+}) correlated significantly with prokaryotic community assembly. The interaction of prokaryotic communities in pit mud ecosystem, and the relationship among prokaryotic communities and environmental factors contribute to higher quality of pit mud in older fermentation pits.

Key words

prokaryotic community, different aged pit mud, co-occurrence patterns, 16S rRNA gene, high-throughput sequencing

Introduction

Chinese strong-flavored liquor (CSFL) is a traditional alcoholic drink, which comprises more than 70% of the production of all distilled liquors in China (Zhao et al. 2013). CSFL is a distilled product from grains fermented (~60 days) in an underground soil pit (about 2 m in width, 3 m in length and 2 m in depth) by using traditional solid-state fermentation (Hu et al. 2016). The inside walls of the pit are covered with fermentation pit mud, which is prepared by incubating the mixture of fermentative bacteria with fresh common soil and water. In brief, the fermentation process is as follows. Firstly, the crushed raw materials (mixture of sorghum, wheat, corn or glutinous rice grains) are mixed in a 4(5):1 ratio (wt/wt) with fermented grains obtained from last fermentation round. Secondly, the mixture is steamed to distil off the liquor, which is collected and stored for maturation and then diluted with water to yield an ethanol content of 40–55% (v/v) before consumption (Hu et al. 2015). The steamed mixture is cooled and then supplied with 10-20% (wt/wt) Daqu-starter, which mainly includes mold and yeast (Tao et al. 2014). Next, the above-mentioned mixture is placed into the pit and then sealed with common mud to naturally ferment for over 60 days. After the fermentation, the fermented grains are taken out of the pit and distilled to make liquor. The process described above is periodically repeated after new fermentation materials are supplied.

It is widely reported that older pits always produce better taste CSFL. Some pits in *Wuliangye* and *Luzhoulaojiao* , the famous CSLF companies in China, have been used for more than hundred years without interruption (Zhao et al. 2013). The quality

of pit mud is the key factor affecting the quality and flavor of CSFL, in addition, prokaryotic communities in pit mud play an essential role in CSFL production (Hu et al. 2015; Tao et al. 2014; Wang et al. 2014; Zhao et al. 2013). Prokaryotic microbes in pit mud synthesize various flavor components such as butyric acid, caproic acid, and ethyl caproate, which are considered to be important for the aromatic style and quality of CSFL (Tao et al. 2014). Culture-independent methods have been used to detect the prokaryotic communities in pit mud. Gram-positive (G^+) bacteria and anaerobic bacteria were dominant in pit mud, and the abundance of (G^+) bacteria increased with the pit ages by using phospholipid fatty acid analyses (PLFA) (Zhao et al. 2013). Recently, high-throughput sequencing has been employed to accurately reveal the overall microbial community in pit mud (Hu et al. 2016; Tao et al. 2014; Wang et al. 2014). Tao *et al* (2014) and Wang *et al* (2014) demonstrated the cellar-age-related changes of prokaryotic community in the pit muds, revealing that long-term fermentation shaped unique prokaryotic community structure. Hu *et al* (2016) analyzed prokaryotic community in the pit mud with different levels of quality (degraded, normal and high quality) based on the 16S rRNA gene sequences, and their study showed that prokaryotic community was correlated with the pit mud quality. Previous studies have determined that CSFL flavor components-producing microbes, such as *Clostridum kluyveri*, increased in the high quality pit mud compared to that in the degraded quality pit mud (Hu et al. 2016). In addition, some non-flavor components-producing microbes such as methanogenic archaea were highest in the high quality pit mud (Hu et al. 2016; Tao et al. 2014). Methanogens

include hydrogenotrophs (e.g., *Methanobacterium*, *Methanobrevibacter*, and *Methanoculleus*) utilizing H_2 and *Methanosarcina* utilizing both hydrogen/CO_2 and acetate (Demirel and Scherer 2008; Vanwonterghem et al. 2014). These non-flavor components-producing microbes may improve the pit mud quality indirectly by cooperating with the flavor components-producing microbes. For instance, hydrogenotrophic methanogens can utilize hydrogen from the production of caproic acid by *Clostridium kluyveri*, so methnaogens control hydrogen pressure and make caproic acid formation more favorable (Hu et al. 2016). It is necessary to determine the correlations among the prokaryotic community to control pit mud quality. Previous studies mostly focused on shift of prokaryotic community structure across a quality or an age gradient in the pit mud to identify the specific microbes controlling the CSFL quality (Hu et al. 2016; Tao et al. 2014; Wang et al. 2014), however, it is poorly reported that direct and indirect interactions among prokaryotic community in pit mud.

To figure out the above-mentioned puzzling questions, we investigated the interactions among prokaryotic taxa in the different aged pit mud to figure out ecological linkages among prokaryote in put mud systems. Our research can provides the scientific evidence to support the practical experience that the quality of older pit mud is better. In our study, we analyzed the prokaryotic communities in pit mud from different aged CSFL pits (1-year-old, 6-year-old and 10-year-old) by high-throughput sequencing of 16S rRNA gene. The aims of this study were to (i) investigate the dynamics of prokaryotic community in different-aged pit mud; (ii) reveal correlations

of the prokaryotic taxa within the pit mud ecosystem; (iii) elucidate the relationships between physicochemical properties of pit mud and the structure of prokaryotic community.

Materials and methods

Pit mud sampling

Pit mud samples were collected from a Chinese strong-flavored liquor distillery in Henan province, China. The pits, used for 1, 6 and 10 years, respectively, were selected for sampling, and three pits were selected for each age. In each pit, five pit mud subsamples were collected from the four corners and the center of the bottom of the pit, respectively, and then mixed immediately. Pit mud for high-throughput sequencing analysis were frozen in liquid nitrogen immediately and stored at -80 °C. The rest of pit mud was transferred to the laboratory on ice and kept at 4 °C for physicochemical analysis.

Physicochemical determinations of pit mud

pH was measured by Mettler Toledo 320-S pH meter (Mettler–Toledo Instruments Co. Ltd., Shanghai, China) using a pit mud-to-water ratio of 1:2.5 (wt/vol). The total N (TN) and organic matter (OM) of the pit mud were determined according to Kjeldahl digestion method (Yuen and Pollard 1953) and dichromate oxidation method (Mebius 1960), respectively. Ammonium (NH_4^+) was extracted in 2mol L^{-1} KCl at a 1:5 ratio (wt/vol), and measured using a Skalar SAN Plus segmented flow analyzer (Skalar Inc., Breda, The Netherlands). Available P (AP) was extracted using sodium bicarbonate and measured with the molybdenum blue method. Available K (AK) was

155 extracted using ammonium acetate for measurement with flame photometry.

156 **DNA extraction**

157 Total DNA from 0.5g pit mud (dry weight) was extracted using FastDNA spin kit for
158 soil (MP Biomedicals, Cleveland, OH, USA), according to the manufacturer's
159 instructions. DNA was extracted in triplicate from each sample of different aged pit
160 mud, and triplicate DNA were pooled for high-throughput sequencing. The quality
161 and quantity of DNA were detected by using a Nanodrop ND-1000UV-Vis
162 Spectrophotometer (NanoDrop Technoloqies, Wilmington, DE, USA), and the DNA
163 was then stored at -20 °C.

164 **Real-time quantitative PCR of 16S rRNA genes**

165 Real-time quantitative analysis of 16S rRNA genes in the total DNA was performed
166 to determine the alteration of absolute numbers of prokaryote in different aged pit
167 muds on a CFX96 Optical Real-Time Detection System (Bio-Rad, Laboratories Inc.,
168 Hercules, CA, USA). The primers of was 515F (GTGCCAGCMGCCGCGG)/ 907R
169 (CCGTCAATTCMTTTRAGTTT) (Zheng et al. 2014) were used for the real-time
170 PCR. The reaction was performed in a 20 μL mixture containing 10.0 μL SYBR
171 Premix Ex Taq (Takara, Dalian, China), 0.5 μL each primer and 1 μL of DNA
172 template. The amplification conditions were as follows: preheating at 95°C for 3min,
173 40 cycles of 95°C for 10s, 55°C for 30s, 72°C for 30s, and increases of 0.5°C every 5s
174 from 65°C to 95 °C for melting curve analysis. The real-time PCR standards were
175 generated using plasmid DNA from one representative clone containing 16S rRNA
176 genes. Amplification efficiency of 97% was obtained with R^2 values of 0.996.

High-throughput sequencing of 16S rRNA genes

High-throughput sequencing of total 16S rRNA gene was performed using the universal primers 515F (GTGCCAGCMGCCGCGG)/ 907R (CCGTCAATTCMTTTRAGTTT). Barcodes unique to each sample were added to the 515F primer to barcode the PCR amplicons, and PCR was performed according to a previously described method (Zheng et al. 2014). PCR products were purified on 1.8% agarose gels and determined by a Nanodrop ND-1000 UV-Vis Spectrophotometer. Sequencing was performed on an Illumina Miseq sequencer (Illumina, San Diego, CA). Pair end sequences were joined with FLASH (Magoc and Salzberg 2011). Raw sequences were imported into the mothur software (Schloss et al. 2009) to generate high quality sequences (read length longer than 200 bp, average quality score more than 25, without ambiguous base). The high quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity with UCLUST algorithm in QIIME (Caporaso et al. 2010). Taxonomic assignment of the high quality sequences were obtained by Ribosomal Database Project (RDP) with a confidence threshold of 80%.

Statistical analysis

Cluster analysis (CA) was performed with the unweighted-pair group method using average linkages (UPGMA) using Bray-Curtis distance in R within the Vegan package. Principal coordinates analysis (PCoA) using weighted UniFrac distance was used to evaluate the overall structural changes of prokaryotic communities in R using the Vegan package. A heatmap was analyzed using the gplots package in R to

compare the top 10 genera in each sample. To construct networks of prokaryotic communities, all possible pairwise Spearman's rank correlations among 68 abundant genera (relative abundance was more than 0.1% of the total genera in at least one sample) was calculated to construct a correlation matrix. Valid co-occurrence patterns as well as negative correlations were identified based on the nine pit mud samples from three ages when statistical significance was less than 0.01, and spearman's correlation coefficient was more than 0.6 and less than -0.6, respectively. Networks analysis was performed using the interactive platform Gephi (Bastian et al. 2009), generating nodes and edges representing genera and significant correlations, respectively. The co-occurrence incidence among different prokaryotic taxa was the ratio of targeted edges to the total edges.

Mantel tests were performed to assess the correlation between prokaryotic communities and environmental variables using R within vegan package. Redundancy analysis (RDA) was performed to conduct the possible relationship between prokaryotic communities at genus level and environmental variables in the R within the vegan package. The statistical significance of the difference between the means of samples was conducted using the SPSS Statistics soft package version 16.0.

Accession number of nucleotide sequences

Sequences have been deposited at the DNA Data Bank of Japan (DDBJ) with accession number DRA005441 for the 16S rRNA genes.

Results

Physicochemical property of different-aged pit mud

pH, NH_4^+, AP and AK contents increased significantly (P<0.05) with pit mud age (Table 1). Compared with 1-year pit mud, the Ca^{2+} contents decreased 1.53-fold in 6-year pit mud and 1.24-fold in 10-year samples, respectively. The OM and TN contents did not significantly change with pit mud age.

High-throughput fingerprinting of overall prokaryotic community in different-aged pit mud based on analysis of 16S rRNA gene sequences

As shown in Table S1, high-quality sequence reads per sample were about 20, 000. The numbers of OTUs per sample obtained based on 97% similarity in 16S rRNA gene sequences and 19500 reads ranged from 5381 to 6873 (Figure S1). Based on the relative abundance of OTUs, prokaryotic communities from different aged pit mud formed two groups (Figure 1A). Group I contained 1-year samples, while group II contained 6-year and 10-year samples. 1-year samples were significantly different from 6-year and 10-year samples. PCoA analysis indicated that pit-mud samples clustered in 1-year, 6- and 10-year samples (Figure 1B). Pit mud age was the main factor in the first principal coordinate axis (PCo1) and contributed 47.9% of the total variation.

The changes in community of the core prokaryotes in different aged pit mud

Based on RDP classification, 76.4% and 23.6% of total 16S rRNA gene sequences were affiliated with bacterial phyla and archaeal phyla, respectively (Table S1). A total of 27 phyla were identified, including 25 bacterial phyla and 2 archaeal phyla (Table S2). The dominant bacterial phyla (average abundance of all the samples >0.5%) were *Firmicutes*, *Bacteroidetes*, *Synergistetes*, *Chloroflexi* and

243 *Actinobacteria*, and these five phyla accounted for 95.2% ~ 97.8% of total bacterial
244 phyla in each sample. The archaeal phylum was mainly affiliated with *Euryarchaeota*,
245 which accounted for 98.5% to 100% of total archaeal phyla in each sample (Table
246 S2).

247 At the phylum level, the relative abundance of 16S rRNA gene assigned to
248 *Firmicutes* was 71.9% in 1-year samples, which decreased dramatically in 6-year and
249 10-year samples (Figure 2). The relative abundance of *Euryarchaeota* and
250 *Synergistetes* increased significantly with pit mud age (Figure 2). For *Euryarchaeota*,
251 relative abundance was 10.9% in 1-year pit mud, and increased to 22.6% and 37.1%
252 in 6-year and 10-year samples, respectively. For *Synergistetes*, relative abundance
253 increased significantly from 0.93% in 1-year pit mud to 4.62% in 10-year pit mud.
254 The relative abundance of *Actinobacteria* showed a slightly decreasing trend during
255 6-year fermentation, whereas it was up to 1.37% in 10-year sample, representing 3.06-
256 and 5.57-fold increases relative to1-year and 6-year pit mud, respectively. The relative
257 abundance of *Bacteroidetes* affiliated 16S rRNA genes was significantly increased
258 from 1-year sample to 6-year sample and 10-year sample, though it showed a
259 decreasing trend from 6-year to 10-year.

260 A total of 381genera were detected in all pit mud samples. The top 10 genera
261 from each pit mud (a total of 29 genera for 9 samples) are displayed in a heatmap
262 (Figure S2). For the 29 genera, the relative abundance of each genus consisted more
263 than 0.10% in at least one of the pit mud sample. These 29 genera belonged to 5
264 phyla, including *Firmicutes*, *Euryarchaeota*, *Synergistetes*, *Chloroflexi* and

Actinobacteria. Moreover, 18 core genera (relative abundance >1% in at least one of the pit mud sample) among these 29 genera were observed, accounting for 86.7%~92.3% in each pit mud sample (Table 2). For the genera of phylum *Firmicutes*, the relative abundance of *Lactobacillus* and *Caloramator* were significantly decreased with pit mud age, which was 5.74- and 6.17- fold lower in 1-year sample than in 10-year samples, respectively (Table 2). However, the relative abundance of most genera in *Firmicutes* detected in 1-year was lower than in older samples. For instance, the lowest relative abundance of the genera *Clostridium*, *Syntrophomonas*, and *Sedimentibacter* was observed in 1-year pit mud, while the highest abundance of the three genera was detected in 6-year pit mud. For the *Euryarchaeota* phylum, the relative abundance of *Methanosarcina* and *Methanobrevibacter* in 1-year pit mud was 5.31- and 3.99-fold higher compared to 10-year samples. Though the significant growth of methanogens *Methanobacterium* was not observed from 1 year to 6 year in the pit mud, the relative abundance of *Methanobacterium* was 4.23-fold higher in 10-year pit mud than that in 6-year pit mud. The relative abundance of the genus *Aminobacterium* assigned into the phylum *Synergistetes* showed significant increase with pit mud age. In phylum *Actinobacteria,* the relative abundance of Unclassfied_*Coriobacteriaceae* displayed a decreasing trend in 1-year pit mud compared to 6-year pit mud, whereas it significantly increased from 0.22% in 6-year pit mud to 1.32% in 10-year pit mud. The relative abundance of Unclassfied_*Porphyromonadaceae*, *Blvii28* and Unclassfied_*BA008* in phylum *Bacteroidetes* increased significantly from 1-year to

6-year, followed by a decrease to 10 years. Moreover, the relative abundance of *T78* in phylum *Chloroflexi* increased from 0.82% in 1-year old pit mud to 3.43% in 6-year old samples and subsequently decreased to 0.21% in 10-year old pit mud.

Though the prokaryotic community shifted in the different aged pit muds, no significant difference of absolute numbers of prokaryotes was observed in different pit mud ages by real-time PCR of 16S rRNA genes (Figure S3).

Network analyses of prokaryotic communities in pit mud

Both positive correlations (R>0.6 and P<0.01) and negative correlations (R<-0.06 and P<0.01) among prokaryotic communities were observed in our study, which were affiliated with 68 prokaryotic genera. Based on positive correlations (R>0.6 and P<0.01), a total of 67 nodes (genera) and 229 edges (pairs of significant correlations) co-occurrence patterns were detected **(**Figure 3A). The average network distance between all pairs of nodes (average path length) and diameter (longest distance between any two nodes) were 3.339 and 8 edges, respectively. The modularity index was 0.707 (more than 0.4), indicating that the network had a modular structure (Hu et al.2016).

There were 12 significant connected phyla , including *Firmicutes* (38 nodes), *Euryarchaeota* (9 nodes), *Bacteroidetes* (8 nodes), *Tenericutes* (2 nodes), *Synergistetes* (2 nodes), *Chloroflexi* (2 nodes),*Chlorobi* (1 nodes), *Spirochaetes* (1 node), *WWE1* (1node), *Crenarchaeota*(1 node), *Actinobacteria* (1 node) and *FBP* (1 node) (Figure 3A). Additionally, ten hubs (highly connected genera, more than eleven edges per node) were detected in the co-occurrence patterns. The hubs in the archaeal

phylum *Euryarchaeota* were the three methanogens, including *Methanosarcina, Methanobrevibacter*, and *Methanocorpusculum* (Figure 3A). Another seven hubs included six genera *Tissierella_Soehngenia*, unclassified *Lachnospiraceae*, *Clostridium*, *Syntrophomonas, Sedimentibacter*, and unclassified *Anaerobrancaceae* in class *Clostridia* in phylum *Firmicutes*, one genus *T78* in class *Anaerolineae* in phylum *Chloroflexi*.

The co-occurrence incidence (the ratio of edges between targeted prokarytic phyla to total edges) among archaeal phyla accounted for 2.62% of co-occurrence among prokarytic phyla, while co-occurrence incidence among bacterial phyla accounted for 72.9% of that among prokarytic phyla. Additionally, the co-occurrence among archaeal phyla and bacterial phyla was also observed in our study, which accounted for 24.5% of co-occurrence among prokarytic phyla (Table S3). These results indicated that the co-occurrence among archaeal phyla in the pit muds was much lower than co-occurrence among bacterial phyla and co-occurrence between bacterial phyla and archaeal phyla.

In the co-occurrence among archaeal phyla, the co-occurrence patterns were all related to *Euryarchaeota*, accounting for 2.62% of co-occurrence among prokarytic taxa (Table S3). Among bacterial phyla, genera from different phyla (interphylum) had significantly higher co-occurrence incidence than those within the same phylum (intraphylum). Interphylum co-occurrence incidence was 36.2% of co-occurrence among prokarytic phyla, such as the co-occurrence incidence between *Frimicutes* and *Bacteroidetes* (10.9%), between *Frimicutes* and *Chlorobi* (2.18%), between

Frimicutes and *Tenericutes* (3.06%), between *Frimicutes* and *Synergistetes* (1.75%), between *Frimicutes* and *Actinobacteria* (4.37%), between *Frimicutes* and *Chloroflexi* (2.62%), between *Bacteroidetes* and *Chlorobi* (1.31%), between *Bacteroidetes* and *Chloroflexi* (1.31%). The co-occurrence among *Frimicutes* and *Bacteroidetes* was highest in the interphylum co-occurrence incidence, especially classes *Clostridia* in *Frimicutes* and *Bacteroidia* in *Bacteroidetes* (Table S4). The intraphylum co-occurrence incidence was 36.7%, and the highest value was from *Firmicutes* (34.5%) (Table S3). The co-occurrence between archaeal phyla and bacterial phyla was observed between *Euryarchaeota* and bacterial phyla such as *Firmicutes*, *Bacteroidetes*, *Synergistetes*, and *Chloroflexi* (Table S3). The highest co-occurrence incidence was between *Frimicutes* and *Euryarchaeota*, which was up to 15.7%. In addition, between the two phyla, class *Clostridia* was strongly correlated with methanogens (classes *Methanomicrobia* and *Methanobacteria*), accounting for 13.5% (Table S4).

Significant negative correlations (R<-0.06 and *P*<0.01) were identified from differently aged pit mud samples, and 26 nodes and 27 edges were detected (Figure 3B). There were 8 significant connected phyla in network analysis, including *Firmicutes* (11 nodes), *Bacteroidetes* (5 nodes), *Euryarchaeota* (3 nodes), *Tenericutes* (2 nodes), *Chloroflexi* (2 nodes),*Synergistetes* (1 node),*Spirochaetes* (1 node), and *Crenarchaeota*(1 node) (Figure 3B). The genera *Ruminococcus*, unclassified_*Porphyromonadaceae* and *Lactobacillus* were hubs (more than 5 edges) mainly connected with the members of classes *Clostridia* and *Bacteroidia*.

Relationships between prokaryotic communities and environmental variables

Redundancy analysis (RDA) was performed to conduct the possible relationship between prokaryotic communities at genus level and environmental variables (Figure 4). The first and second axis explained 73.1% and 19.1% of the variation in prokaryotic communities and environmental factors, respectively. Mantel test indicated that pH, NH_4^+, AK, AP and Ca^{2+}were significantly correlated with prokaryotic communities at genus level (P<0.05). Pearson's test indicated that pH was strongly positively correlated with the core genera in methanogens, such as *Methanosarcina* and *Methylobaterium*, and the core genera in class *Synergistia*, such as *Aminobacterium*, while significant negative correlation between pH value and relative abundance of representatives of the class *Bacilli*, especially the genus *Lactobacillus*, was observed in our study (Table S5). The content of NH_4^+, AP and AK had positive effect on the population of the phyla *Euryarchaeota*, *Synergistetes* and some core genera in phylum *Firmicutes* (Table S5). The content of Ca^{2+} has no significant positive effect on the relative abundance of the core prokaryotic taxa in our study, while it strongly negatively correlated with the genera *Syntrophomonas, Sporanaerobacter* and *Methanobrevibacter* (Table S5).

Discussion

It is widely recognized that prokaryotic microbes in pit mud significantly affect the quality of CSFL, and the older pits always produce liquor with better taste (Hu et al. 2016; Liang et al. 2016; Luo et al. 2014; Tao et al. 2014). Previous studies have focused on shifts of diversity and structure of prokaryotic communities, however,

interactions of prokaryotic taxa in different-aged pit mud is poorly understood. Our results contribute to provide microbial mechanisms underlying the pit mud-age-related changes and reveal the interactions of prokaryotic taxa in different aged pit mud.

The core prokaryotic community were composed of eighteen genera (Table 2), consisting of fermentative bacteria, syntrophs and methanogens. These taxa also dominated in the pit mud from Jiangsu and Sichuan Province, China, as analyzed by high-throughput sequencing (Hu et al. 2016; Tao et al. 2014), suggesting that these genera are widespread in pit mud across China. Thirteen core genera of these eighteen genera were identified, namely, *Lactobacillus*, *Aminobacterium*, *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, *Ruminococcus*, *Caloramator*, *Clostridium*, *Syntrophomonas*, *Sedimentibacter*, *Blvii28*, *T78* and *vadinCA11*. *Lactobacillus* produces lactic acid as major end metabolite by utilizing fermentable carbohydrates (Dalié et al. 2010). Increased abundance of *Lactobacillus* may destabilize the pit mud, which is a core microbe indicator for degraded pit mud (Hu et al. 2016). *Lactobacillus* was most abundant in 1-year-old pit mud, which was the lowest pH environment. The relative abundance of *Lactobacillus* significantly decreased with pit mud age (Table 2), indicating the improved quality of pit mud with ages. In our study, the most abundant genus in the class *Synergistia* was *Aminobacterium*, which grew significantly during ten years' fermentation. *Aminobacterium* fermented ammonia acids such as serine, glycine, and threonine as substrate, and the end products included acetate, which can be reduced by hydrogen to ethanol (Baena et al. 2000;

Hamdi et al. 2015). Because CSFL production is an ethanol-producing progress, the most efficient substrate for caproate-acid synthesis in CSFL is generally regard as ethanol (Zhu et al. 2015). Thus, *Aminobacterium* in the class *Synergistia* can contribute to the formation of caproic-acid in CSFL production. The ammonia acid fermentation by *Aminobacterium* must be co-cultured with hydrogen-consuming methanogens to reduce hydrogen stress (Baena et al. 2000; Hamdi et al. 2015). Methanogens were mainly composed of hydrogenotrophs (*Methanobacterium* and *Methanobrevibacter*) that utilize H_2 and *Methanosarcina* that utilize both hydrogen/CO_2 and acetate in our study. Caprotic acid formations are hydrogenogenic under anaerobic condition (Ding et al. 2010), thus hydrogenotrophic methanogens can facilitate caproic acid production by reducing hydrogen stress when co-cultured with .caproic acid-producing microbes. Therefore, the interaction between methanogens and other microbes, such as *Aminobacterium* in the class *Synergistia,* can facilitate the formation of caproic-acid and improve the stabilities of the complex microbial community The increased abundance of the Methanogens (*e.g.* genera *Methanosarcina* and *Methanobacterium*) and *Amioiabacterium* in the older pit mud may be beneficial to the interaction among them (Hu et al. 2016). These results may provide a reasonable explanation for the practical experience that pit mud quality generally improved with pit mud age.

The network analysis indicated that positive correlations of prokaryotic phyla, which were mainly belonging to phylum *Firmicutes* (mainly class *Clostridia*), phylum *Bacteroidetes* (mainly class *Bacteroidia*) and phylum *Euryarchaeota* (mainly class

419 *Methannobacteria* and *Methanomicrobia*) (Table S4). These putative synergetic
420 relationships were also observed in the pit mud across a quality gradient from Jiangsu
421 province, China (Hu et al. 2016). The co-occurrence among archaeal phylum
422 (*Euryarchaeota*) only contributed 2.62% of total prokaryotic phyla co-occurrence
423 incidence in the pit mud, while the co-occurrence incidence among bacterial phyla
424 was highest in the pit muds (Table S4). For the co-occurrence patterns among
425 bacterial taxa, the co-occurrence incidence between classes *Clostridia* and
426 *Bacteroidia* was highest (Table S4). Both *Clostridia* and *Bacteroidia* can contribute to
427 the production of organic acids such as acetic acid, butyric acid and caproic acid
428 (Zhang et al. 2015). Additionally, the caprotic acid formations by *Clostridia* and
429 *Bacteroidia* produced hydrogen under anaerobic condition, so these bacteria must
430 culture with hydrogenotroph to reducing hydrogen stress (Ding et al. 2010). The
431 archaeal phylum *Euryarchaeota*, especially methanogens, was highly correlated with
432 bacterial phyla in our study. Mutual collaboration has been observed among bacteria
433 such as *Clostridia, Bacteroidia* and methanogens. Methanogens (e.g.,
434 *Methannobacteria* and *Methanomicrobia*) can enhance caproic acid production by
435 reducing hydrogen stress and volatile fatty acids such as acetate, lactate when they
436 cooperated with caproic-acid-producing bacteria (e.g., *Clostridia* and *Bacteroidia*)
437 (Alsaker et al. 2010; Demirel and Scherer 2008; Vanwonterghem et al. 2014). The
438 archaeal phylum *Euryarchaeota* didn't produce CSFL flavor components such as
439 caproic acid directly, however, *Euryarchaeota* can contribute to the flavor
440 components production when they cooperated with bacterial phyla that produced

441 flavor components, indicating that the co-occurrence among bacterial phyla and
442 archaeal phyla were important for the CSFL quality.

443 Network analysis indicated that *Lactobacillus* showed strong negative
444 correlation with *Clostridia*, *Methanomicrobia*, *Bacteroidia* and *Synergistia* (Figure
445 3B).The existence of *Lactobacillus* may lead to the scarcity of hydrogen, thereby
446 inhibiting the growth of methanogens (Zhang et al. 2015). Furthermore, the pH
447 decline may limit the growth of non-acid-resistant prokaryotes such as *Clostridia* and
448 methanogens (Hu et al. 2016). *Lactobacillus* produces various bacteriocins,
449 preventing the growth of Gram-positive bacteria (*e.g. Clostridia*) (Cintas et al. 2001).
450 The increased abundance of *Lactobacillus* likely inhibited the growth of
451 caproic-acid-producing bacteria and methanogens, thereby leading to pit mud
452 degradation. In our study, the relative abundance of *Lactobacillus* decreased
453 significantly while some members of *Clostridia*, *Bacteroidia*, and methanogens that
454 contribute to pit mud quality and stability increased significantly with pit mud age. It
455 suggests that the maturing process of pit mud led to a well-balanced prokaryotic
456 community by long-term mutual collaborations and interactions among different
457 prokaryotic taxa. We propose that microbial mechanisms are at the basis of the
458 relationship between the age and quality of pit mud.

459 The environmental factors pH, NH_4^+, AP and AK showed an increasing trend
460 with pit mud age, which was consistent with a previous study (Tao et al. 2014). RDA
461 analysis indicated that the environmental factors pH, NH_4^+, AP and AK had
462 significant effects on prokaryotic communities. It has been reported that a strong

463 reduction in activity of bacterial community was caused by deviations of 1.7 pH units
464 (Fernandez and Baath 2010). Significant effects of pH on prokaryotic community may
465 contribute to the narrow pH range for the growth of most bacteria (Rousk et al. 2010).
466 The pH value significantly increased with pit mud age (4.75 in 1-year pit mud to 5.69
467 in 10-year pit mud) in our study, which was consistent with previous studies (Liang et
468 al. 2016; Tao et al. 2014). The significant shift of pH value contributed to shape the
469 prokaryotic community composition in pit mud. For instance, pH was negatively
470 correlated with the abundance of genus *Lactobacillus* (Table S5). *Lactobacillus*
471 dominated at pH 4.75 and correlated significantly negative with pH, mainly due to
472 their acid-resistant properties (Dalié et al. 2010). Moreover, the strong positive
473 correlation between pH value and core genera in methanogens, *Methanosarcina* and
474 *Methanobacterium*, were also observed (Table S5), which may be explained by that
475 the suitable range of pH for most species in the core genera *Methanosarcina* and
476 *Methanobacterium* were at 5.5 to 8.5 (Battumur et al. 2016; Shimizu et al. 2015).
477 Thus the higher pH environment in older pit mud was more suitable for the growth of
478 methanogens. The strong positive correlation between pH value and the genus
479 *Aminobacterium* in class *Synergistia* may be due to that the optimum pH for the
480 growth of most species in *Aminobacterium* was at near-neutral pHs (Hamdi et al.
481 2015). NH_4^+content significantly positively correlated with the prokaryotic
482 community. One explanation could be that NH_4^+ has a direct effect on pH, thus,
483 indirectly influencing the prokaryotic community structure (Tao et al. 2014). The
484 other explanation is that NH_4^+ can be used as N-source, directly influencing the

prokaryotic community (Zheng et al.2014). Available phosphorus and potassium significantly influenced the composition of the prokaryotic community (Figure 4). Increased content of P and K in older pit mud, as observed in our study, has been reported previously (Zhang et al. 2015). With respect to P, phosphate-solubilizing bacteria may have been responsible for the increased P content during the fermentation process (Liao et al. 2010; Rodríguez and Fraga 1999). Ca^{2+} content in the pit mud, which was lower in the older pit mud, significantly influenced the prokaryotic community (Figure 4). The explanation may be that calcium lactate, produced by *Lactobacillus*, can result in pit mud compaction, thereby inhibiting the growth and activity of functional bacteria (Zhao et al. 2012).

Conclusion

Taken together, the pit mud ecosystem harboured a complex prokaryotic community, which was strongly associated with the pit mud age. The alteration of prokaryotic communities with pit mud ages, the interaction of prokaryotic communities in the different aged pit mud ecosystem, and the relationship between prokaryotic communities and environmental factors was identified in our study. These findings represent scientific evidence to support the practical experience that the quality of pit mud was higher in older fermentation pits, thus contributing to quality stabilization of pit mud.

Acknowledgements

This work was financially supported by the National Science Foundation of China (41501276), Open Fund for State Key Laboratory of Soil and Sustainable Agriculture

(Y812000008), the Doctoral Science Research Foundation of Zhengzhou University of Light Industry (2014BSJJ073), and Open Fund for the Key Laboratory of solid state fermentation for the production of strong-aroma liquor of Chinese light industry (2017JJ012). The authors thank our lab colleagues for helpful discussions.

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Table 1 Physicochemical properties of the pit mud

	pit mud from different aged pits (year)*		
Variable	1-year	6-year	10-year
pH (H_2O)	4.75 ± 0.09 C	5.10 ± 0.07 B	5.69 ± 0.16 A
NH_4^+(mg g^{-1})	1.29 ± 0.26 B	1.70 ± 0.20 AB	2.19 ± 0.54 A
TN (mg g^{-1})	22.9 ± 6.57 A	20.8 ± 0.20 A	23.8 ± 5.44 A
OM (%)	29.7 ± 6.11 A	25.6 ± 6.55 A	25.6 ± 2.34 A
AP(mg g^{-1})	1.71 ± 0.02 C	4.21 ± 0.04 B	5.74 ± 0.73 A
AK(mg g^{-1})	4.55 ± 0.07 B	4.80 ± 0.14 B	5.66 ± 0.15 A
Ca^{2+} (%)	0.72 ± 0.07 A	0.47 ± 0.02 B	0.58 ± 0.07 B

* All data are presented as means ± standard deviations (n=3). Values with different letters indicate a significant difference (P<0.05) using analysis of variance.

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637 **Table 2 Relative abundances of core prokaryotic genera in pit mud samples***

	Relative abundances of core prokaryotic communities in the different aged pit mud, % §		
phylum and genus	1-year	6-year	10-year
Firmicutes			
Lactobacillus	40.0 ± 0.97 A	11.5 ± 3.75 B	6.97 ± 2.17 B
Ruminococcus	15.6 ± 0.37 A	4.54 ± 2.91 B	15.0 ± 2.62 A
Caloramator	8.57 ± 0.13 A	2.55 ± 1.47 B	1.39 ± 0.08 B
Clostridium	1.44 ± 0.14 C	3.42 ± 0.05 A	1.84 ± 0.12 B
Syntrophomonas	1.35 ± 0.15 C	6.43 ± 1.80 A	2.52 ± 0.13 B
Sedimentibacter	1.16 ± 0.04 A	2.30 ± 1.43 A	1.22 ± 0.12 A
Unclassified *Clostridia*	0.40 ± 0.05 B	0.55 ± 0.15 B	1.38 ± 0.37 A
Unclassified *OPB54*	0.06 ± 0.00 A	0.75 ± 0.21 A	1.36 ± 1.56 A
Euryarchaeota			
Methanosarcina	3.50 ± 0.34 B	8.07 ± 4.26 B	18.6 ± 6.78 A
Methanobacterium	3.37 ± 0.35 B	2.60 ± 0.24 B	11.0 ± 5.01 A
Methanobrevibacter	2.19 ± 0.36 B	10.3 ± 2.09 A	8.74 ± 0.28 A
vadinCA11	0.51 ± 0.02 A	1.58 ± 1.47 A	1.00 ± 1.31 A
Bacteroidetes			
Unclassfied *Porphyromonadaceae*	7.98 ± 0.68 C	19.8 ± 1.80 A	15.3 ± 2.73 B
Blvii28	2.13 ± 0.12 B	7.55 ± 1.94 A	0.05 ± 0.05 C
Unclassfied *BA008*	1.06 ± 0.05 B	2.77 ± 0.13 A	0.03 ± 0.03 C
Synergistetes			
Aminobacterium	0.86 ± 0.04 C	2.60 ± 0.33 B	4.36 ± 0.89 A
Chloroflexi			
T78	0.82 ± 0.09 B	3.43 ± 0.15 A	0.21 ± 0.19 C
Actinobacteria			
Unclassfied *Coriobacteriaceae*	0.37 ± 0.07 B	0.22 ± 0.05 C	1.32 ± 0.22 A
sum	92.3 ± 0.71	86.7 ± 0.65	90.6 ± 3.52

638 *The core genera were defined that the genera with relative abundances were more than 1% of total prokaryotic
639 communities in at least one sample.
640 §All data are presented as means ± standard deviations (n=3). Values with different letters indicate a significant
641 difference (P<0.05) using analysis of variance.

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Figure captions

Figure 1 Cluster analysis of prokaryotic communities in the pit mud based on Bray-Curtis distances (A) and principal of coordinate analysis (PCoA) using weighted UniFrac distance matrices (B). In (A), Triplicate samples with same pit mud age are shown as "yr-R1" to "yr-R3".

Figure 2 Relative abundances of targeted bacterial reads in total reads retrieved from different aged pit muds at phylum level. The above taxa represented occurred at more than 1% abundance in at least one sample.

Figure 3 Networks of co-occurring prokaryotic genera in pit mud based on correlation analysis. A connection indicates a statistically significant (P<0.01) strongly positive correlation (A) Spearman's R>0.6 or a negative correlation (B) Spearman's R<-0.6. Different phyla were represented by different colors. The size of each node is proportional to the number of connections, and the thickness of each connection between two nodes is proportional to the value of Spearman's correlation coefficients of >0.6 or R<-0.6.

Figure 4 Redundancy analysis of pit mud physicochemical properties and prokaryotic community across an age gradient in pit mud, including 1-year, 6-year and 10-year pit muds. The arrows indicated the direction and magnitude of measurable variables associated with prokaryotic community structures.

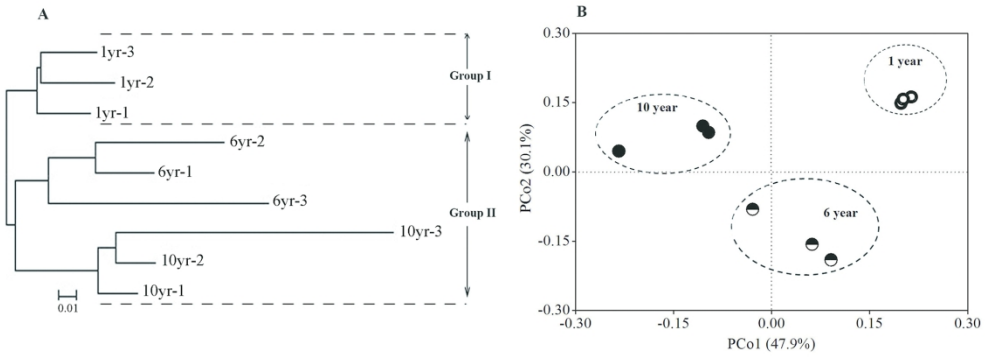

Figure 1 Cluster analysis of prokaryotic communities in the pit mud based on Bray-Curtis distances (A) and principal of coordinate analysis (PCoA) using weighted UniFrac distance matrices (B). In (A), Triplicate samples with same pit mud age are shown as "yr-R1" to "yr-R3".

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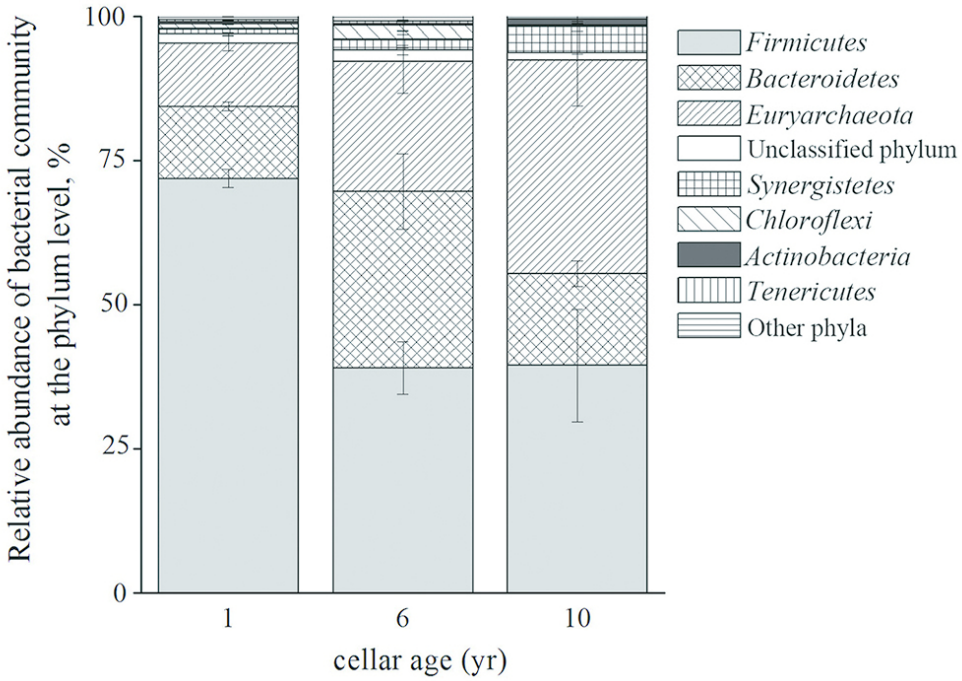

Figure 2 Relative abundances of targeted bacterial reads in total reads retrieved from different aged pit muds at phylum level. The above taxa represented occurred at more than 1% abundance in at least one sample.

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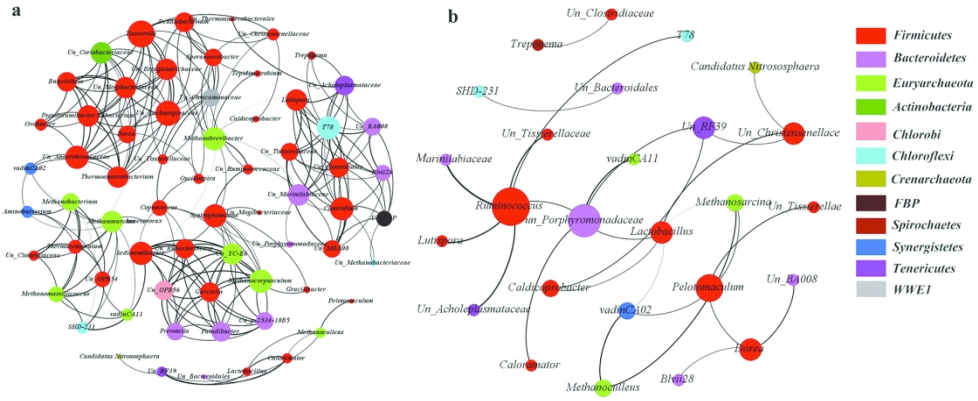

Figure 3 Networks of co-occurring prokaryotic genera in pit mud based on correlation analysis. A connection indicates a statistically significant (P<0.01) strongly positive correlation (A) Spearman's R>0.6 or a negative correlation (B) Spearman's R<-0.6. Different phyla were represented by different colors. The size of each node is proportional to the number of connections, and the thickness of each connection between two nodes is proportional to the value of Spearman's correlation coefficients of >0.6 or R<-0.6.

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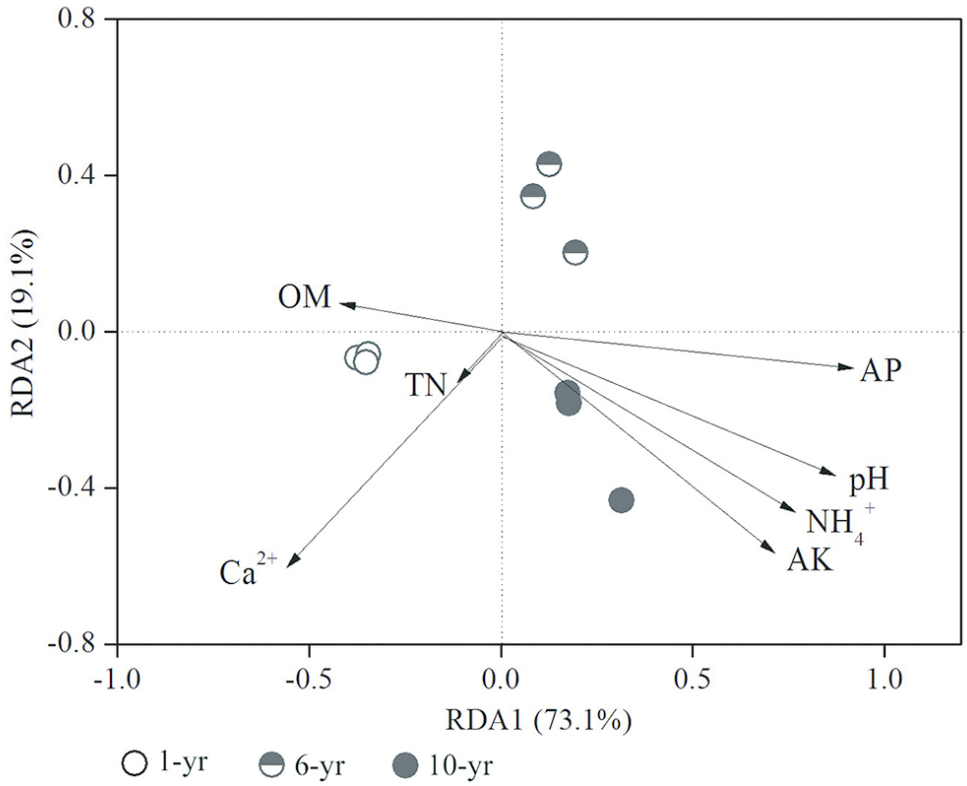

Figure 4 Redundancy analysis of pit mud physicochemical properties and prokaryotic community across an age gradient in pit mud, including 1-year, 6-year and 10-year pit muds. The arrows indicated the direction and magnitude of measurable variables associated with prokaryotic community structures.

86x69mm (300 x 300 DPI)