

## Characterization of Microbial Diversity and Community Structure in Fermentation Pit Mud of Different Ages for Production of Strong-Aroma Baijiu

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### Abstract

In the traditional fermentation process of strong-aroma Baijiu, a fermentation pit mud (FPM) provides many genera of microorganisms for fermentation. However, the functional microorganisms that have an important effect on the quality of Baijiu and their changes with the age of fermentation pit (FP) are poorly understood. Herein, the Roche 454 pyrosequencing technique and a phospholipid fatty-acid analysis were employed to reveal the structure and diversity of prokaryotic communities in FPM samples that have been aged for 5, 30, and 100 years. The results revealed an increase in total prokaryotic biomass with an FP age; however, Shannon's diversity index decreased significantly ( $p < 0.01$ ). These results suggested that a unique microbial community structure evolved with uninterrupted use of the FP. The number of functional microorganisms, which could produce the flavor compounds of strong-aroma Baijiu, increased with the FP age. Among them, *Clostridium* and *Ruminococcaceae* are microorganisms that directly produce caproic acid. The increase of their relative abundance in the FPM might have improved the quality of strong-aroma Baijiu. *Syntrophomonas*, *Methanobacterium*, and *Methanocorpusculum* might also be beneficial to caproic acid production. They are not directly involved but provide possible environmental factors for caproic acid production. Overall, our study results indicated that an uninterrupted use of the FP shapes the particular microbial community structure in the FPM. This research provides scientific support for the concept that the aged FP yields a high-quality Baijiu.

**Key words:** strong-aroma Baijiu, fermentation pit mud, microbial community, Roche 454 pyrosequencing, PLFAs

### Introduction

Strong-aroma Baijiu is a unique liquor that is clear and transparent and comprises of water, ethanol, and flavor compounds. The flavor compounds include acids, alcohols, esters, carbonyl, and phenolic compounds, etc. (Liu and Sun 2018; Wu et al. 2019; Zhao et al. 2019). Although the content of flavor compounds is less than 2%, it determines the consumers' acceptance and preference of Baijiu (Zhao et al. 2018). Microorganisms produce most flavor compounds during fermentation. Strong-aroma Baijiu has a unique production process and fermentation vessels (Fig. 1). The fermentation vessel is called a mud pit (Li et al. 2017; Liu and Sun 2018). The mud pit is a similar cuboid pond (Ding et al. 2015),

just below the horizon and surrounded by special mud that is called a fermentation pit mud (FPM). The fresh FPM is produced via a complicated process using natural yellow soil that contains the abundance of iron oxide and aluminum oxide, Daqu, the fermented exudate that is called Huangshui, and Baijiu (Sun et al. 2017; Liu et al. 2019). During the fermentation process, the FPM interacts with Huangshui and fermentation grain (Li et al. 2017). The fermentation grain was called Zaopei, and it is composed of sorghum, rice husks, and Daqu. The FPM gradually matures when the fermentation process is carried out round after round. The content of flavor substances in the produced liquor is gradually increasing until it becomes relatively stable (Tao et al. 2014; Zheng et al. 2015; Wang et al. 2017). In general

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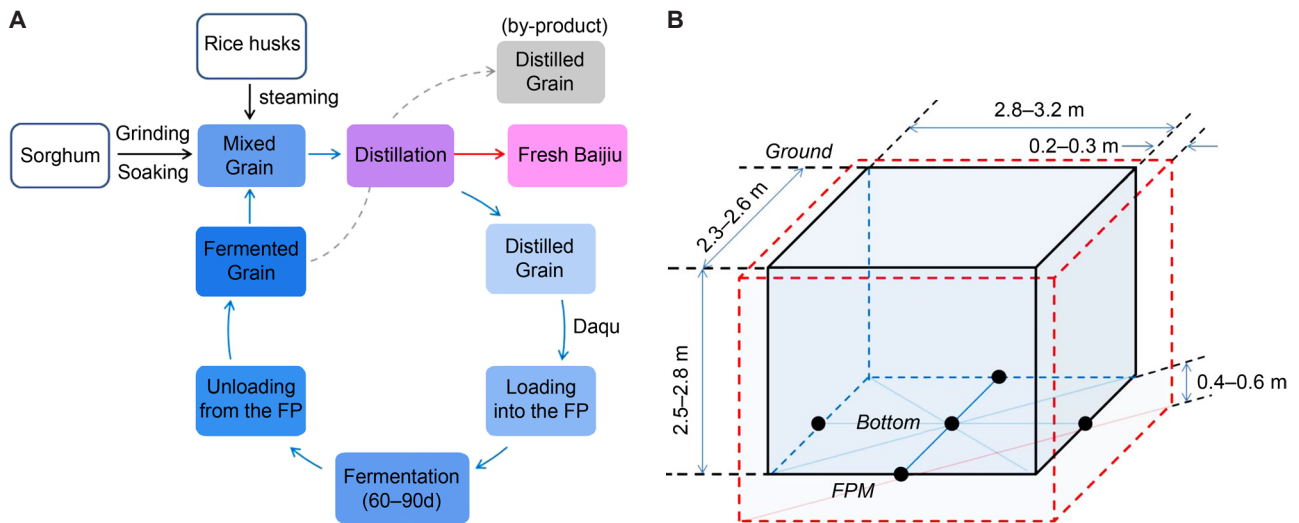


Fig. 1. The production (A) and the FP (B) of strong-aroma Baijiu.

A: The concentration of rice husks is about 20% of sorghum (dry weight); the main chemical composition of the fresh grain (sorghum and rice husks) are starch (48.1–50.4%), protein (5.3–8.3%), fat (3.2–3.7%), cellulose (8.3–10.2%), lignin (4.8–6.4%), pentosan (3.2–3.4%), tannin (0.1–0.5%), etc; the concentration of fresh grain is about 20% of mixed grain (dry weight); the concentration of Daqu is about 20% of sorghum; during fermentation, the moisture content of the grain is about 55–60%. B: The solid circle (●) represent sampling sites of FPM, the FP is surrounded by FPM.

the quality of Baijiu is improved when the fermentation pit (FP) age is more than five years (Zhao et al. 2012), and becomes relatively stable when the FP age is about 30 years (Tao et al. 2014).

During strong-aroma Baijiu brewing, multiple of microorganisms co-exist in the FP. The FPM contained plentiful of microbes that can produce the flavoring compounds (Deng et al. 2012; Zhao et al. 2012; Liu et al. 2014; Luo et al. 2014). Therefore, the quality of Baijiu partly depends on the microbes from the FPM (Tao et al. 2014; Li et al. 2017; Xu et al. 2017). In general, the PFM in the aged PF contains more microbes that can produce the flavoring compounds. (Hu et al. 2016; Liu et al. 2017; Zhang et al. 2017). In China, the people regard the aged FP as a living artifact, and it can apply for urban or national cultural heritage to protect when the FP age is more than 30 or 100 years. The most famous is *Luzhou Laojiao* national cultural heritage FP group, especially the 1573 FP group that began construction in 1573 AD (Liu et al. 2017). The aged FP can produce high-quality Baijiu (Ding et al. 2014; Tao et al. 2014; Yao et al. 2015; Xu et al. 2017). However, the key factors to produce high-quality Baijiu are microorganisms that are more conducive to the yielding of the flavor compound in the FPM (Ding et al. 2014; Hu et al. 2015; Liu et al. 2017; Chai et al. 2019). Therefore, analysis of the microbial community in the FPM of different ages is intentional and may detect the significant functional microorganisms.

With the techniques of molecular biology and the detection methods developed, the unveiling of microbial communities in environmental samples become quite an easy task. The composition of microbial

communities in samples such as soils (Torsvik and Øvreås 2002), ocean water (DeLong 2009), hot springs (Chapelle et al. 2002), gut (Chapelle et al. 2002), and FPM (Tao et al. 2014; Li et al. 2017; Chai et al. 2019) has been reported in succession. High-throughput sequencing technology is one of the most used techniques for analyzing of microbial communities. It could be used widely in the studies of Baijiu, such as analysis of the microbial communities of fermentation starters (Wang et al. 2017) or fermented grains (Chai et al. 2019), FPM (Tao et al. 2017). The determination of the content of phospholipid fatty acid (PLFA) is one of the most popular techniques for analyzing of total biomass in a sample (Green and Scow 2000; Zheng et al. 2013; Ding et al. 2015). Therefore, pyrosequencing and PLFAs were employed to assess the structure of prokaryotic communities within the FPM and to reveal the changes within these communities with age during the FPM maturation. In addition, the relationship between environmental factor variables and prokaryotic community structure and diversity in the FPM was revealed. It would be promising to find out the functional microorganisms that produced the flavor compounds of strong-aroma Baijiu.

## Experimental

### Materials and Methods

**Materials.** UltraClean Soil DNA Isolation Kit was purchased from MOBIO (USA). QIAquick Gel Extraction Kit was purchased from QIAGEN (USA).

Quant-iT™ PicoGreen™ dsDNA Assay Kit was acquired from Invitrogen (China). DNA polymerase, dNTPs, and DNA Marker were purchased from Takara (Japan). DNA sequencing was carried out at the Roche GS Junior sequencing platform.

**Samples collection.** The FPM samples were obtained from the renowned strong-aroma Baijiu producer from Luzhou, Sichuan province, China. Samples of the FP that had been utilized for 5, 30, and 100 years were sampled in triplicate, with five FP samples being collected in each FP when a round of fermentation process is just over. The sampling points were located at the bottom of the FP in the midpoint of the four sides and the intersection of the diagonal (Fig. 1). Each sample (20 g) was frozen at  $-20^{\circ}\text{C}$  and shipped to the Sichuan University of Science and Engineering, Yibin, China on dry ice for analysis of the microbiome.

**Chemical analysis.** A gravimetric approach was used to measure the FPM moisture, with soil being collected and immediately dried at  $60^{\circ}\text{C}$  for 48 h. A method previously detailed by Mehlich was used for the humic acid measurements (Mehlich 1984), while the Kjeldahl method was used to quantify total nitrogen content (Tao et al. 2014). The levels of  $\text{NH}_4^+$  in samples were measured via a sodium salicylate approach (Tao et al. 2014). Ammonium fluoride and hydrochloric acid were used to extract phosphorus (Sun et al. 2017), which was measured by SpectraMax 190 Microplate Reader (Molecular Devices, USA). Total acidity was measured via 0.1 M NaOH titration, as previously described (Wherry 1920; Zhang et al. 2017). Primary organic acids (the levels of caproic, acetic, butyric, and lactic acids) were measured with an ion chromatograph (Metrohm 761 Compact IC, Switzerland) that had a conductivity detector as well as an ion exclusion column (Metrosep Organic Acids 6.1005.200, Switzerland), as previously detailed (Rozendal et al. 2006). The available calcium was determined using ICP-OES (Agilent, USA) (Górecka et al. 2006).

**DNA extraction and PCR amplification.** The samples of the FPM were homogenized by mixing. DNA within a  $\sim 0.5$  g oven-dried mud samples were extracted with the UltraClean Soil DNA Isolation Kit following the manufacturer's instructions. Extraction was conducted on ice with a 200  $\mu\text{l}$  elution volume, after which a Nanodrop ND-2000 spectrophotometer (Nanodrop, USA) was used to quantify DNA levels. Also, 0.8% agarose gel electrophoresis was used to assess the DNA sample integrity, with  $0.5\times$  TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) being used as a buffer.

The community of the FP was prepared via a combination of equally sized 10  $\mu\text{l}$  samples of five sampling isolates from the same pit. The DNA mixture from each FP was amplified, respectively, using the two-primer pairs specific for the V2-V3 and V6-V8 regions of

the 16S rRNA gene. For V2-V3, 109F (Großkopf et al. 1998) was a forward primer: 5'-ACK GCT CAG TAA CAC GT-3' and 518R (Ovreås et al. 1997): 5'-ATT ACC GCG GCT GCT GG-3' was a reverse primer. For V6-V8, 968F: 5'-AAC GCG AAG AAC CTT AC-3' was a forward primer, and 1401R: 5'-CGG TGT GTA CAA GAC CC-3' (Sánchez et al. 2007) was a reverse primer. In addition, forward primers contained the Titanium A adapter sequence together with sample-specific 9-bp sample barcodes. Amplification reactions were conducted in a 50  $\mu\text{l}$  volume containing 50 ng DNA together with 0.4  $\mu\text{M}$  of specific primers, 5 U *Ex Taq*™ DNA polymerase, 1  $\times$  Ex Taq Buffer, and 0.2 mM dNTP. Thermocycler settings were as follows: 4 min at  $94^{\circ}\text{C}$ ; 25 cycles of  $94^{\circ}\text{C}$  for 30 s,  $56^{\circ}\text{C}$  for 30 s, and  $68^{\circ}\text{C}$  for 80 s, and 10 min at  $72^{\circ}\text{C}$ . The amplified PCR products were separated via 1% agarose gel electrophoresis, then, their purification via QIAquick Gel Extraction Kit (QIAGEN, USA) was performed based on the directions provided.

**Pyrosequencing and data analysis.** The amplified sequences of the 16S rRNA gene prepared as above were then sequenced with a Roche 454 FLX Titanium sequencing platform. The QIIME pipeline was then used to process the raw read sequences, as in previous studies (Wang J et al. 2017). Briefly, those sequences that were either  $< 200$  bp or  $> 600$  bp long were excluded from analyses, after which all high-quality sequences underwent operational taxonomic unit (OTU) clustering via USEARCH (v7.0.1090) (Edgar 2013), with a 97% similarity threshold. The sequences representatives for each OTU were obtained. An OTU was only considered valid if a minimum of five reads in the present study were associated with it. The UCHIME (v4.2.40) algorithm (Edgar et al. 2011) was used for chimera filtration. The optimized sequences were used for OTU alignment, and OTU abundance in each sample was then analyzed. The SILVA web-based tools (<http://www.arb-silva.de>) were used for representative OTU sequence taxonomic classification (Quast et al. 2012).

**PLFA extraction and analysis.** The FPM samples were freeze-dried and grounded using a ball mill to a particle size of fewer than 10  $\mu\text{m}$  prior to analysis. A modified version of a three-step protocol was then used for PLFA extraction (Bligh and Dyer 1959). Briefly, methanol, chloroform, and water were used to extract lipids from the soil, after which a silicic acid column was used to separate out phospholipids, neutral lipids, and glycolipids. A gas chromatograph (Agilent 6890) equipped with a 19091B-102 column (25.0 m  $\times$  200  $\mu\text{m}$   $\times$  0.33  $\mu\text{m}$ , Agilent Technology, USA) and a flame ionization detector (FID) was then used for the alkaline methanolysis of phospholipids. GC settings for this analysis were: a  $250^{\circ}\text{C}$  inlet temperature, a 10:1 split ratio, and a 1 ml/minute flow of hydrogen

Table I  
PLFA markers used for taxonomic groups.

| Taxonomic group        | PLFA group                 | Specific PLFA markers   | Reference                    |
|------------------------|----------------------------|---|------------------------------|
| Bacteria               | Multiple groups            | Sum of i15:0, a15:0, i16:0, i17:0, a17:0, cy17:0, cy19:0, 16:1 $\omega$ 7, 18:1 $\omega$ 7, and 17:1 $\omega$ 9 | (Frostegård and Bååth 1996)  |
| Gram-positive bacteria | Branched PLFAs             | Sum of i15:0, a15:0, i16:0, i17:0, and a17:0  | (O'Leary 1988)               |
| Gram-negative bacteria | Cyclopropyl and mono PLFAs | Sum of cy17:0, 16:1 $\omega$ 7, 18:1 $\omega$ 7, and 17:1 $\omega$ 9  | (Moore-Kucera and Dick 2008) |
| Actinomycetes          | 10Me-PLFAs                 | Sum of 10Me16:0, 10Me17:0, and 10Me18:0   | (Moore-Kucera and Dick 2008) |
| Anaerobes              | Cyclopropyl                | Sum of cy17:0, cy19:0 $\omega$ 7, cy19:0 $\omega$ 9   | (Li et al. 2017)             |

as a carrier gas. The oven was warmed for 2 minutes at 140°C, and the temperature was then raised by 5°C per minute up to a final 250°C temperature where it was maintained for 5 minutes. Peak areas were then compared to those of an internal C19:0 reference standard (Fluka, Switzerland) in order to quantify PLFAs. Fatty acids that had a < 0.5% overall relative abundance were omitted from this data set. Besides, PLFA nomenclature was designated according to a previous report (Moore and Dick 2008). The abundance of certain microbial groups was assessed by using specific PLFAs as biomarkers (O'Leary 1988; Frostegård and Bååth 1996; Moore-Kucera and Dick 2008; Li et al. 2017) (Table I), whereas PLFA 16:0 is present within all species of bacteria, plants, and fungi, and was therefore not considered to be a group-specific PLFA (Ding et al. 2015).

**Data analysis.** All data were means  $\pm$  standard deviation from triplicate analyses, and were compared via ANOVAs.  $P < 0.05$  was the significance threshold. SPSS 17.0 was used for all statistical analyses. The redundancy analysis (RDA) and microbial community analysis were performed using a program of RStudio (v1.0.136).

## Results and Discussion

**Chemical properties of the FPM.** The physico-chemical properties of the FPM are shown in Table II. The moisture increased in the FPM with age, and it may imply that there were differences in the microbial metabolic activity. The samples containing higher moisture might represent higher microbial metabolic activity. The available phosphorus can be an indirect indicator of biomass; it increased from 5 to 30 years and then stabilized. This pattern could also represent changes in biomass. There was no significant difference in total acidity; however, the content of caproic acid increased, and the content of lactic acid significantly declined with the FP age ( $p < 0.05$ ). Caproic acid is produced by microbial fermentation. The high caproic acid content indicated that the FPM contained more numerous microorganisms producing caproic acid. Lactic acid mainly comes from Huangshui, and it was produced during fermentation, and its content was similar in FP of different ages. The content of lactic acid in the FPM was variable, indicating that there was a different abundance of microorganisms that could use lactic

Table II  
Basic physicochemical properties of fermentation pit mud.

| Variable                            | Values for fermentation pit age (yr) <sup>a</sup> |                  |                  |
|-------------------------------------|---|------------------|------------------|
|                                     | 5   | 30               | 100              |
| Moisture(%)                         | 35.15 $\pm$ 6.42                                  | 39.62 $\pm$ 1.11 | 44.97 $\pm$ 2.83 |
| Humic matter (%)                    | 10.54 $\pm$ 1.59                                  | 13.82 $\pm$ 0.09 | 10.90 $\pm$ 0.52 |
| Total acidity(%)                    | 1.54 $\pm$ 0.09                                   | 1.81 $\pm$ 0.05  | 1.13 $\pm$ 0.15  |
| Acetic acid(mg/g)                   | 3.98 $\pm$ 0.36                                   | 3.44 $\pm$ 0.46  | 4.21 $\pm$ 0.67  |
| Lactic acid(mg/g)                   | 94.22 $\pm$ 1.30                                  | 28.81 $\pm$ 7.05 | 21.33 $\pm$ 3.15 |
| Butyric acid(mg/g)                  | 2.87 $\pm$ 0.34                                   | 2.36 $\pm$ 0.26  | 2.79 $\pm$ 0.38  |
| Caproic acid(mg/g)                  | 15.15 $\pm$ 5.12                                  | 29.62 $\pm$ 4.19 | 45.97 $\pm$ 5.13 |
| Total nitrogen(%)                   | 0.94 $\pm$ 0.09                                   | 1.41 $\pm$ 0.08  | 2.16 $\pm$ 0.11  |
| NH <sub>4</sub> <sup>+</sup> (mg/g) | 14.12 $\pm$ 1.77                                  | 21.02 $\pm$ 2.03 | 25.32 $\pm$ 1.66 |
| Available phosphorus(mg/g)          | 4.91 $\pm$ 0.52                                   | 9.15 $\pm$ 0.78  | 9.15 $\pm$ 0.41  |
| Available calcium(mg/g)             | 9.51 $\pm$ 1.02                                   | 8.18 $\pm$ 1.91  | 7.41 $\pm$ 0.52  |

<sup>a</sup>All data are presented as means  $\pm$  standard deviations (n = 3)



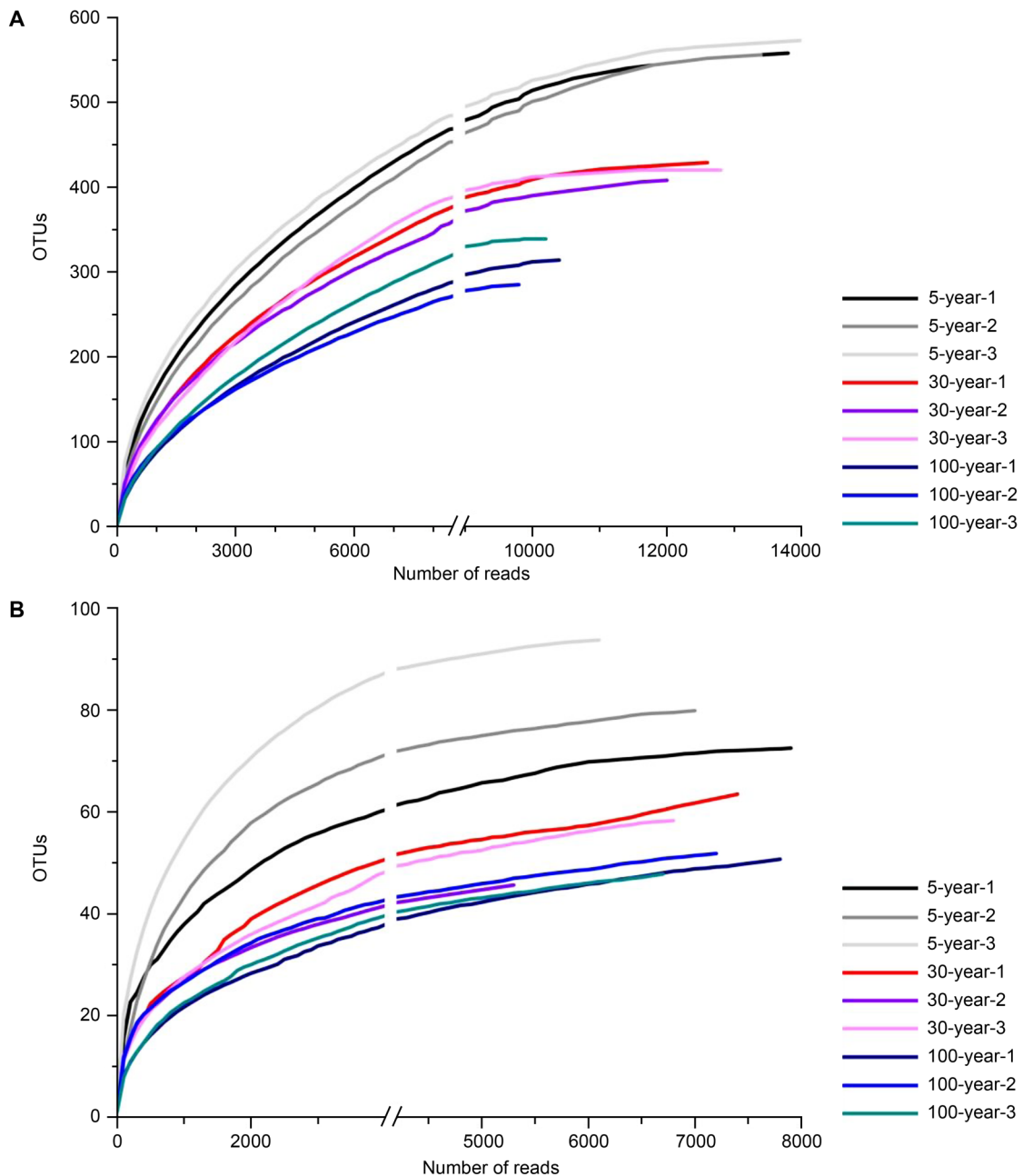


Fig. 2. OTUs rarefaction curves at a 97% sequence similarity cutoff. OTUs from the amplicon libraries of bacteria (A), and archaea (B).

acid. The content of  $\text{NH}_4^+$  increased in the FPM with age; it may imply that old FPM might contain more microorganisms metabolizing amino acids to produce  $\text{NH}_4^+$ . Total nitrogen increase could be understood as an accumulation process that included a dynamic balance of nitrification and nitrogen loss. The available calcium decrease could be considered as a gradual loss process when compared to Huangshui.

**The prokaryotic community structure and diversity.** Each of the FP samples generates two amplicon libraries by amplification of V2-V3 and V6-V8 regions. Eighteen amplicon libraries were sequenced using the Roche 454 Junior platform, yielding 2.2 Gb of data, of

which 34% passed Q30. After quality control, denoising, and chimera removal, we obtained 211,740 reads, with an average 377.4 bp read length. A total of 1,089 OTUs (952 OTUs belong to bacteria, 137 OTUs belong to archaea) were obtained based on a 3% dissimilarity in the sequences of the 16S rRNA gene, considering those OTUs with  $\geq 5$  sequences to be valid ones. Rarefaction analyses revealed a good representation of all prokaryotic communities within these samples, as all the curves approached a saturation plateau (Fig. 2).

Compared with archaea, the bacteria occupy an absolute advantage on OTUs. As the age of the FPM increases, the diversity of microorganisms decreases.

Table III  
Prokaryotic diversity indices based on a cutoff of 97% similarity of the 16S rRNA gene sequences<sup>a</sup>.

| FPM age (yr) | Bacteria     |              |             | Archaea     |              |               |
|--------------|--------------|--------------|-------------|-------------|--------------|---------------|
|              | Chao1        | Observed OTU | Shannon     | Chao1       | Observed OTU | Shannon       |
| 5            | 634.0 ± 84.4 | 583 ± 13     | 2.49 ± 0.56 | 97.3 ± 12.2 | 75 ± 7       | 1.374 ± 0.049 |
| 30           | 501.6 ± 64.6 | 419 ± 11     | 2.16 ± 0.31 | 78.5 ± 6.3  | 53 ± 5       | 1.159 ± 0.068 |
| 100          | 435.8 ± 62.1 | 312 ± 27     | 1.93 ± 0.34 | 74.2 ± 4.7  | 48 ± 4       | 1.068 ± 0.087 |

<sup>a</sup>All data are presented as means ± standard deviations (n = 3)

Table IV  
The preference of sequencing results based on different variable regions V2-V3 and V6-V8<sup>a</sup> of the 16S rRNA gene.

| Variable region | FPM age (yr) | Total       |          | Bacteria    |          | Archaea     |        |
|-----------------|--------------|-------------|----------|-------------|----------|-------------|--------|
|                 |              | reads       | OTU      | reads       | OTU      | reads       | OTU    |
| V2-V3           | 5            | 10977 ± 684 | 126 ± 12 | 1267 ± 104  | 51 ± 9   | 9710 ± 562  | 75 ± 7 |
|                 | 30           | 10562 ± 513 | 100 ± 11 | 1009 ± 84   | 47 ± 8   | 9553 ± 410  | 53 ± 5 |
|                 | 100          | 11172 ± 751 | 75 ± 6   | 938 ± 53    | 27 ± 5   | 10233 ± 720 | 48 ± 4 |
| V6-V8           | 5            | 18893 ± 886 | 594 ± 17 | 17826 ± 844 | 583 ± 13 | 1067 ± 89   | 11 ± 3 |
|                 | 30           | 17409 ± 395 | 428 ± 15 | 16543 ± 354 | 419 ± 11 | 866 ± 68    | 9 ± 2  |
|                 | 100          | 16894 ± 806 | 319 ± 24 | 15537 ± 758 | 312 ± 20 | 1357 ± 133  | 7 ± 2  |

<sup>a</sup> All data are presented as means ± standard deviations (n = 3)

This result reflected in the rarefaction curve that the total number of OTUs in the sample decreases (Fig. 2). The lowest diversity was observed in the 100-year samples. Shannon's diversity index decreased significantly with the FP age ( $p < 0.05$ ) from 5- to 30-year old FPM, and it became constant in the 30- to 100-year old FPM. Richness levels (OTUs) of bacteria significantly decreased in the 5-, 30-, and 100-year samples ( $p < 0.05$ ). For archaea, it decreased in the 5-year to 30-year aged samples and were similar in the 30-year to 100-year aged samples (Table III). In general, the microbial community structure changed in both periods, but the change was faster in the period from 5 to 30 years than from 30 to 100 years. Although the variable of the number was similar, the latter period takes more time (70 years). These results indicated that the microbial community decreases rapidly and then gradually stabilizes. It could be the adaptation pattern of microorganisms to this unique habitat of the FP (the enriched organic matter, acidity, and lack of oxygen). The adaptive microorganisms enriched gradually, and formed the dominant species, and eventually shaped a stable community structure.

#### Phylogenetic composition of the FPM community.

The significant differences were observed in sequencing results using different variable regions (V2-V3, V6-V8) of the 16S rRNA gene (Table IV). The results showed that most of the reads (about 90.2%) belonged to archaea when sequencing was performed with the V2-V3. However, most of the reads (about 93.8%)

belonged to bacteria when sequencing was performed within the V6-V8. These results indicate that the variable regions have a preference for some types of microorganisms. The variable region V2-V3 is more suitable than V6-V8 for analyzing community of archaea, and V6-V8 is more suitable than V2-V3 for analyzing community of bacteria in the FPM samples. Thus, different primers may lead to different results. Therefore, the choice of suitable primers based on the characteristics of the microbial structure is essential to obtain the objective results. There are abundant microbial species in the FPM, including bacteria and archaea. What kind of primers is most suitable for the particular environment samples? This issue requires further research.

There were 952 OTUs that belonged to bacteria, of which 95.9% were relative of low abundance (<1%), and 21.8% (208) OTUs were present in all samples. 137 OTUs belonged to archaea, of which 93.4% were relative of low abundance (<1%), and 20.4% (28) OTUs were present in all samples. These results showed that although there were many types of microorganisms, there were also the dominant microorganisms in the FPM. These dominant microorganisms may be functional microorganisms of the FPM.

Most bacteria belonged to five phyla *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria*, and *Synergistetes*. The two most dominant phyla of bacteria were *Firmicutes* (75.8%), and *Bacteroidetes* (5.6%) (Fig. 3). Only *Euryarchaeota* was observed within archaea in all FPM samples (data not show).

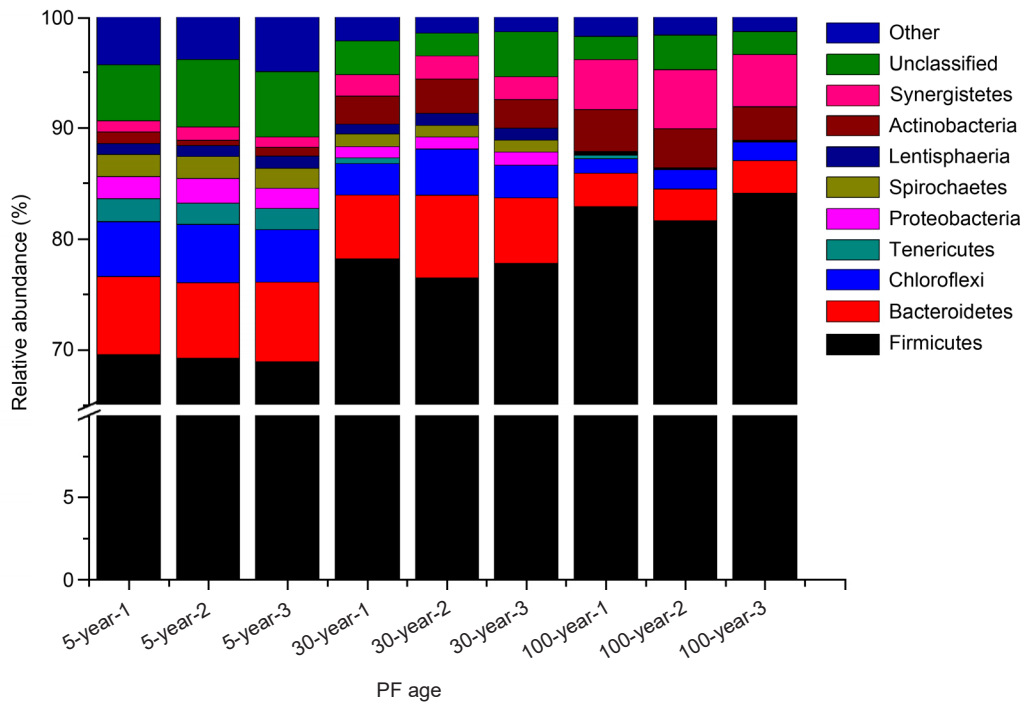


Fig. 3. Phylum level prokaryotes taxonomic classification in the FPM samples as determined with the RDP classifier using an 85% confidence threshold.

At the genus level, most bacteria belonged to 29 genera (Fig. 4A), 11 of them are core genera. The core genera were those present within all samples and had a relative abundance of more than 1.0%. The core genera belonged to *Firmicutes* (*Lactobacillus*, *Ruminococcaceae*, *Bacillus*, *Syntrophomonas*, *Clostridium*, *Carnobacterium*, and *Sporanaerobacter*), *Bacteroidetes* (*Petrimonas* and *Proteiniphilum*) *Actinobacteria* (*Celulomonadaceae*) and *Chloroflexi* (*Anaerolineaceae*). Most archaea belonged to seven genera (Fig. 4B); six of them were core genera: *Methanobacterium*, *Thermoplasmatales*, *Methanoculleus*, *Methanosata*, *Methanobrevibacter*, and *Methanocorpusculum*. These core genera constituted 53–77% of total abundance.

**Microbial community structure changes with the FP age.** *Firmicutes* were the dominant phyla; its relative abundance increased with the FP age. It is worth noting that all the seven core genera of *Firmicutes* increased in number with the FP age. This result showed that *Firmicutes* could adapt to the special environment of the FP. This adaptation may benefit from their special stress-resistant structure, such as thick cell walls. It may also advantage from their diverse metabolic patterns. *Actinobacteria* relative abundance increases with the FP age. The core genus of *Acinetobacter*, *Celulomonadaceae*, may be involved in cellulose metabolism (Stackebrandt and Schumann 2015). Although there was cellulose in the FP, the harmful environmental factors such as acidity and lack of oxygen may be the reasons for hindering its rapid increase. *Synergistetes* did not contain core genera, but its relative

abundance had increased significantly. It also contained only one genus, *Aminobacterium*, which might be involved in amino acid metabolism (Baena et al. 2015). The abundant amino acids in the environment provided favorable conditions for the increase of its relative abundance. *Bacteroidetes* includes two core genera, *Proteiniphilum* and *Petrimonas*; their relative abundance decreases with the PF age. *Proteiniphilum* might be involved in protein metabolism (Whitman et al. 2015b), *Petrimonas* could metabolize glucose through fermentation (Whitman et al. 2015a), and the reason for their reduction needs further study. *Chloroflexi* contained a core genus, its relative abundance decreased with the FP age. The core genus, *Anaerolineaceae*, might be involved in carbohydrate and protein metabolism (Yamada and Sekiguchi 2020), the product included hydrogen. The high partial hydrogen pressure in the FP might be the reason for its reduction. The relative abundance of the other four phyla decreased with age. All speculations about metabolism mentioned above were based on the Bergey's Manual of Systematics of Archaea and Bacteria.

Regarding archaea, the relative abundance of two core genera *Methanobacterium* and *Methanocorpusculum* increased with the FP age, and the other four core genera, *Methanoculleus*, *Methanobrevibacter*, *Methanosata*, and *Thermoplasmatales*, decreased with the FP age. *Methanobacterium* and *Thermoplasmatales* had the highest relative abundance. However, their trends of change were in the opposite. This change might be the result of competition among microbial genera.

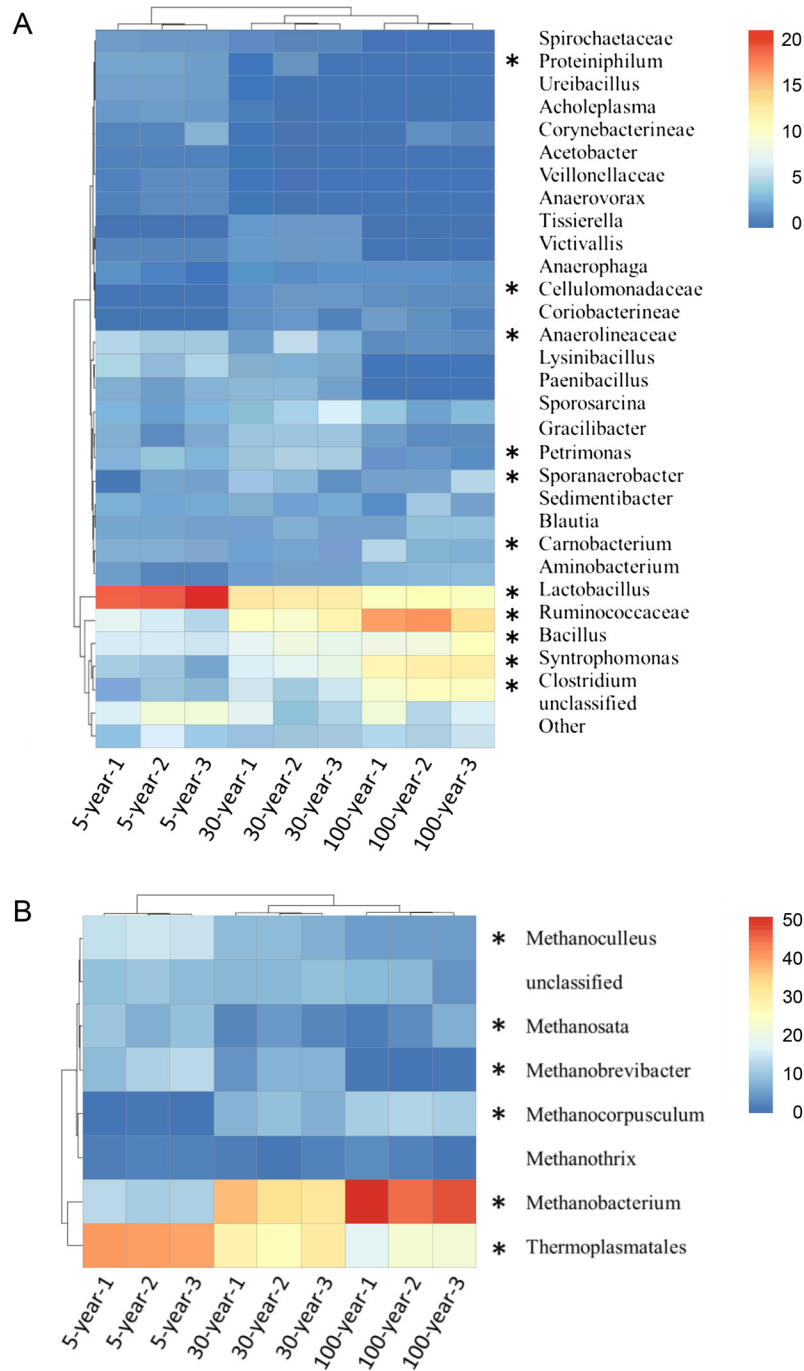


Fig. 4. The relative FPM prokaryotic genera abundance, based on Roche 454 pyrosequencing. It includes the genera of bacteria (A) and genera of archaea (B). Variations in  $\log_2$  scale-based genera abundance are shown by the scale bar, with “\*” indicating the genera shared among all FPM samples that had the relative abundance >1%.

*Thermoplasmatales* might be inferior in the competition because it lacks a true cell wall (Langworthy 2015).

**Quantitative Analysis of Specific Microbial Groups Based on PLFAs Detection.** Microbial biomass was assessed based on PLFAs concentrations in the FPM. A total of 30 PLFAs were identified (Fig. 5). The results show that the concentrations of most PLFAs in the FPM were different in the samples of different ages. It indicates that there was a difference in the microbial community structure in the FPM of different

ages. This result is a validation of the sequencing results and once again shows that the community structure changes with FP age.

The concentrations of total PLFAs increased with the pit age (Fig. 6). This result suggested that the total biomass increased with age. However, microbial diversity decreased with age (Table III). These results suggested that the number of specific microbial species had increased. The changes in the contents of microbes, such as Gram-positive bacteria, anaerobes, and actinomy-



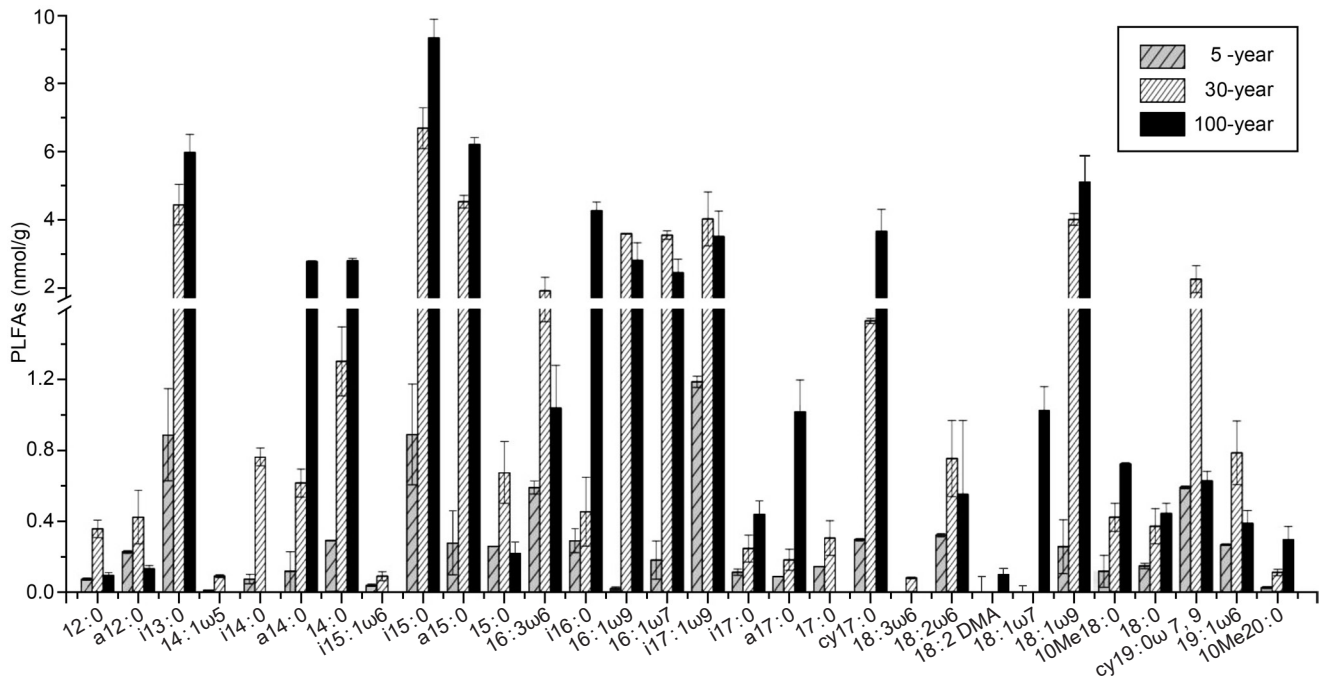


Fig. 5. Concentration of phospholipid fatty acids (PLFAs) in different FPM samples. The concentration of each sample was determined by an internal standard (19:0). Error bars indicate standard deviations ( $n = 3$ ).

ceces were similar to the changes of total PLFA (Fig. 6). The probable reason that the biomass of Gram-positive bacteria increased with the FP age is that these groups of microorganisms have thick cell walls and strong resistance to stress. This result is consistent with changes in the structure of the microbial community. There are 11 core bacterial genera, six of them belong to Gram-positive, and five of them belong to Gram-negative (Ezaki 2015; Hammes and Hertel 2015a; Hammes and Hertel 2015b; Hernandez-Eugenio et al. 2015; Logan and Vos 2015;

Rainey et al. 2015; Sekiguchi 2015; Stackebrandt and Schumann 2015; Whitman 2015a; Whitman 2015b; Yamada and Sekiguchi 2020). The relative abundance of the seven core bacterial genera increased with the FP age, there were five genera belonging to Gram-positive and two genera belonging to Gram-negative. The relative abundance of four core bacterial genera decreases with age; there were three genera of Gram-negative and one genus of Gram-positive. Environmental screening may be responsible for the relative abundance of the

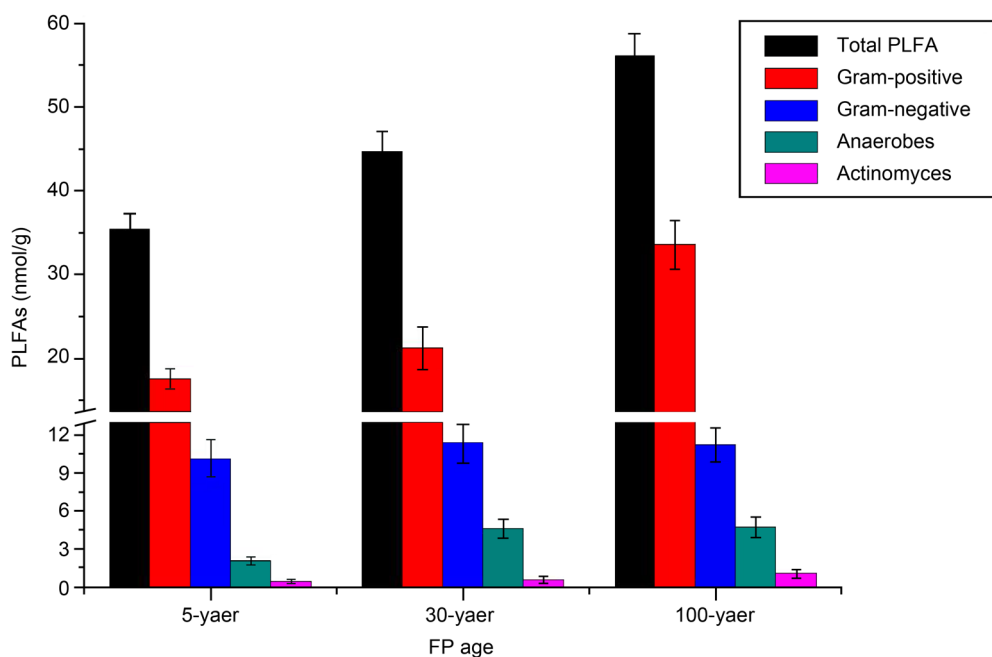


Fig. 6. Concentrations of phospholipid fatty acids (PLFA) in different samples at the microbial groups level. Error bars show standard deviations ( $n = 3$ ).

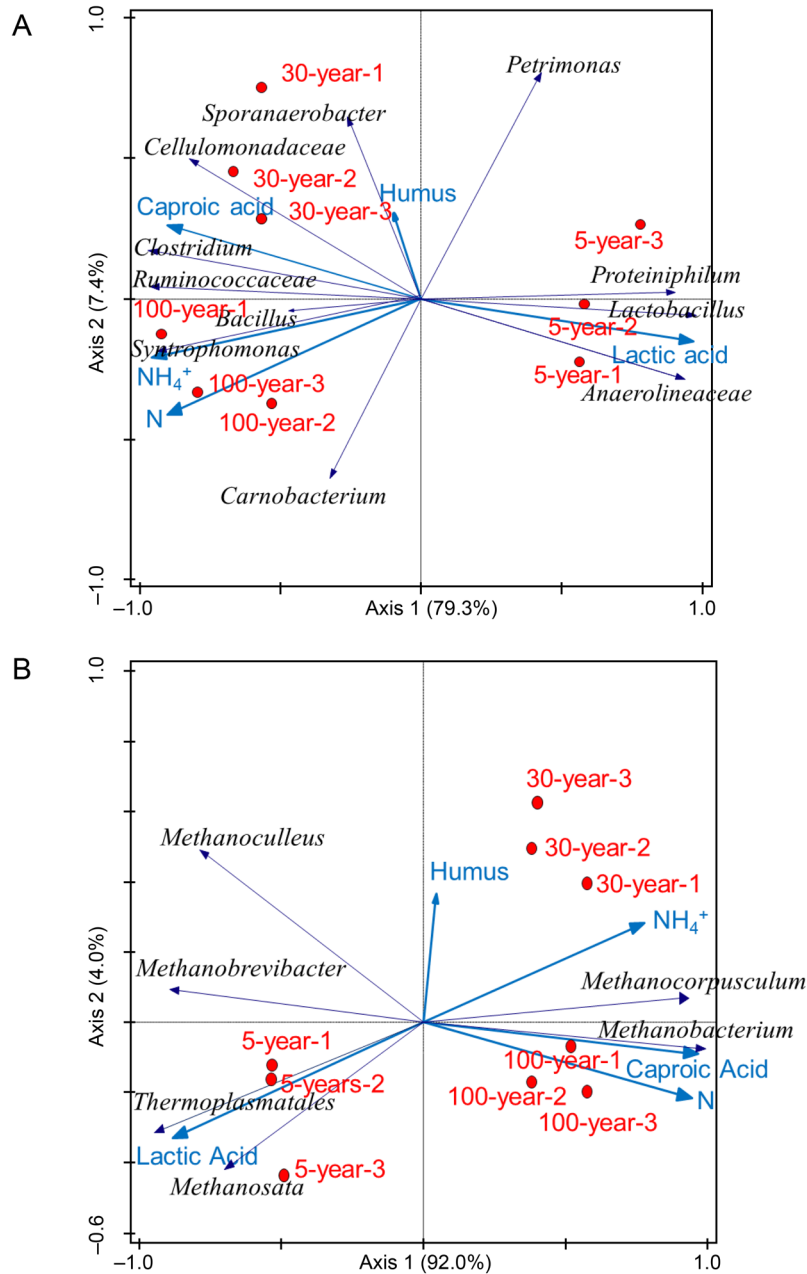


Fig. 7. Prokaryotic community redundancy analysis (RDA) based upon Roche 454 pyrosequencing and chemical properties. The dominant community structure-related genera and the direction/magnitude of chemical properties are represented by arrows. It includes the genera of bacteria (A) and genera of archaea (B).

anaerobic group. During long-term fermentation, in the FP, an anaerobic environment occurs. The reason that the relative abundance of actinomycetes increases with the FP age needs further study, and the relative abundance of core genus, *Cellulomonadaceae*, increased with the FP age, and it might be a key factor.

**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. 7). The two axes with respect to bacteria community differentiation explained 86.7% of the variation, while these two axes explained

96% of the archaeal variation, suggesting the remarkable correlation between prokaryotic community structure and environmental factors. Lactic acid levels mainly correlated positively with prokaryotic communities in the 5-year samples. However, caproic acid and NH<sub>4</sub><sup>+</sup> levels mainly correlated with those in the 30- and 100-year samples. Humic acid and TN levels mainly correlated with those in the 30- and 100-year samples, respectively.

The content of lactic acid correlated positively with *Lactobacillus*, and negatively correlated with *Clostridium*, *Ruminococcaceae*, and *Syntrophomonas*. These results suggested that lactic acid might be produced by *Lactobacillus*, and *Clostridium*. *Ruminococcaceae* and

*Syntrophomonas* may be involved in the process of lactic acid consumption. The content of caproic acid correlated positively with *Clostridium*, *Ruminococcaceae*, *Syntrophomonas*, *Methanobacterium*, and *Methanocorpusculum* abundance, and negatively correlated with *Lactobacillus* and *Thermoplasmatales* levels. *Clostridium* is a classic microorganism that produces caproic acid using ethanol (Rainey et al. 2015). *Ruminococcaceae* can produce caproic acid from lactic acid, such as *Ruminococcaceae bacterium* CPB6 (Yin et al. 2016). *Syntrophomonas* can use carboxylic acid to produce acetic acid and hydrogen. *Syntrophomonas* dependence on caproic acid may be the reason for its positive correlation with caproic acid content. A high partial pressure of hydrogen accompanied the production of caproic acid. This condition is also beneficial to *Methanobacterium* (Boone 2015), and *Methanocorpusculum* (Chong and Boone 2015). The symbiotic interactions between them need further study. The content of humic acid correlated positively with *Cellulomonadaceae*. Humus produced via the degradation of lignin and cellulose, and its content in the 30-year samples was significantly higher than those in the 5-year and 100-year (Table II). It is worth noting that the trend of changes in the number of *Cellulomonadaceae* is similar to that of humus level. *Cellulomonadaceae* may be involved in cellulose metabolism (Stackebrandt and Schumann 2015). The content of TN and  $\text{NH}_4^+$  affected the relative abundance of various microbial genera; they involved amino acid metabolism, nitrification, and denitrification. In addition, the nitrogen source, as the main nutrient element for microbial growth and development is closely related to the microbial community.

The FPM provides a large number of microorganisms for the fermentation of strong-aroma Baijiu. It involved protein and amino acid, carbohydrate, lignin and cellulose metabolism, as well as hydrogen, acid and methane production, etc. The most relevant to the quality of strong-aroma Baijiu is the production of caproic acid. *Clostridium* and *Ruminococcaceae* are microbes that directly produce caproic acid. Increasing their relative abundance in the FPM could improve the quality of strong-aroma Baijiu. In addition, the simplest way to increase the relative abundance of these two genera of microorganisms is to inoculate them in large quantities into the FPM. Nevertheless, this may not necessarily be an effective method. Although inoculation with microorganisms can temporarily increase relative abundance, there is a risk of decline. The strict monitoring of ecological factors makes the evolution of microorganisms beneficial to these two groups; thus, forming the dominant populations can provide better results. What ecological factors do they need? This issue needs further study. *Syntrophomonas*, *Methanobacterium*, and *Methanocorpusculum* may also be benefi-

cial to caproic acid production. These genera are not directly involved, but provide possible environmental factors for caproic acid production.

## Conclusions

These results offer insight into microbial community structural diversity within the FPM used for producing strong-aroma Baijiu as determined via Roche 454 pyrosequencing and PLFA. Our results demonstrate that the variable regions have a preference for the specific groups of microorganisms in the FPM, and sequencing with the appropriate variable region can make the results more objective. The complex functional populations of microorganisms inhabit the FPM ecosystem, and the microbial community structure in the FPM changes with the FP age. The total prokaryotic biomass in the FPM increased with the FP age; however, Shannon's diversity index decreased significantly ( $p < 0.01$ ). These results suggested that a unique microbial community structure evolved with uninterrupted use of the FP. The results of RDA correlations indicate that *Clostridium* and *Ruminococcaceae* are genera that directly produce caproic acid. Increasing their relative abundance in the FPM could improve the quality of strong-aroma Baijiu. The control of ecological factors makes the community succession of microorganisms beneficial to these two groups, which could promote the quality of strong-aroma Baijiu. However, the way of control of these ecological factors requires further research.

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## Authors' contributions

Jie Deng conceived and designed the experiments; Xu-Jia Wang performed the experiments; Hong-Mei Zhu analyzed the data; Zhi-Qiang Ren completed the manuscript; Zhi-Guo Huang and Chun-Hui Wei gave important suggestions.

## Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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