The Effect of Spatial Location of Fermentation Pit on Prokaryotic Community Diversity in Pit Mud for Chinese Strong-Flavor Baijiu Production

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The effect of spatial location of fermentation pit on prokaryotic community diversity in pit mud for Chinese strong-flavor Baijiu production

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Abstract

Background

Chinese strong-flavor baijiu (CSFB) accounts for more than 70% of all Chinese liquor markets. Diverse microbes in pit mud found in the fermentation pit play a key role in CSFB production. However, the effect of spatial location on the diversity and structure of the microbial community in pit mud is still poorly understood

Results

Prokaryotic microbes in different pit mud（4- and 40-year）were analyzed by using Illumina MiSeq sequencing of 16S rRNA gene. The samples were collected from pit mud that was 4 and 40 years old in the top, middle, and under parts of the cellar walls and at the bottom of them. The results showed there was no significant difference ($p>0.05$) in the physicochemical factors, the Observed OTU, and α-diversity between the pit mud of two different ages. The index of the 4-year-old pit mud was lower (except for total acid, which was higher than) than that of the 40-year-old pit mud. The pH, total acid, Observed OTU, Chao 1, ACE in the 4-year-old pit mud (in the top and middle part of the cellar wall) and the 40-year-old-pit mud(in the top part of the cellar wall) had significant ($p<0.05$) difference. At the phylum level, Firmicutes (56.70%) and Bacteroidetes (26.56%) accounted for more than 80% of the overall level. For the 4-year-old pit mud sample, the dominant genuses at each location were *Proteiniphilum*, *Lactobacillus*, and *Caproiciproducens*, while for the 40-year-old pit mud, there were 10 common genuses accounted for more than 50% of the dominant genuses in different pit mud belong to Clostridia. The RDA analysis results showed that 85.71% of the dominant bacteria positively correlated with pH, and especially the

most correlated with the total acid and available phosphorus, so its content was significantly related to the dominant bacteria ($p<0.01$). Therefore, it inferred that the total acid and available phosphorus were the main physicochemical factors that affected the spatial distribution of prokaryotic microbial communities in the pit mud of cellars from the liquor distillery.

Conclusions

Comparing the young pit mud and the old pit mud, the structure and physicochemical factors of the prokaryotic microbial community in pit mud from Henan liquor-making company changed, and the changes occurred in the spatial location of different pits. Clostridia, which accounted for more than 50% of all types of pit mud, were responsible for the effect of total acid and available phosphorus on the microbial community. Based on the above, it provides a theoretical basis for Henan liquor-making companies in maintaining pit mud.

Keywords: fermentation pit mud; spatial heterogeneity; microbial community; physicochemical factors; Chinese strong-flavor Baijiu.

Background

Chinese strong-flavor baijiu (CSFB) is one of the four flavors of liquor in China. Under the joint action of the environment, Daqu, and microorganisms in the cellar, Chinese strong-flavor baijiu was obtained through a series of complex substances and energy metabolism[1] of bacteria. With the acclimation and development of pit mud microorganisms, a unique microbial community has gradually formed in a few years or hundreds of years, and the unique cellar aroma of CSFB depends on the unique microbial community within these pit mud[2]. Besides, there were differences in the living environment of microorganisms in different locations of the pit pool, and typical spatial heterogeneity existed in the pit mud microbial community. These differences affected the production and metabolism of microorganisms in the pit mud [3]. Therefore, it is necessary to study the cellar mud of different ages and in different spatial locations, and use a systematic method to analyze the distribution characteristics of the microbial community in pit mud.

In recent years, with the use of pyrosequencing technology, Tao et al. [6] analyzed the structures of prokaryotic microbial communities in various ages (1, 10, 25, and 50 years) of pit mud, and found the cellar mud needed at least 25 years to reach maturity; Using metagenomic sequencing, Guo et al. [17]found the microorganisms Euryarchaeota and Bacteroidetes in cellar mud increased in 50, 140, 220, and 440 years as the age of the cellar increased; Liu et al. [7] first reported the *Caproiciproducens* for Chinese Baijiu production in pit mud by using Illumina MiSeq sequencing technology; Li[4]and Hu[5] and others concluded that the composition of pit mud microbial community was significantly affected by environmental variables. At present, there are many studies on pit mud from distilleries in Sichuan, Guizhou, and other provinces[1, 2,3,5,6,7], most of them are about the analysis of the diversity

of pit mud microbial community in different pit ages, qualities, and geographical locations, few related studies on the distribution characteristics of the microbial community of CSFB mud from distilleries in Henan province. Zhang Huimin et al. [8] found there was a certain difference in the composition of microbial community between the young and the old cellar mud. This shows that there is a correlation between the distribution and the composition of the microbial community in pit mud. Therefore, in this study, we collected samples from a 4- and 40-year-old pit mud occupied on the top, middle, under, and bottom cellar wall in a CSFB company in Henan. Illumina MiSeq sequencing technology was used to analyze the distribution characteristics of the cellar mud microbial community. It provided plentiful information for the study of the microecology of pit mud in Henan liquor distilleries and also provided a theoretical basis for the specific influence of different ages and spatial locations of pit mud in its distribution of microbial communities.

Results

Physicochemical factors of different pit mud

In Table 1, compared with the 40-year pit mud, the water content, pH value, ammonium nitrogen, and available phosphorus content of the 4-year-old pit mud were lower but its total acid content was significantly higher ($p<0.05$). There was a significant difference ($p>0.05$) neither in the moisture content of different aged pit mud nor in the physicochemical factors of 40-year-old pit mud in different locations. In comparison with the 40-year-old pit mud, the pH value of the 4-year-old pit mud was lower ($p<0.05$) (on the top and middle cellar wall) but the same at the bottom while its total acid content at all locations was higher ($p<0.05$). Furthermore, the

102 contents of NH4+ and AP were the lowest in the top 4-year-old pit mud. In summary,
103 the physicochemical factors of the top 4-year-old pit mud were different from those of
104 the under 4-year-old pit mud and the 40-year-old pit mud.

105 **Table 1.** Physicochemical factors, number of OTUs of prokaryotic microbial communities, and α-diversity in pit mud from different pit

Variable	4-year				40-year			
	4T	4M	4U	4B	40T	40M	40U	40B
Moisture（%）	37.1±1.92a	40.1±1.34a	40.32±1.67a	37.8±0.66a	37.49±6.33a	41.93±4.22a	44.54±3.28a	38.63±0.93a
pH	4.17±0.07a	4.09±0.29a	5.27±0.92ab	6.33±2.65abc	8.64±0.07c	8.44±0.28c	7.26±1.17bc	8.26±0.22c
Total acid（mg•g^{-1})	10.86±0.66c	9±0.09c	3.41±2.19b	2.17±1.76ab	0.31±0a	0.31±0.22a	1.32±0.55ab	0.31±0a
NH4+(mg•100g^{-1})	38.45±5.98a	46.85±16.39ab	136.43±40.24abc	206.99±110.44c	171.56±49.38bc	186.79±35.37c	173.91±43.34bc	116.44±42.91abc
AP(mg•100g^{-1})	1.84±0.25a	5±2.87a	11.01±1.72ab	14.09±8.36ab	12.89±12.87ab	21.21±15.38ab	27.9±4.65b	13.69±10.75ab
OTU	392±9.9a	405.5±62.93a	706±182.43ab	663±156.98ab	1079.5±434.87bc	1242.5±200.11c	1148±111.72bc	1065±93.34bc
Chao 1	431.19±5.46a	551.75±131.88a	846.89±191.28ab	832.89±69.62ab	1310.61±568.21bc	1500.39±236.49c	1430.29±148.98bc	1368.53±71.08bc
ACE	436.54±7.56a	527.27±83.37a	867.98±213.43ab	855.1±100.04ab	1357.09±620.61bc	1560.85±239.45c	1487.28±155.43bc	1415.26±77.1bc
Shannon	3.72±0.39ab	3.22±0.52a	3.93±0.19ab	4.03±0.07ab	4.24±0.48b	4.66±0.27b	4.09±0.62ab	4.12±0.31ab
Simpson	0.94±0.03a	0.85±0.12a	0.95±0a	0.96±0a	0.93±0.04a	0.97±0.01a	0.93±0.05a	0.95±0.02a

106 [1] Data are presented as means ± standard deviations (n=2). Values with different letters within a row are significantly different statistically ($P<0.05$).

Alpha diversity of microbial communities in different pit muds

As shown in Table 1，the number of OTUs each sample obtained, based on 97% similarity in 16S rRNA gene sequences, ranged from 392 to 1242. Chao 1 index was between 431.19 and 1500.39. The Shannon index was between 3.22 and 4.66. From the analysis of pit age, both the number of OTUs and the alpha diversity index of the 4-year-old pit mud were lower than those of the 40-year-old pit mud. And the OTU, Chao 1, and ACE in the top 4-year-old pit mud were significantly ($p<0.05$) lower than those in the top 40-year-old pit mud. The Shannon index of the middle 4-year-old pit mud was significantly lower ($p<0.05$) than that in the top 40-year-old pit mud. It inferred that the diversity of microbial species was low in 4-year-old pit mud. With the deepening of the pit mud, there was no significant difference in alpha diversity between the 4-and the 40-year-old pit mud at different locations, but certain differences in the change law existed. In the alpha diversity index, the 4-year-old pit mud at the top location was lower while the 40-year-old pit mud at the top location was higher than that at the under location.

Beta diversity of microbial communities in different pit muds

Clustering of pit mud samples based on UniFrac analysis

The cluster analysis of species UniFrac (with species and its abundance taken into account) showed that the samples were classified into two categories(Fig. 1). The under and bottom locations of 4-year-old pit mud were divided into Cluster I (except 4B1), other locations into Cluster Ⅱ, but for the 40 -year-old cellar, all locations of

pit mud were assigned to Cluster I. There were 3 subclusters in cluster I. Subclusters ① and ② contained 40-year-old pit mud samples, clearly distinguishing two 40-year-old pit pools. Sub-cluster ③ mainly contained samples of 4-year-old pit mud at the under and bottom locations of the cellar. The comprehensive analysis showed that the microbial community structure of the under 4-year-old pit mud was more similar to that of the 40-year-old pit mud.

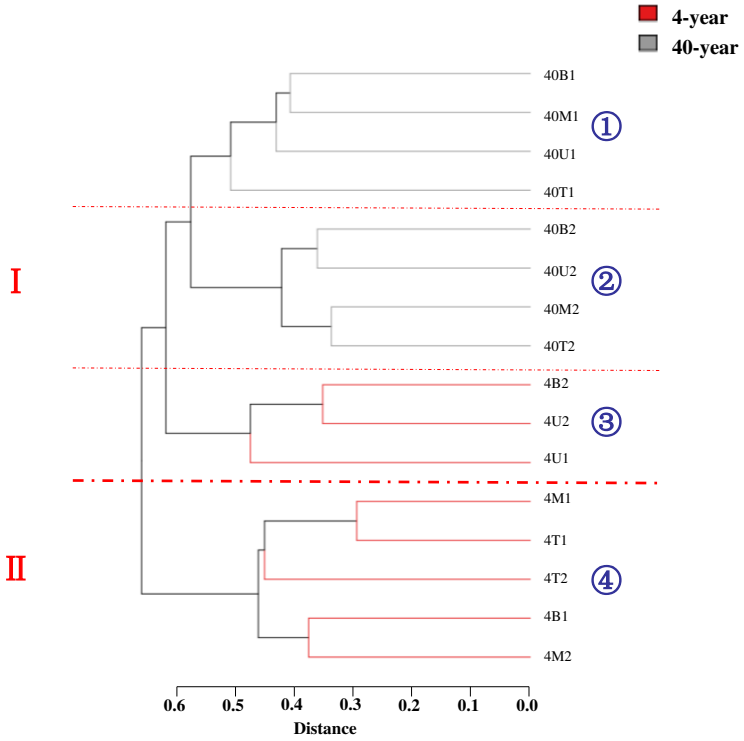

Fig 1. UniFrac distance cluster analysis based on pit-mud flora at different positions in 4-years and 40-years pits

Prokaryotic microbial community composition of different pit mud

At the phylum level, a total of 20 bacteria and 1 archaea (Euryarchaeota) were detected. More than 97% of the total abundance of samples were classified into 3 primary dominant phyla (relative abundance ≥1%, marked with red solid lines in Fig. 2) and 6 secondarily dominant phyla(abundance ≥1% at least each sample, marked with a solid green line in Figure 2). The relative abundances of the dominant phyla were as follows: Firmicutes (56.70%), Bacteroidetes (26.56%), Synergistetes (5.28%),

and Euryarchaeota (3.45%), Proteobacteria (1.81%), Chloroflexi (1.48%), Actinobacteria (1.44%), Cyanobacteria (1.34%), Tenericutes (1.01%). As against 4- and 40-year-old pit mud, the microflora structure changed significantly (Fig. 2). In the top 4-year-old pit mud, the abundances of the nine dominant phyla were all greater than 1%, while in the top 40-year-old mud, Only Firmicutes dominated(67.90%) and Actinobacteria (0.71%), Cyanobacteria (0.001%) and Tenericutes (0.54%) were all less than 1%.

Except for the pit mud in the top part of the cellar wall, there was no significant difference in the microbial structure of the pit mud at the same sampling location, and the relative abundance was different. The pH, TA, and NH4+ content (Table 1) in the 4-year-old pit mud were significantly different from those in the 40-year-old pit mud.

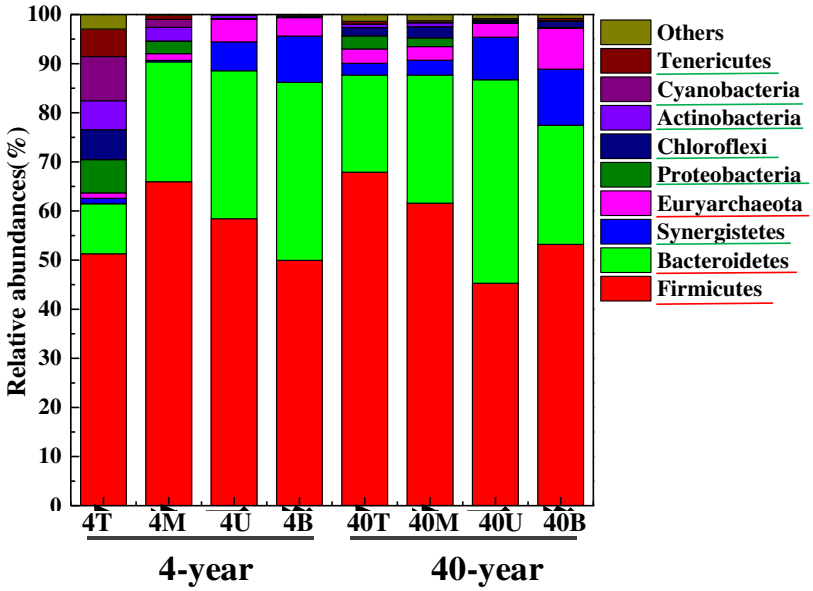

Fig 2. Composition of prokaryotic microorganisms in pit mud at the level of phylum

At the genera level, 301 genera were found. In this study, the top 10 genera (Top 10, 38 in total) of each sample were selected to reveal the diversity of microbial community composition in the pit mud from the genus classification level (Fig. 3). The top 10 genera of each sample contained dominant genera(relative abundance> 1%,

14 with red underline). The samples were classified into three clusters: the top 4-year-old pit mud, the under 4-year-old pit mud, and the 40-year-old pit mud. Cluster ① was a large one alone and clusters ② and ③ were also large, which was consistent with the results in Fig. 1.

In area i (Fig. 3) in the circle, Lactobacillus was the dominant genus in 4-year-old pit mud, while the abundance was less than 1% in 40-year-old pit mud. 8 species of bacteria including Mycoplasma, Bifidobacterium, etc. (except Lactobacillus and Bacteroides) were dominant in the top 4-year-old pit mud. However, its abundance was not detected in the 40-year-old pit mud. Bacteroides were the dominant genus only in the top 4-year-old pit mud, and *Fastidiosipila* and *DMER64* were dominant only in 40-year-old pit mud. Both 4- and 40-year-old pit mud had unique dominant bacterial genuses. The eight genera in *Christensenellaceae_R-7*, *Caproiciproducens*, *Clostridium_sensu_stricto_12*, and the area ii in the circle (Fig. 3) were 4 and 40 years old. The analysis of the locations of pit mud showed us such a result that with the depth of the cellar only 36% of the dominant genuses in the 4-year-old cellar mud did not change significantly, while 79% of those in the 40-year-old cellar mud not. As the depth of the pit deepened, The abundance of the genus of fungi in area i as well as *[Ruminococcus]_gauvreauii* and *Mesotoga* genus decreased, while the abundance of the regional genus bacterium, *Aminobacterium,* and *Methanoculleus* genus in the 4-year-old pit mud increased. However, with the depth of the cellar, the abundance of the 40-year-old pit mud did not change significantly. The genus *Clostridium_12* always decreased. Therefore, it was inferred that its abundance was greatly affected by the locations of the pit mud.

Fig 3. Heat map analysis based on Top 10 genera in each sample

The dominant bacteria of the samples (content ≥1%) belonged to 11 classes. The first dominant class was Clostridia, whose dominant genera belonging to Clostridia were 46.15% in 4-year-old pit mud, 54.39% in 40-year-old pit mud, and 56.67% in both pit mud.

The dominant genuses (3) in different locations (Fig. 4a) of the 4-year-old pit mud outnumbered those in different locations (Fig. 4b) of the 40-year-old pit mud. The endemic genuses were the most in the 4-year pit mud (16 species), whereas fewer in the 40-year pit mud (6 species). The physicochemical factors of the top 4-year-old pit mud were significantly different from those of other groups ($p<0.05$) (Table 1), but there was no significant difference at all locations of the 40-year-old pit mud. It implied that the differences in physicochemical factors resulted in different

predominant genuses that caused the differences in the spatial distribution of 4-and 40-year-old pit mud.

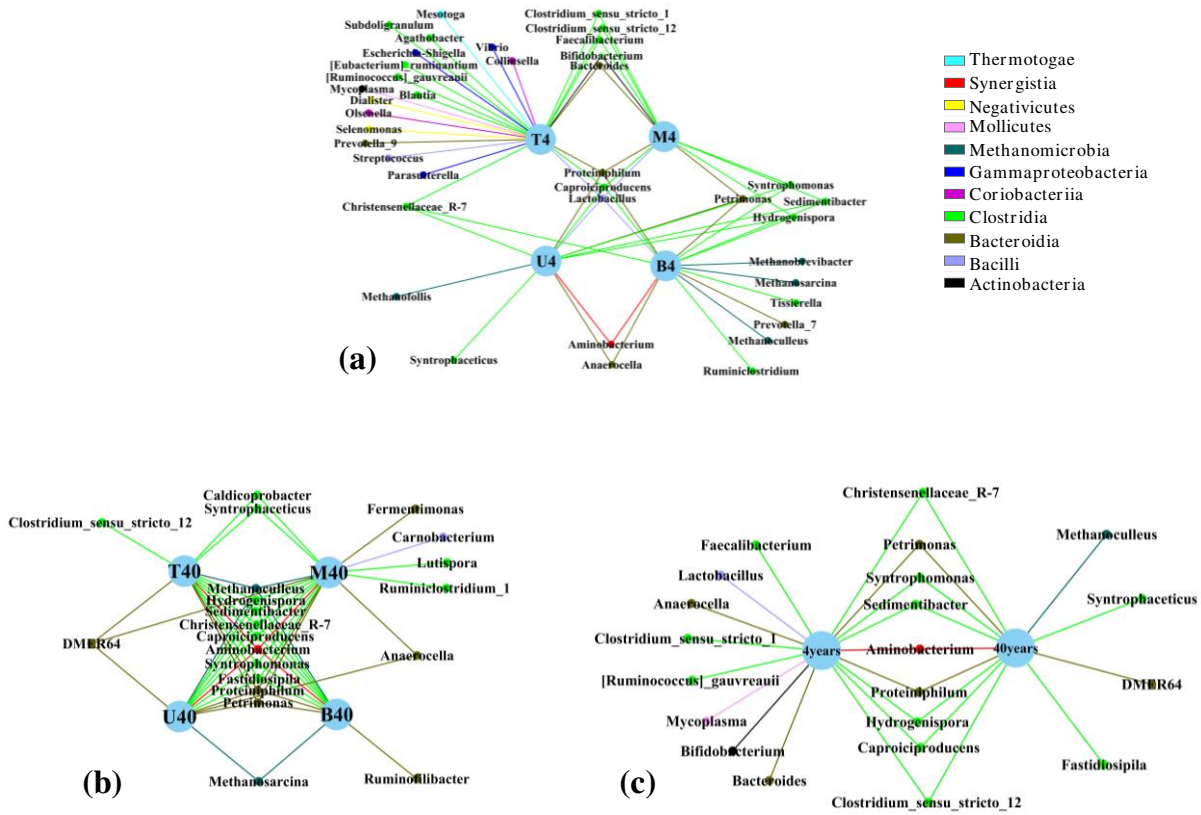

Fig 4. Distribution of dominant genera of different pit mud.

(a) shows the distribution characteristics of the 4-year pit mud's dominant position. (b) shows the distribution characteristics of the dominant genera of pit mud for 40-year. (c) shows the distribution characteristics of pit mud dominance in 4 and 40 years.

Network analyses of prokaryotic microbial communities in pit mud

Both positive (R>0.6 and P<0.01) and negative correlations (R<-0.06 and P<0.01) among prokaryotic communities, which were affiliated with 65 prokaryotic genera, were observed in this study. Based on positive correlations (R>0.6 and P<0.01), 42 nodes (genera) and 89 edges (pairs of significant correlations) co-occurrence patterns were detected (Fig. 5a). All nodes belonged to 12 classes, mainly concentrated in Clostridia (50% of the total number of nodes), Bacteroides (19.05%), Methanobacteria (7.14%), and Gammaproteobacteria (4.76%), and Bacillus (4.76%).

There were 12 hubs (nodes with more than 10 edges) in the co-occurrence network, which were the rare genera: *Subdoligranulum*, *Prevotella-9*, and *Parasutterella*, *Mycoplasma*, *Faecalibacterium*, *EscherichiaShigella*, *Collinsella*, *Clostridium_sensu_stricto_1*, *Blautia*, *Shuang Bifidobacterium*, *Bacteroides,* and *Bacillus* (Figure 6a). They belonged to 6 classes: Clostridia (4), Bacteroidia (2), Gammaproteobacteria (2), Mollicutes(1), Actinobacteria (1), and Coriobacteriia (1). The total content of the 40-year pit mud hub (0.047%) was much lower than that of the 4-year pit mud hub (11.7%).

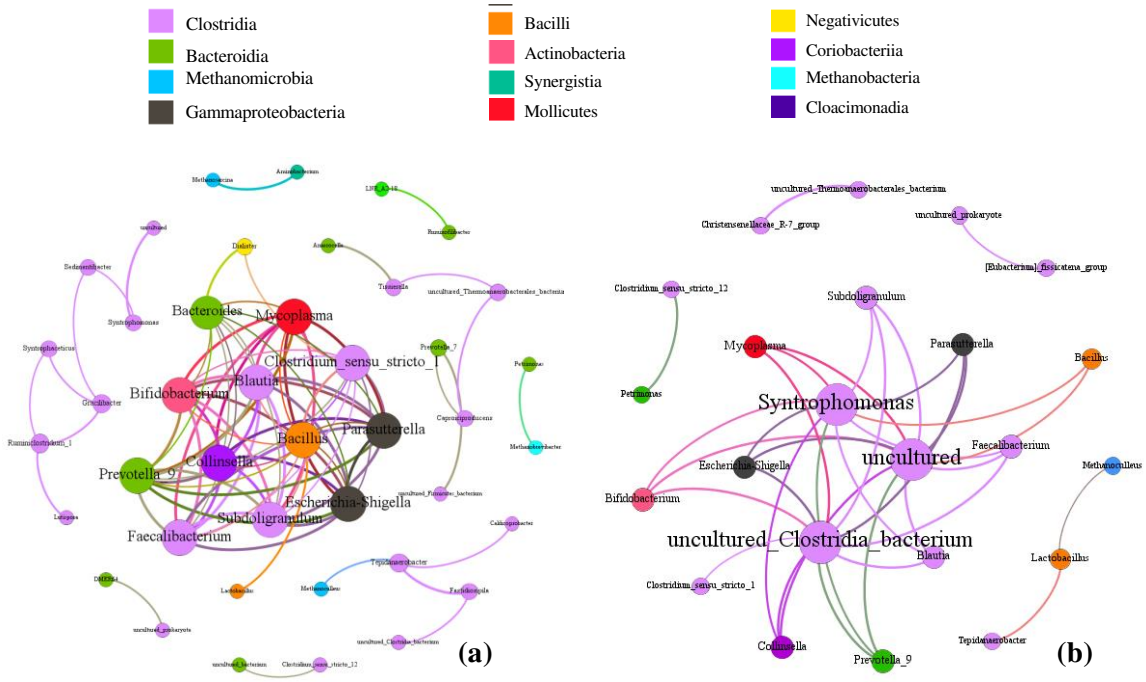

Fig 5. Networks of co-occurring prokaryotic genera in pit mud based on correlation analysis. (a) A connection indicates a statistically significant (P<0.01) strongly positive correlation Spearman's R>0.8; (b) a negative correlation Spearman's R<-0.8. Different phyla were represented by different colors. 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 >0.8 or R<-0.8.

Based on the negative correlation network, 23 nodes and 36 edges belonged to 8 classes. Microorganisms with negative correlation in the pit mud mainly occurred in the genus *Syntrophomonas*, unclassified genus, and other genuses (Fig. 5b). Based on

the positive correlation network, *Syntrophomonas had* a negative correlation with most hubs.

Redundancy analysis of prokaryotic communities and environmental variables（RDA）

Redundancy analysis (RDA) was performed to study the possible relationship between prokaryotic communities at the genus level and environmental variables(Fig 6). The first and second axis respectively explained 31.76% and 15.44% of the variation in prokaryotic communities and environmental factors. The samples showed the distribution and clustering of 4-year-old pit mud were more affected by its location in the cellar, whereas that of the 40-year-old pit mud was less affected. The 40-year-old pit mud from different locations of the cellar could be clustered together and its prokaryotic microbial community had obvious spatial structure characteristics, which were greatly affected by the age of the pit mud.

Conditional Term Effects was used to analyze comprehensively how each physicochemical factors contributed, which was total acid (30.9%), AP (15.5%), pH (6.1%), moisture content (2.9%), and NH4+ (2.4%), had influenced the flora structure (Fig 6a), The total acid and AP had the greatest influence on the prokaryotic microbial community and showed a significant correlation ($P<0.01$). There was no significant correlation between pH, water content, and ammonium nitrogen ($P>0.05$).

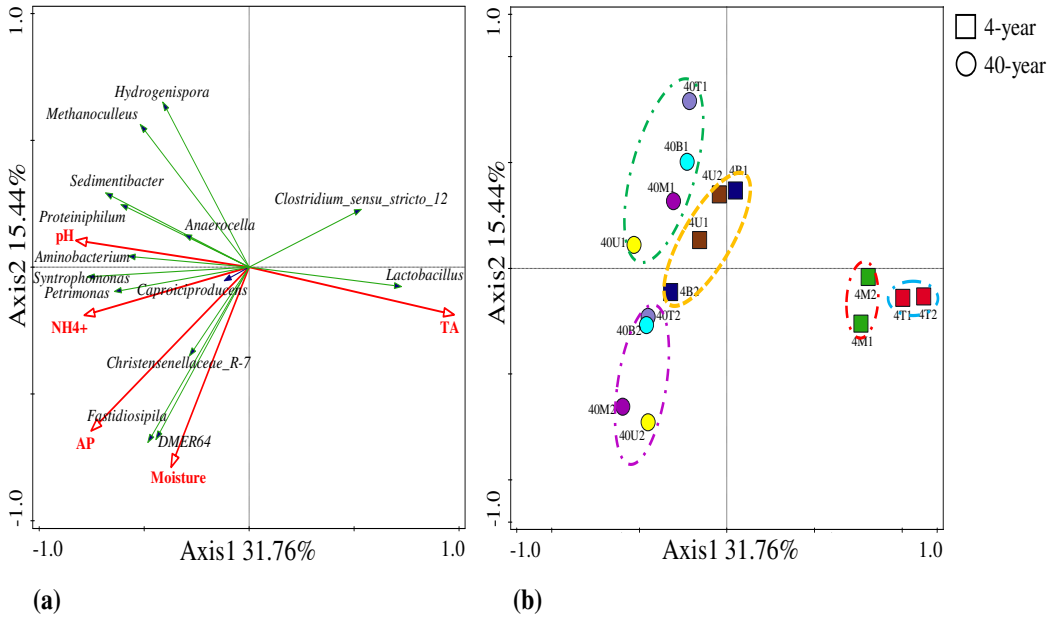

Fig 6. Redundancy analysis (RDA) of dominant microorganisms and physicochemical factors in pit mud. (a) Shows the Species and environmental variables. (b) Shows the samples.

Discussion

It is widely recognized that prokaryotic microbes in pit mud affect the quality of CSFB, and the older pit mud always produces liquor with a better taste [5, 6]. Previous studies have been focused on shifts of diversity and structure of prokaryotic communities; however, interactions of prokaryotic taxa in different-aged and different spatial locations of pit mud are poorly understood. Related studies have shown the NH4+ content of pit mud increases with its quality. N is an indispensable quick-acting component of microbial growth and reproduction, as it is conducive to the degradation of toxic and harmful substances from microorganisms [9]; the effective phosphorus content and the biomass of microorganisms in soil and the number of metabolites are positively correlated [10]. The pH value is positively correlated with the bacteria content in the pit mud ($p<0.01$) [11]. In this study (Table 1), pH, NH4+,

and AP in 40-year-old pit mud were higher than that in 4-year-old pit mud. It was inferred the 40-year pit mud had a higher maturity and better quality. It was consistent with the research results of Tao [6]and Zhang[8]. The alpha diversity index of the top pit mud was lower than that of the under pit mud(Table 1). On the contrary, the 40-year top pit mud alpha diversity index is higher than the under pit mud. This was inconsistent with the results of Zhang and Wang et al[20,24]. Zhang et al. [8]concluded that the diversity of pit mud on the wall of old cellars (cellar age>50 years) was lower than that at the bottom. Wang's [24] research results showed that the alpha diversity of the 6-year pit mud was higher than that of the 16-year pit mud. Thus, it was inferred that the difference might be caused by the age and the origin of the pit mud.

Except for the pit mud on the top cellar wall, there was no significant difference in the microbial structure of the pit mud in the same sampling location, only the relative abundance was different (Fig 2). The pH, total acid, and NH4+ content of 4-year-old pit mud on the top cellar wall were significantly different from those of 40-year-old pit mud. Physicochemical factors had an impact on the microbial community structure of pit mud [6,8,18]. The reason for the change in the microbial structure of the top pit mud of different aged cellars was that the physicochemical factors of the cellar mud used repeatedly for a long time had changed. The two dominant phyla at different locations of pits were Firmicutes and Bacteroidetes, which was consistent with the results of most relevant studies in China [8,12,13,14]. The single microbial composition in the under pit mud mainly included Firmicutes, Bacteroidetes,

Synergistetes, and Euryarchaeota, with a relatively simple structure. The microbial diversity of pit mud on the top cellar wall was higher than that on the under cellar wall. With the depth of the pit, the abundance of Synergistetes and Euryarchaeota increased while Proteobacteria decreased.

According to the heat map in Fig 3, the relevant literature summarized [14,15,16] that the abundance of *Lactobacillus* decreased significantly with the improvement of the quality of the pit mud and the growth of the pit age, which is the same as the results shown in this study. *Hydrogenispora*, *Sedimentibacter,* and *Clostridium* were the main Clostridia genus present in the pit mud [23], and their advantages were prominent in the aging and mature pit mud [15]. The correlation of pit mud quality with *Sedimenlibacter and Aminobacterium* was positive [18] and in high-quality pit mud [5], *Methanoculleus* was the dominant genus.

In summary, it was inferred, from the level of microbial community structure, that the quality of the 40-year-old pit mud and the under 4-year-old pit mud in this study was better than that of the top 4-year-old pit mud.

The network analysis indicated the positive correlations of prokaryotic class, which mainly belonged to class Clostridia (Fig 5). *Mycoplasma* had the highest relative content in the top 4-year-old pit mud, which had been rarely reported in the past [17,18, 19, 20]. The genus *Petrimonas* and *Syntrophomonas* associated with the degraded pit mud did not appear in the hub [17]. A total of three larger independent modules and five independent modules emerged in the co-occurring network (Figure 5a). This was caused by the fact that the nodes or hubs that could connect the modules had little or no content. The connection between the hub and the micro-ecological

311 stability of the pit mud was an important factor affecting the entire microbial flora
312 network in the environment, and changes in its type and content could lead to
313 fragmentation of the network [28]. *Syntrophomonas* had a negative correlation with
314 most hubs in the positive correlation network (Fig 5b). However, the results of Hu et
315 al. showed that *Lactobacillus* was negatively correlated with most genuses[5]. GUO
316 [17]showed that the content of *Syntrophomonas* in degraded pit mud was significantly
317 ($p<0.05$) higher than that of normal pit mud. Following this, WU[22] and others
318 further confirmed that *Syntrophomonas* was detected in the third-class pit mud, but
319 not in the first- and second-class pit mud. This indicated that the genus
320 *Syntrophomonas* might accelerate the imbalance of the microbial community of the
321 pit mud, the reduction of the robustness of the bacterial community, and the
322 degradation of the pit mud.

323 RDA analysis indicated that the environmental factors, TA and AP, had significant
324 ($P<0.01$) effects on prokaryotic communities(Fig 6). Lactic acid could cause the
325 formation of ferrous lactate, calcium lactate, and other crystals in the pit mud, which
326 could significantly affect the growth of the genus of bacteria in the pit mud and lead
327 to the aging of the pit mud [20]. AP provides fast-acting P nutrients for the growth and
328 reproduction of microorganisms in the environment and has a significant impact on
329 the microbial community beta diversity. This further indicated that total acid and AP
330 had a greater influence on the distribution of microbial communities in the pit mud.
331 85.71% of the dominant genera were distributed on the left side of the central axis of
332 Axis1, but only two genera, *Clostridium_sensu_stricto_12* and *Lactobacillus,* were
333 distributed on the right side and were positively correlated with total acid (Fig. 6a).
334 *Lactobacillus* was positively correlated with total acid content and negatively with pH.

Because *Lactobacillus* metabolites were mainly lactic acid, the accumulation of lactic acid could lead to an increase in total acid content and a decrease in pH, which in turn gave rise to a deterioration in the quality of pit mud.

Methanoculleus can symbiosis with caproic acid bacteria such as *Clostridium* and promote the production of caproic acid by its bacteria[20]. *Aminobacterium* and *Sedimentibacter* have the function of degrading amino acids and improving ammonium nitrogen[13]. *Caproiciproducens* have a negative correlation with *Lactobacillus*. Studies have confirmed that medium- and long-chain fatty acids (such as caproic acid) produced by caproic acid bacteria can inhibit *Lactobacillus* [21]. In addition, *Fastidiosipila* mainly uses acetic acid and butyric acid as the main metabolites [25]. At present, the bacterium is rarely reported in the pit mud[26], and the genus *Christensenellaceae* is mainly found in the human intestine [27].

Conclusions

This study systematically compared the structures and spatial distribution characteristics of prokaryotic microbial communities between 4- and 40-year-old pit mud from Henan CSFB company. The predominant phyla of pit mud were Firmicutes, Bacteroidetes, Synergistetes, Euryarchaeota, Proteobacteria, Chloroflexi, Actinobacteria, Cyanobacteria, and Tenericutes, among which Firmicutes was the most dominant 14 genera, such as *Proteiniphilum*, *Hydrogenispora*, *Lactobacillus,* were principal. The proportion of endemic bacteria was higher in 4-year-old pit mud, and the number of endemic bacteria in 4-year-old pit mud on the top cellar wall was

the highest. In 40-year-old pit mud, the dominant bacterial genuses were common in each of its locations, and the unique bacterial genuses were few. The 4-year-old pit mud on the top and under cellar wall was well distinguished, whereas the 40-year-old pit mud was well clustered. In older pits, the microbial community was less affected by the sampling locations of the pit mud. The total acid and AP ($P<0.01$) might be the main environmental factor affecting the spatial distribution of the prokaryotic microbial community of pit mud.

Methods

Materials

The pit-mud samples were taken from a well-known liquor-producing company (Jiahu Distillery Group Co., Ltd., E113°21′15"~113°44′53", N33°7′50" ~33°30′22") in Henan Province, China. For sampling, two types of fermentation pits under normal production with different aged pit mud including young (4 years, XJ) and old (40 years, LJ) pit mud were randomly selected. Two types of pit muds were selected for each above type, and four locations varying in depth of each pit were selected, which were the top (T), middle (M), under(U), and bottom (B) cellar wall (Fig. 7). Two subsamples were collected.

Among them, 2 normal cellars (XJ 1 and XJ 2) and 2 old cellars (LJ 1) were randomly selected. LJ 2, LJ3, and LJ 4), and samples of pit mud were collected at the four locations of each cellar: the top(T), middle (M), under (U), and the bottom(B) cellar

walls. (Figure 1). 16 representative samples (Table 2) were stored at -30℃ for future use.

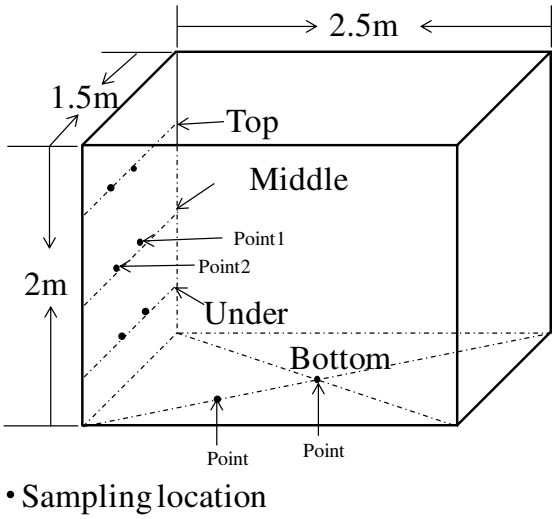

Fig 7. Collection Sample Location of pit mud

Table 2. Collect samples and numbers of pit mud

Pit mud of aged	Top	Middle	Under	Bottom
4-year	4T1、 4T2	4M1、 4M2	4U1、 4U2	4B1、 4B2
40-year	40T1、40T2	40M1、40M2	40U1、40U2	40B1、40B2

Determination of physicochemical factors

Referring to drying methods and potentiometric methods employed by Li Junhui and others to measure the water content and pH value of pit mud (Li et al., 2018), the acid and alkali neutralization titration method was used to determine the total acid content (Wang et al., 2012). The indigo blue colorimetric method was used to detect the content of ammonium nitrogen in pit mud (Nanjing Institute of Soil Sciences., 1987). The effective phosphorus content in pit mud was quantified referring to the NY/T1121.7-2014 standard (Agricultural Industry Standard of the People's Republic of China., 2014). Reagents included sodium hydroxide, phenolphthalein, potassium hydrogen phthalate, concentrated sulfuric acid (ρ=1.84g/mL), hydrochloric acid

(=1.19g/mL), phenol, potassium nitroso ferricyanide, sodium perchlorate, chlorine Ammonium chloride, potassium antimony tartrate, ammonium molybdate, ammonium fluoride, boric acid (purchased from China National Pharmaceutical Group Chemical Reagent Co., Ltd.). All reagents were analytically pure. UV BlueStar A ultraviolet spectrophotometer (Leiber Tech Instruments, Beijing, China). TGL-20M high-speed refrigerated centrifuge (Lu Xiangyi Centrifuge Instrument Co., Ltd., Shanghai, China). PB-10 PH meter (sartorius, Germany). MP200A Precision Electronic Balance (Liangfeng Instrument Co., Ltd., Shanghai, China). 101-1 Electric blast drying oven (Zhongxing Weiye Instrument Co., Ltd., Beijing, China). DF-1 collector types constant temperature magnetic stirring pot (Jintan Zhongda Instrument Factory, Jiangsu, China).

DNA extraction and Illumina MiSeq sequencing of 16S rRNA genes

Magen HiPure Soil DNA Kit was used under the guidance of the step-by-step instructions to extract DNA from the pit mud samples, and the Qubit® dsDNA HS Assay Kit was used to detect the DNA concentration. The upstream primer 5'-CCTACGGRRBGCASCAGKVRVGAAT-3' and downstream primer 5'-GGACTACNVGGGTWTCTAATCC-3' were used to amplify the V3 and V4 regions of the 16S rRNA gene of prokaryotes. PCR amplification reaction system: TransStart Buffer 2.5 μL, dNTPs 2 μL, TransStart Taq DNA 0.5 μL, template 20 ng, upstream and downstream primers 1 μL each, make up the volume to 25 μL with ddH2O (double distilled water), reaction conditions: pre-denaturation 94℃ 3 min,

Denaturation 94°C 5 s, annealing 57°C 90s, extension 72°C 10s, final extension 72°C 5min, 24 cycles. Qualified PCR products were entrusted to Jinweizhi Biotechnology Co., Ltd. (Suzhou, China) for Illumina MiSeq sequencing library construction and double-end sequencing.

Statistical analysis

The Illumina MiSeq sequence was controlled using Cutadapt (v1.9.1), Vsearch (1.9.6), and Qiime (1.9.1) software. These quality controls included sequence splicing, removal of sequences containing N, primers, and linkers, bases with a quality value <20 and sequences with a length of <200 bp, and chimeras. VSEARCH (1.9.6) was used for sequence clustering for sequences that had passed quality control, where the similarity was set at 97% and classified as 1 OUT. The species taxonomic analysis on the representative sequences of OTU was performed by using the RDP classifier (Ribosomal Database Program) Bayesian algorithm, and the community composition of each sample was studied under classification levels of different species. Based on the OTU, a random leveling method for the sample sequence was used to calculate the alpha diversity indexes of the samples Shannon, Chao1, etc., the complete analysis of variance and clustering were performed using SPSS (IBM19) software. The software Origin9.0 was applied to draw a histogram of the percentage of phylum in the advantages of pit mud samples (relative abundance was more than 1%). HemI and software Canoco 5 were used to draw Heat maps and RDA maps separately.

Data availability

The sequencing data were submitted to the Sequence Read Archive (SRA) of the NCBI database under BioProject PRJNA646030.

Abbreviations

CSFB: Chinese strong-flavor Baijiu

NH4+: Ammonium Nitrogen

AP: Available phosphorus

OTU: Operational taxonomic units

RDA: Redundancy analysis

Authors' contributions

All authors did the experiments and analyzed the data. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest

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