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Deep sequencing reveals changes in prokaryotic taxonomy and functional diversity of pit muds in different distilleries of China

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Abstract

Purpose: The microbial community in the pit mud correlated closely with the quality of the final product of Chinese strong-flavored Baijiu (CSFB). However, environmental conditions and brewing processes can vary by region and distilleries. This may lead to differences in microbial composition and function in pit mud. Therefore, revealing the features of the pit mud microbial community structure and functions of different distilleries will provide key information for understanding the diversity and difference of microbes in the brewing of CSFB, which will be beneficial for the improvement of the quality of pit mud and CSFB in the future.

Methods and results: Illumina MiSeq sequencing of 16S rRNA gene amplicons was used to analyze the similarities and differences in microbial community structure and function in pit muds of different distilleries located in Shihezi (Xinjiang), Xiangyang (Hubei), and Yibin (Sichuan). At the genus level, *Clostridium*, *Lactobacillus*, *Aminobacterium*, *Petrimonas*, *Syntrophomonas*, *Methanoculleus*, *Syntrophaceticus*, *Sedimentibacter*, *Caloramator*, *Ruminococcus*, *Bacillus*, *Methanosarcina*, and *Garciella* were the dominated genera of pit muds. There were great differences in the composition of microorganisms in pit muds used by different distilleries. The significantly enriched prokaryotic microbiotas of pit muds collected in the distilleries of Xiangyang were mainly affiliated with *Bacillus*, *Lactobacillus*, and *Croceifilum*, and the relative abundance of methanogens, such as *Methanomicrobia* and *Methanobacteria*, were only significantly enriched in the pit mud collected from the distilleries of Yibin ($P < 0.05$). Functional analysis indicated that the difference of microbial composition in pit mud will further lead to significant differences in various metabolic functions.

Conclusion: The compositions and functions of dominant microorganisms in pit mud used for the production of CSFB by different enterprises across regions in China were greatly different, and there was a close relationship between the compositions and functions of microorganisms in pit mud. Therefore, it may be an effective method to improve CSFB fermentation processes by directionally regulating the microbial community functions of pit mud using specific strains.

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Introduction

Chinese liquor, vodka, rum, tequila, brandy, and whiskey are the six most popular distilled liquors of the world. Of them, Chinese strong-flavored Baijiu (CSFB) is one of the most important traditional distillates, accounting for >70% of the total liquor consumption in China (Liu et al. 2017). The raw materials for making CSFB include grains, typically sorghum or a mixture of corn, rice, millet, and wheat (Zheng and Han 2016). CSFB is fermented under anaerobic conditions in cellars (approximate dimensions: length, 2.0–3.5 m; width, 2.0–3.0 m; and depth, 2.3–2.5 m) lined with pit mud, and the process takes 60–90 days. The prokaryotes present in pit mud break down the macromolecules from the raw material into peptides and monosaccharides, producing aromatic compounds responsible for the flavor of the product (Li et al. 2011; Wang et al. 2017; Xiang et al. 2019). Both recent studies and long-term production practices have revealed that the microbial community in pit mud correlates closely with the quality of the final product (Tao et al. 2017; Liu et al. 2020). However, environmental conditions and brewing processes can vary by region and distilleries. This may lead to differences of microbial composition in pit mud. Therefore, revealing the features of the pit mud microbial community structure and functions of different distilleries will provide key information for understanding the diversity and difference of microbes in CSFB brewing, which will be helpful for the improvement of CSFB production technology in the future.

Classical microbiological methods have provided early insight into the predominant bacteria involved in CSFB fermentation. The development of the next-generation DNA sequencing technologies has revolutionized microbial ecology studies in recent years by allowing high-throughput analysis. Technological progress has allowed more efficient and accurate identification of the microbial communities and functional capacities in different niches, including Daqu (a Chinese traditional fermentation starter) (Xie et al. 2020), soil (Fan et al. 2020), and parts of the body, such as the gut (Hou et al. 2020) and skin (Rainer et al. 2020). At present, this technology has also been applied in some studies related to pit mud microbes (Tao et al. 2017; Chen et al. 2020). However, these studies often collect pit mud samples from specific distilleries located in the same region. As a result, the commonalities and differences of microorganisms in pit mud of different distilleries across regions in China have not been reported yet.

In this study, pit mud samples used for CSFB production were collected from different distilleries located in Shihezi (Xinjiang Province) and Xiangyang (Hubei Province). The community structure and functional characteristics of prokaryotes present in pit muds were analyzed by using Illumina MiSeq sequencing technology. In addition, we integrated and examined sequencing data of pit mud samples taken from Yibin (Sichuan Province) (http://www.ncbi.nlm.nih.gov/bioproject/?term= PRJNA597727) (Chen et al. 2020), a region known for the production of high-quality CSFB in China. This study could deepen our understanding of the compositions and functional characteristics of the prokaryotic community in pit mud from different distilleries in China and help further improve the quality of pit mud and CSFB.

Materials and methods

Sample collection

Pit mud samples were collected from Shihezi and Xiangyang. Shihezi is far from the ocean, and air from the ocean is does easily to reach this region. Therefore, there is less precipitation and a dry climate. Xiangyang, in turn, is characterized by the transition from north to south and the consideration of east and west. It belongs to the north subtropical monsoon climate with moderate humidity.

The CSFB distilleries of Shihezi and Xiangyang selected for this study had been established for a long time. However, on the whole, the fermentation cellars of distilleries in Xiangyang collected in this study were younger than those of Shihezi. The collected samples in these fermentation cellars showed characteristics typical of normal pit mud. It does not show degradation of earth color or ester aroma, and it has a moist, soft, and uniform texture. Samples were collected according to the stratified random method (Zheng et al. 2013), and the sampling sites were based on the method adopted by a previous study (Chen et al. 2020). In brief, samples were collected from three depths in each cellar (upper layer, 10 cm below the cellar surface; middle layer, 1.1 m below the cellar surface; and lower layer, collected after removing a 2–3-cm layer of pit mud from the cellar bottom). The sampling procedure is illustrated in Fig. S1. A total of 30 samples were collected from ten distilleries in Shihezi and 27 samples were collected from nine distilleries in Xiangyang. Two subsamples were collected from each layer using a soil core sampler (AMS Inc., USA); 100 g of each subsample was obtained and thoroughly mixed. All samples were kept in ice boxes and transported from the distilleries to the laboratory at low temperatures (transportation time

<48 h). In the laboratory, they were stored at −20°C until further analysis.

DNA extraction

DNA was extracted from each sample using the FastDNA SPIN kit for soil (Mpbio, USA) following the manufacturer's instructions. The quality of extracted DNA was checked using 1% agarose gel electrophoresis (Beijing Liuyi Biological Technology Co., Ltd. China) and spectrophotometry (ratio of optical density at 260 and 280 nm) using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, USA). All extracted DNA samples were stored at −20°C until further analysis.

PCR amplification

The V3–V4 region of the 16S ribosomal RNA (rRNA) gene was amplified, as previously described (Chen et al. 2020), using the forward 341F (5′-CCTAYGGGRBG-CASCAG-3′) and reverse 806R (5′-GGACTACHVGGG TWTCTAAT-3′) primers (Yu et al. 2005). The primers contained a set of seven nucleic acid barcodes, which were used to distinguish samples in subsequent analyses. The PCR involved the following steps: 2 min at 95°C; 30 cycles of 20 s at 95°C, 30 s at 55°C, and 30 s at 72°C; final extension at 72°C for 5 min; and cooling at 4°C. The amplicons were sequenced at Majorbio (Shanghai, China) using the Illumina MiSeq system.

Bioinformatics analyses

Primers and barcode sequences were removed from high-quality reads via in-house Python scripts. Furthermore, based on barcodes, the reads were divided into different sample groups. After merging all sequencing data, including the data of pit mud samples taken from Yibin (Chen et al. 2020), the Quantitative Insights Into Microbial Ecology (QIIME) package (version 1.9.1) (Caporaso et al. 2010) was applied to perform bioinformatics analysis. The specific steps were similar to those from our previous study (Hou et al. 2020). Briefly, UCLUST (Edgar 2010) was employed to classify high-quality sequences into operational taxonomic units (OTUs) at an identity threshold of 97% similarity, while ChimeraSlayer (Haas et al. 2011) was used to remove potential chimeric sequences from the OTU representative set. Singleton OTUs (OTUs with only one sequence) were removed from all datasets. Based on the information extracted from Greengenes (version 13.8) (Desantis et al. 2006), the Ribosomal Database Project (RDP, Release 11.5) (Cole et al. 2007), and SILVA (Version 138) (Quast et al. 2013), each OTU was assigned to the lowest possible taxonomic level on the basis of a minimum bootstrap threshold of 80% (Hou et al. 2015). The OTU table was subsampled correspondingly to adjust the sampling depth using the "multiple_rarefactions.py" program from the QIIME pipeline. Alpha- and beta-diversity were calculated based on the de novo taxonomic tree constructed by the representative chimera-checked OTU set using Fast-Tree (Price et al. 2009). The α-diversity indices, such as the number of observed OTUs and the Shannon diversity index, were calculated in 1000 iterations of rarefied OTU tables in QIIME with the minimum sequencing depth among all study subjects. The functional profiles of prokaryotes in pit mud samples were predicted based on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al. 2013) software and the COG database.

Statistical analyses

Statistical analyses were performed using R software version 4.0.2 (https://www.r-project.org/). Nonparametric Mann–Whitney or Kruskal–Wallis tests were used to ascertain the statistical significance of between-group differences in α-diversity indices, prokaryotic composition, and functional categories. False discovery rate values were estimated using the Benjamini-Yekutieli method to control for multiple testing (Benjamini and Yekutieli 2001). P values less than 0.05 were considered statistically significant. To evaluate the prokaryotic community structure in different samples, principal coordinate analysis (PCoA) was carried out based on both the weighted and unweighted UniFrac distances derived from the phylogenetic tree (Lozupone and Knight 2005). The extent of compositional differences between groups was determined using permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) tests based on UniFrac distances, which examine the significance of inter- and intra-group variations (pseudo F-statistic) via permutations of group assignment. Canonical analysis of principal coordinates (CAP) was conducted using MATLAB software version 2016. The linear discriminant analysis effect size (LEfSe) method was used to determine taxa of prokaryotic biomarkers among the pit mud samples from different regions (LDA score ≥ 3) (Segata et al. 2011). Procrustes analysis was employed to reveal the correlation between the microbial community and functional profiles. Figures were plotted using the R ggplot2 (Wickham 2016) and gplots packages (https://github. com/talgalili/gplots).

Results

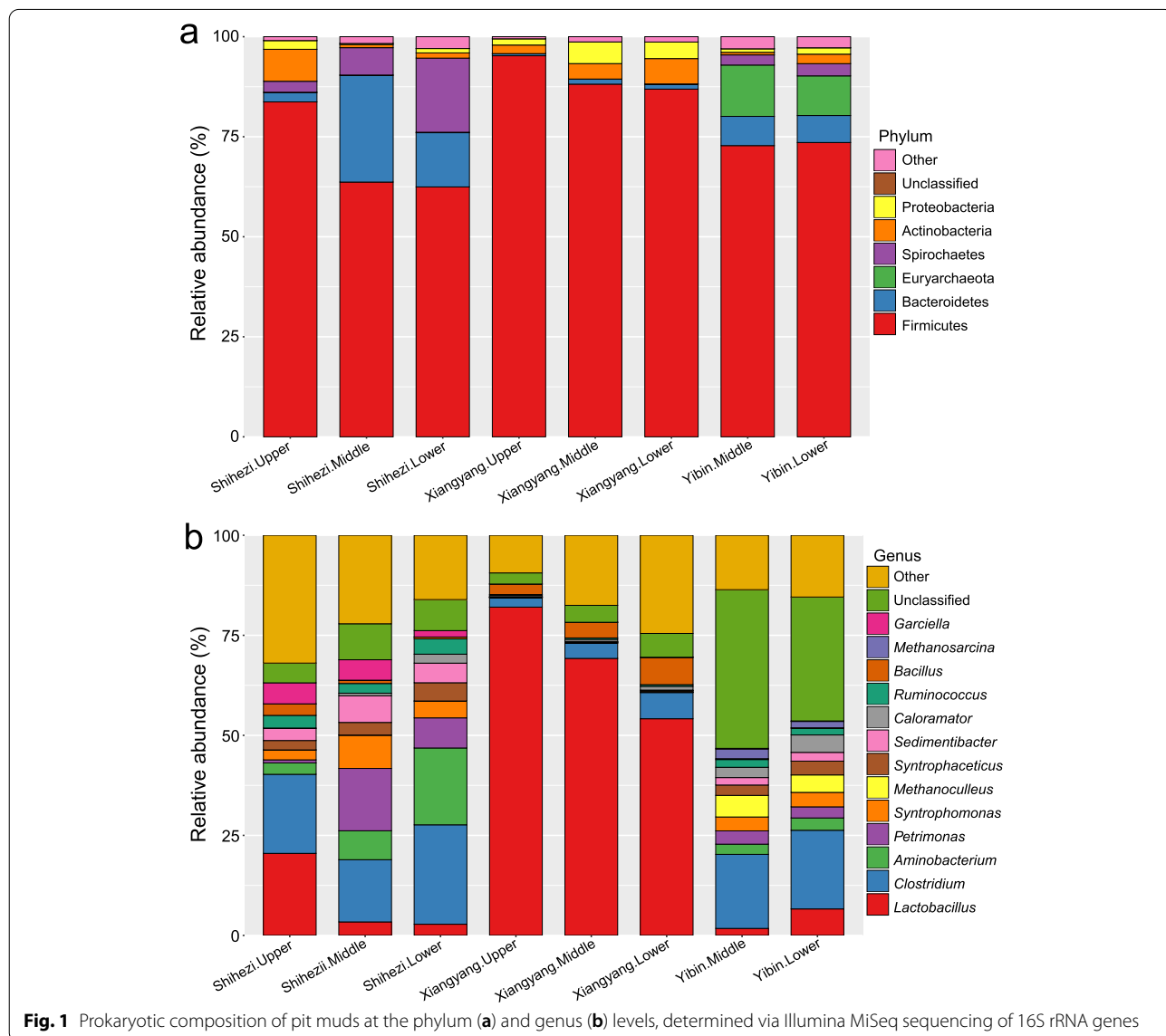### Sequence coverage of prokaryotic communities across samples from Shihezi and Xiangyang

We generated a dataset that included 2,127,407 high-quality reads of the V3–V4 regions of prokaryotic 16S rRNA genes present in samples from Shihezi and Xiangyang; there were 37322 ± 7789 high-quality reads (mean

± SD, range 16,519–54,860) per sample. At a high identity cutoff level of 97% sequence similarity, 67,896 OTUs were detected. After removing singleton OTUs, the average number of OTUs per sample was 2484 (range, 712–5496; SD=1243). Based on the information extracted from the RDP, Greengenes, and SILVA databases, each OTU was assigned to the lowest taxonomic level using homologous sequence alignment and clustering. Overall, 5.54% of the sequences could not be assigned to any genus level. Quantification of α-diversity revealed that the Shannon−Wiener diversity curves (rather than the rarefaction curves) reached saturation (Fig. S2), indicating that the obtained sequence depth for all samples was sufficient, although other new phylotypes may be identified by further sequencing.

Prokaryotic composition of all pit mud samples

The prokaryotic community structure of pit mud samples was analyzed at the phylum and genus levels. In total, 68 phyla and 1044 genera were identified from all pit mud samples. Six phyla showed a relative abundance >1%: Firmicutes (76.0%), Bacteroidetes (7.5%), Euryarchaeota (6.3%), Spirochaetes (3.8%), Actinobacteria (2.5%), and Proteobacteria (1.7%) (Fig. 1a). Firmicutes appeared to be the most prevalent phylum in all samples, while Euryarchaeota were mainly present in samples collected from Yibin.

At the genus level, there were obvious differences among the dominant genera in pit muds collected from different regions and distilleries (Fig. 1b). Specifically, there were fifteen and thirteen genera with an average relative abundance >1% in pit muds of Shihezi and Yibin,

Fig. 1 Prokaryotic composition of pit muds at the phylum (**a**) and genus (**b**) levels, determined via Illumina MiSeq sequencing of 16S rRNA genes

respectively. However, there were only eight genera with an average relative abundance >1% in pit muds of Xiangyang. The predominant genera in pit mud samples from all distilleries were as follows: *Lactobacillus* (19.1%), *Clostridium* (16.1%), *Aminobacterium* (3.9%), *Petrimonas* (3.6%), *Syntrophomonas* (3.2%), *Methanoculleus* (2.7%), *Syntrophaceticus* (2.4%), *Sedimentibacter* (2.3%), *Caloramator* (2.2%), *Ruminococcus* (1.8%), *Bacillus* (1.4%), *Methanosarcina* (1.1%), and *Garciella* (1.0%). Interestingly, *Methanoculleus* and *Methanosarcina* were mainly present in pit mud samples from Yibin.

Presence of core fermentation-associated prokaryotes in pit mud samples

To identify the core prokaryotes that were most likely to be involved in fermentation, the OTUs that were shared by ≥95% of pit mud samples in at least one region were defined as core OTUs (cOTUs). Overall, 129 OTUs met this threshold (Fig. S3). Among them, 18 cOTUs were found in all samples, of which nine showed annotation results for *Clostridium*; other cOTUs showed annotation results for *Lactobacillus*, *Ruminococcus*, and *Bacillus*. The cumulative relative abundances of cOTUs in the upper, middle, and lower layers of pit mud from Shihezi were 1.9%, 3.3%, and 19.4%, respectively, while in Xiangyang, these values were much higher (49.3%, 59.9%, and 70.4%, respectively). However, the proportions of cOTUs in the middle and lower layers of pit mud from Yibin were 0.3% and 0.4%, respectively. Notably, many cOTUs that were only found in pit mud samples from Shihezi and Yibin but were missing from Xiangyang samples showed annotations for *Sedimentibacter*, *Caldicoprobacter*, *Tissierella*, *Syntrophaceticus*, and *Petrimonas*. Nevertheless, pit mud samples collected from Xiangyang had many unique cOTUs, several of which had annotations for *Lactobacillus*, *Clostridium*, *Bacillus*, Micromonosporaceae_unclassified, *Ralstonia*, *Thermoactinomyces*, and *Hydrogenispora*. Therefore, the community structure of prokaryotes in Xiangyang pit mud collected in this study was more special.

Differences in α-diversity based on distilleries and depth

Prokaryotic species richness and diversity were assessed based on the number of observed OTUs and the Shannon diversity index, respectively (Fig. 2). Pit mud samples collected from Yibin had the highest α-diversity, followed by those from Shihezi and Xiangyang; the α-diversity indices were significantly different among the regions ($P<0.05$). In addition, the α-diversity increased with increasing depth in pit mud samples from Xiangyang, and the α-diversity was significantly higher in lower-layer pit mud than in upper-layer pit mud ($P<0.05$). There was

no significant difference in the α-diversity between the middle and lower layers of pit mud samples from Yibin.

Comparison of prokaryotic community structure in pit mud samples

To evaluate the variation in prokaryotic community structure in pit mud samples from different distilleries and depths, we used PCoA based on unweighted and weighted UniFrac distances (Fig. 3a, b). When using unweighted UniFrac distances, score plots generated from principal components 1 and 2 showed a clear clustering pattern associated with the collected regions. PCoA based on weighted UniFrac distances showed that the pit mud samples from Xiangyang were clearly distinct from those of Shihezi and Yibin, but that there was some overlap between samples from the latter two regions. There was also a clear overlap between samples obtained from different depths in the same region.

To further compare the influence of distilleries and sampling depth on prokaryotic community structure, the sampling regions and depths were used as grouping factors in PERMANOVA. The results showed that the calculated F value was greater when grouping was performed according to sampling regions ($F = 36.75$) than when it was performed according to sampling depth ($F = 6.35$). This indicated that the influence of sampling distilleries on prokaryotic community structure was greater than that of sampling depths. These results were confirmed by calculations performed using CAP (Fig. 3c), as samples from the same region were found to be clustered together.

Differences in the microbial composition of pit mud samples from different distilleries and depths

To compare the differences in microbial composition in distilleries located in different regions, LEfSe analysis was used to identify the taxonomic prokaryotes with significant abundance differences among the three regions (Fig. 4). Due to the lack of upper pit mud samples in Yibin, the analysis was only based on the middle and lower pit mud samples in the region. The significantly enriched prokaryotic microbiotas in Yibin were mainly affiliated with *Methanomicrobia* and *Methanobacteria* of Euryarchaeota. The significantly enriched prokaryotic microbiotas in Xiangyang were mainly affiliated with *Bacillus*, *Lactobacillus*, and *Croceifilum*. The significantly enriched prokaryotic microbiotas in Shihezi were mainly affiliated with *Clostridium*, *Ruminococcus*, *Fermentimonas*, *Petrimonas*, *Hazenella*, *Aminobacterium*, *Syntrophomonas*, *Garciella*, and *Sedimentibacter* of the classes Bacteroidia, Bacilli, Clostridia, and Spirochaetia. Based on the Kruskal–Wallis tests, we found that the unclassified prokaryotes in Yibin accounted for 30%,
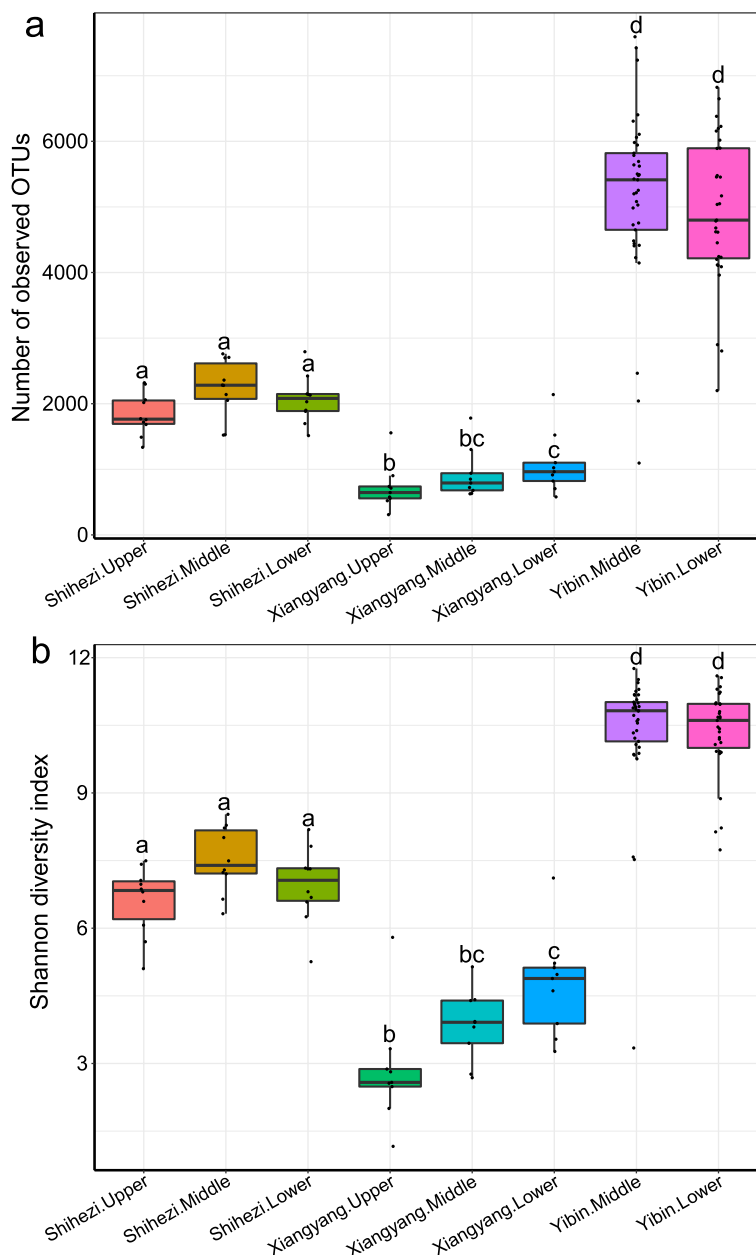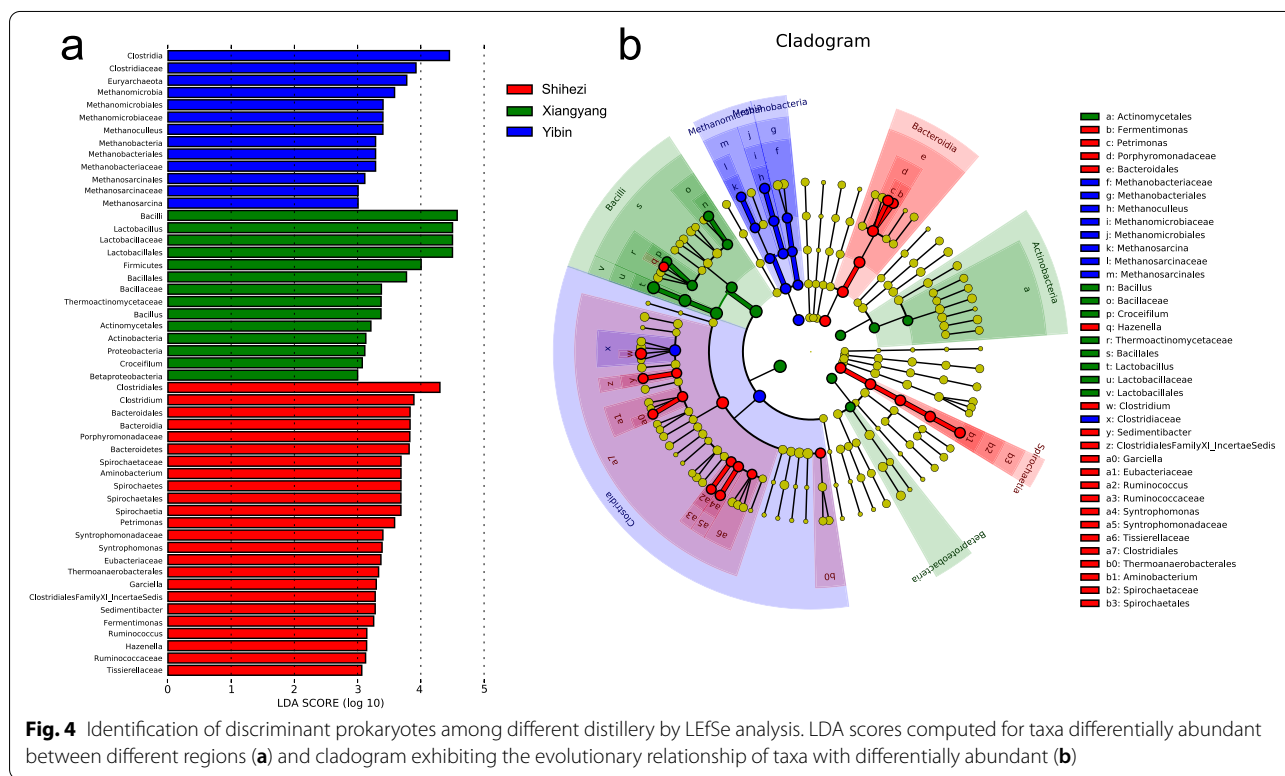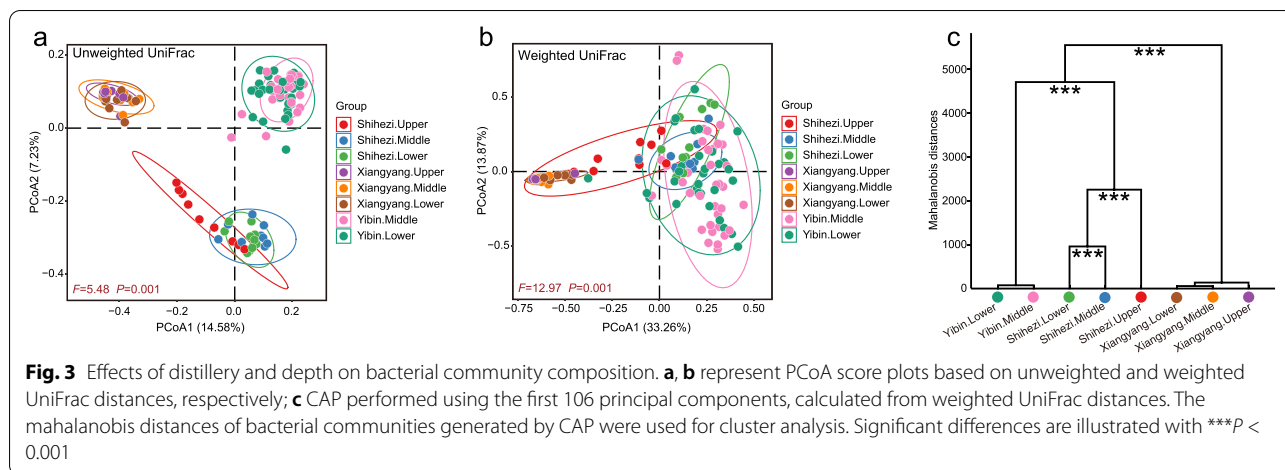
Fig. 2 Comparison of α-diversity indexes (**a** number of observed OTUs; **b** Shannon diversity index) for bacteria in samples collected from different regions and depths. Dots represent specific values for each sample

which was significantly higher than those in Shihezi and Xiangyang.

In order to reveal the influence of depth on prokaryotic composition, we compared the relative abundance of groups present at an abundance >0.1% in samples obtained from Shihezi and Xiangyang using paired Kruskal–Wallis tests (Table 1). For samples from Shihezi, the abundance of 21 genera varied significantly across different depths. The genera showing a decrease in relative abundance with an increase in depth included *Lactobacillus, Hazenella, Bacillus, Rummeliibacillus, Paenibacillus, Dehalobacterium*, and *Desulfotomaculum*, while those that showed an increase in relative abundance with increasing depth included *Aminobacterium, Cloacibacillus*, and *Gracilibacter*. The relative abundance of other prokaryotic genera did not change regularly with depth. In Xiangyang, only the relative abundance of three genera changed

Fig. 3 Effects of distillery and depth on bacterial community composition. **a**, **b** represent PCoA score plots based on unweighted and weighted UniFrac distances, respectively; **c** CAP performed using the first 106 principal components, calculated from weighted UniFrac distances. The mahalanobis distances of bacterial communities generated by CAP were used for cluster analysis. Significant differences are illustrated with ***$P <$ 0.001

Fig. 4 Identification of discriminant prokaryotes among different distillery by LEfSe analysis. LDA scores computed for taxa differentially abundant between different regions (**a**) and cladogram exhibiting the evolutionary relationship of taxa with differentially abundant (**b**)

significantly with increasing depth. Among these genera, the relative abundance of *Lactobacillus* decreased, while that of *Clostridium* and *Ruminococcus* increased with increasing depth. Using Mann–Whitney tests, we found that the relative abundance of *Lactobacillus*, *Tissierella*, *Nocardia*, *Caloribacterium*, and *Ralstonia* in the lower-layer samples from Yibin was significantly higher than that in the corresponding middle-layer samples (*P*<0.05).

Functional analysis of the microbial community in pit mud samples

Using PICRUSt software and the COG database, 4675 putative COGs were identified from all pit mud samples in the present study. In addition to the poorly characterized categories R and S, COG categories related to cell growth, such as G (carbohydrate transport and metabolism), J (translation, ribosomal structure, and biogenesis), L (replication, recombination, and repair), E (amino acid transport and metabolism), and K (transcription),
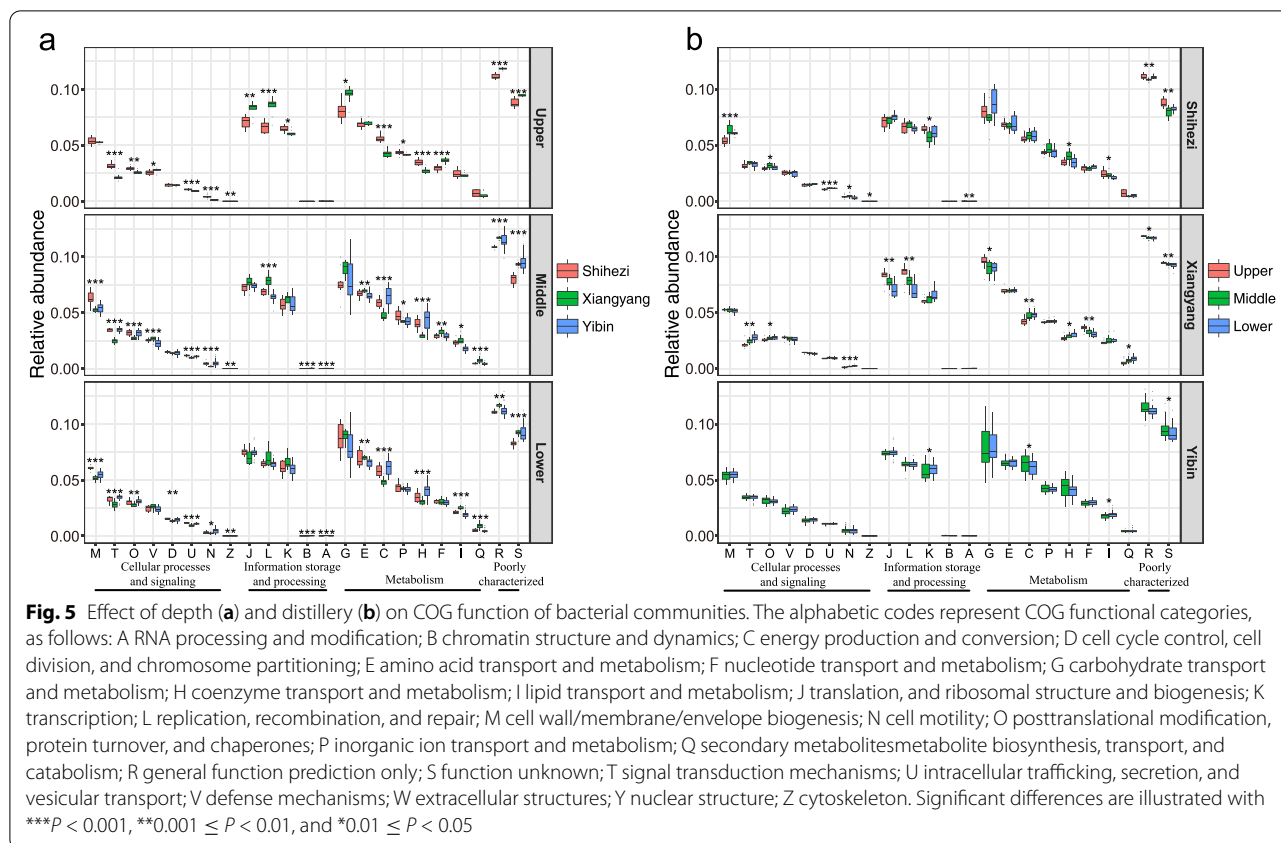
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Table 1 Genera showing significant differences in abundance at different depths in the three regions

	Genus	Upper	Middle	Lower	*P* value
Shihezi	*Lactobacillus*	20.51 ± 0.4	3.37 ± 5.02	2.79 ± 1.83	0.008
	Hazenella	7.33 ± 0.35	0.06 ± 1.82	0.03 ± 0.76	0.019
	Bacillus	2.88 ± 1.79	0.83 ± 1.78	0.45 ± 5.56	0.038
	Rummeliibacillus	1.89 ± 5.63	0.11 ± 0.98	0.04 ± 1.12	0.021
	Paenibacillus	1.05 ± 0.44	0.09 ± 1.13	0.05 ± 1.27	0.017
	Dehalobacterium	0.58 ± 0.82	0.40 ± 1.57	0.19 ± 0.68	0.012
	Desulfotomaculum	0.32 ± 1.74	0.04 ± 1.18	0.03 ± 0.80	0.021
	Petrimonas	0.76 ± 5.32	15.58 ± 0.03	7.53 ± 0.01	< 0.001
	Fermentimonas	0.65 ± 0.83	6.88 ± 0.40	2.80 ± 0.13	< 0.001
	Syntrophomonas	2.48 ± 0.28	8.30 ± 0.55	4.19 ± 0.50	0.006
	Sedimentibacter	3.00 ± 0.94	6.68 ± 0.05	4.90 ± 0.02	0.007
	Proteiniphilum	0.40 ± 0.31	1.53 ± 0.10	1.02 ± 0.24	0.025
	Tepidimicrobium	0.36 ± 0.23	0.76 ± 0.15	0.29 ± 0.06	0.022
	Microbispora	1.06 ± 0.4	0.09 ± 0.06	0.09 ± 0.03	0.005
	Tepidanaerobacter	0.12 ± 0.39	0.64 ± 0.04	0.41 ± 0.02	0.003
	Lutispora	0.21 ± 0.51	0.45 ± 0.08	0.23 ± 0.05	0.038
	Thermoactinomyces	0.59 ± 0.05	0.02 ± 0.17	0.07 ± 0.13	0.004
	Aminobacterium	2.85 ± 0.04	7.23 ± 0.09	19.20 ± 0.07	0.003
	Cloacibacillus	0.36 ± 0.23	0.38 ± 0.01	0.72 ± 0.03	0.005
	Gracilibacter	0.06 ± 0.03	0.29 ± 0.10	0.30 ± 0.10	0.021
Xiangyang	*Lactobacillus*	82.05 ± 3.64	69.23 ± 3.71	54.15 ± 5.14	0.002
	Clostridium	2.38 ± 0.42	3.87 ± 0.81	6.58 ± 0.61	< 0.001
	Ruminococcus	0.15 ± 0.03	0.36 ± 0.18	0.43 ± 0.10	0.025
Yibin	*Lactobacillus*	-	1.75 ± 1.11	6.62 ± 2.48	< 0.001
	Tissierella	-	0.39 ± 0.06	0.48 ± 0.05	0.031
	Nocardia	-	0.1 ± 0.03	0.44 ± 0.16	0.037
	Caloribacterium	-	0.11 ± 0.04	0.19 ± 0.06	0.049
	Ralstonia	-	0.06 ± 0.03	0.19 ± 0.06	< 0.001

were found to be predominant across all samples. Comparative analysis of the relative abundance of COG categories in samples from the same depth in each region (Fig. 5a) showed that COG categories Z (cytoskeleton) and U (intracellular trafficking, secretion, and vesicular transport) were significantly more abundant in samples collected from distilleries of Shihezi than in those from the other two regions. In addition, COG category R at all depths and categories L and F (nuclear transport and metabolism) in the upper and middle layers were significantly more abundant in samples from Xiangyang than in those from the other two regions. Yibin samples were significantly more enriched for the COG categories N (cell motility), C (energy production and conversion), H (coenzyme transport and metabolism), B (chromatin structure and dynamics), and S than those from the other two regions. It is worth noting that the relative abundance of COG functional categories N, C, H, and B in samples from Xiangyang was the lowest among the three regions.

Paired Mann–Whitney or Kruskal–Wallis tests were used to compare COG functional categories in samples from different depths in the three regions (Fig. 5b). For Shihezi, functional category U increased significantly in abundance as sample depth increased, and functional category I decreased with increasing depth. For Xiangyang, the relative abundance of functional categories N, H, O (posttranslational modification, protein turnover, and chaperones), T (signal transduction mechanisms), C, and Q (secondary metabolites biosynthesis, transport, and catabolism) significantly increased with increasing sample depth, while that of functional categories L, S, R, G, F, and J decreased with increasing depth. For Yibin, we found that the abundance of functional categories K and I was significantly higher in the lower layer, while that of categories C and S was significantly higher in the middle layer.

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Fig. 5 Effect of depth (**a**) and distillery (**b**) on COG function of bacterial communities. The alphabetic codes represent COG functional categories, as follows: A RNA processing and modification; B chromatin structure and dynamics; C energy production and conversion; D cell cycle control, cell division, and chromosome partitioning; E amino acid transport and metabolism; F nucleotide transport and metabolism; G carbohydrate transport and metabolism; H coenzyme transport and metabolism; I lipid transport and metabolism; J translation, and ribosomal structure and biogenesis; K transcription; L replication, recombination, and repair; M cell wall/membrane/envelope biogenesis; N cell motility; O posttranslational modification, protein turnover, and chaperones; P inorganic ion transport and metabolism; Q secondary metabolitesmetabolite biosynthesis, transport, and catabolism; R general function prediction only; S function unknown; T signal transduction mechanisms; U intracellular trafficking, secretion, and vesicular transport; V defense mechanisms; W extracellular structures; Y nuclear structure; Z cytoskeleton. Significant differences are illustrated with ****P* < 0.001, ***0.001 ≤ *P* < 0.01, and *0.01 ≤ *P* < 0.05

Relationship between prokaryotic microbiotas and functions in pit mud samples

To identify co-occurrence patterns among the dominant genera (i.e., those with an average relative abundance >1%) and potential functions of particular genera, we used Spearman's rank correlation analysis on abundance matrixes of selected prokaryotic genera and COG functional categories (Fig. 6a, b). There were close correlations between prokaryotic genera. In addition, prokaryotic genera also have close correlations with COG functional categories. Notably, the dominant genera in pit mud samples could be divided into two groups: one represented by *Lactobacillus* and *Bacillus* and the other containing all the remaining dominant genera. There was a significant negative correlation between the abundance of the two groups, but the abundance of genera within the same group showed a positive correlation with each other (Fig. 6a). There was also a significant negative correlation between the relative abundance of the first group and the COG categories B, U, T, N, O, H, and C, while there was a significant positive correlation with the COG categories L, I, E, Q, V, and K (Fig. 6b). The correlation between the relative abundance of the second group and the abovementioned COG functional units was the complete opposite to the relationship observed for the first group. Procrustes analysis was employed to reveal the consistency of two-dimensional shapes produced by the superimposition of ordination analyses from taxonomic abundance and functional profiles, and it turned out to be extremely significantly related (*P* = 0.001) (Fig. 6c).

Discussion

In the present study, we revealed that the dominant phylum in pit mud was Firmicutes, which is consistent with previous reports (Zheng et al. 2013; Liang et al. 2016). *Clostridium* and *Lactobacillus* were the dominant genera in all pit mud samples. However, the relative abundance of these two genera was quite different among samples from different distilleries. In addition, half of the cOTUs that were shared by samples from the three regions were also annotated as *Clostridium*, indicating that the genus exists widely in these regions. *Clostridium* are strictly anaerobic and play a key role during liquor production (Hu et al. 2016). First, they influence interspecies hydrogen transfer and make a synergistic contribution to metabolism, thereby maintaining the stability of the microbial community (Zheng et al. 2013). Second, they can synthesize caproic acid using sugar, starch, and cellulose as substrates (Dürre 2016; Cavalcante et al. 2017). After reacting with ethanol, caproic acid could form ethyl
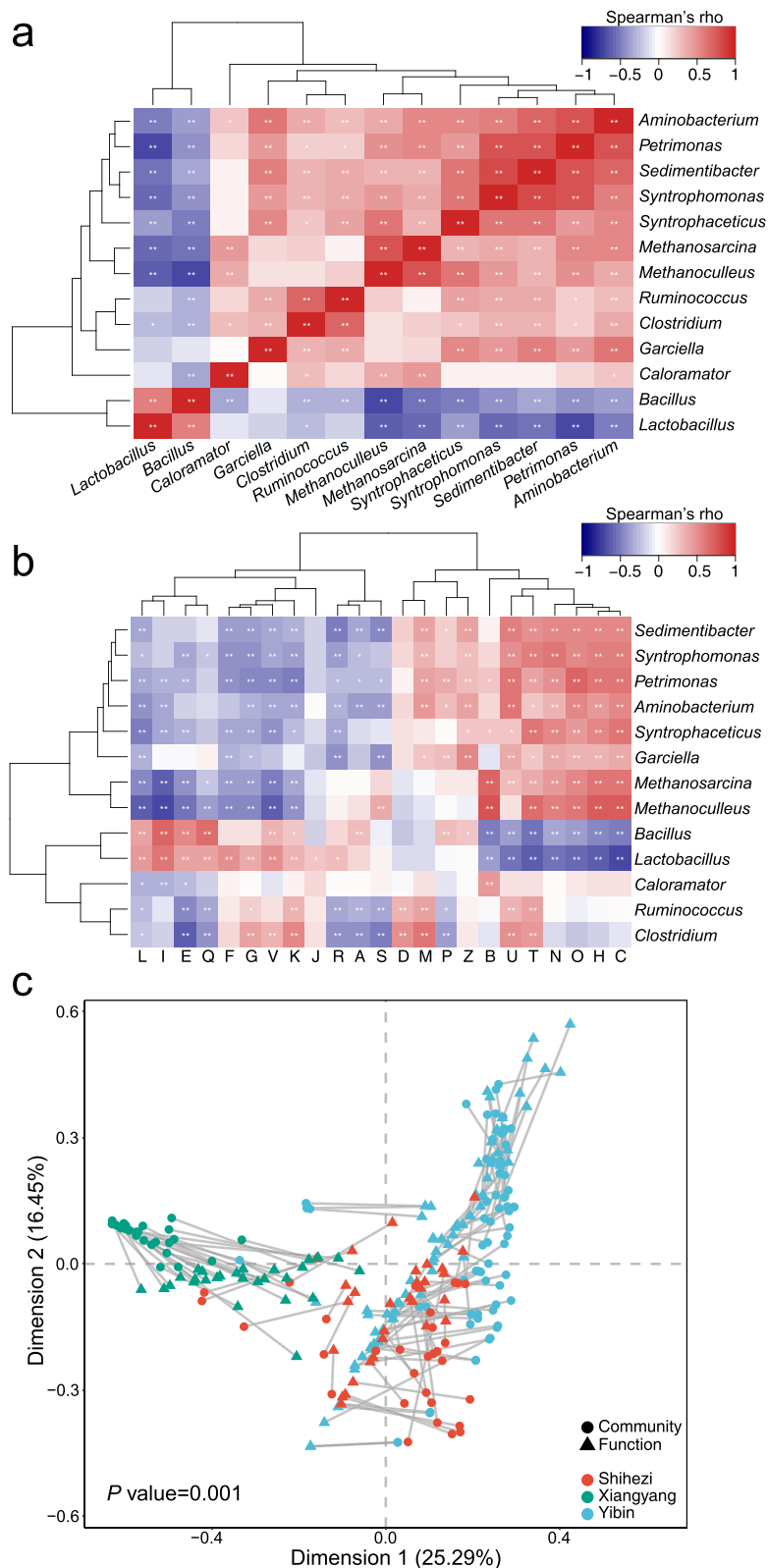
Fig. 6 Spearman's correlation between the main bacteria in pit mud samples (**a**) and between bacteria and COG functional categories (**b**). Procrustes analysis of the correlation between microbial community and functional profiles (**c**). Significant differences are illustrated with **P < 0.01 and *0.01 ≤ P < 0.05

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butyrate and ethyl caproate, the main flavor components of CSFB, through enzyme catalysis and nonenzyme catalysis, respectively (Hu et al. 2015). Therefore, a certain abundance of *Clostridium* spp. in pit mud is very important for ensuring the excellent flavor of CSFB products.

As another dominant group, *Lactobacillus* also plays an important role in CSFB production. Lactic acid, the predominant metabolite of *Lactobacillus*, has been identified as the precursor of ethyl lactate, which is also a major flavoring compound in CSFB (Fan and Qian 2005; Yang et al. 2020). In addition, *Lactobacillus* reduces the unfavorable characteristics of liquor, as lactic acid reduces the sensory impact of ethanol, enhancing its mellowness and prolonging its aftertaste (Yang et al. 2019). However, an overabundance of *Lactobacillus* in fermented grains can lead to the production of excess lactic acid and, consequently, excess ethyl lactate. Once the ethyl lactate content surpasses that of ethyl hexanoate, the aroma is reduced, the main body flavor is suppressed, and the flavor loses its balance, with a stuffy sweetness appearing (Li 2010). It is worth noting that *Lactobacillus* was present in high abundance in all samples from the distilleries of Xiangyang, irrespective of the depth of collection, and this was verified based on the results of cOTUs. This may be related to the fact that the fermentation cellars in Xiangyang collected in this study were relatively young. To improve the quality and flavor of CSFB in Xiangyang, more attention should be given to the richness of *Lactobacillus* in pit mud.

Bacillus was present in pit mud samples from all three regions. It was the third richest genus in the samples collected from Xiangyang distilleries, and its richness was obviously higher than that of pit mud samples collected from Shihezi and Yibin. *Bacillus* can produce amylase, glucoamylase, and cellulase (Li et al. 2014; Yang et al. 2017), which degrade cellulose, starch, and protein into substances that can be used for subsequent fermentation. In addition, *Bacillus* in pit mud can produce specific flavor compounds, such as 2,3-butanediol, 3-hydroxy-2-butanone, 2-methylpropionic acid, and 3-methylbutanoic acid, which are beneficial and improve the flavor of the final product (Zhang et al. 2013; Meng et al. 2015).

The results for α-diversity in this study confirmed that there were significant differences in the community structure of prokaryotes in samples from different distilleries and that samples collected from Yibin and Xiangyang had the highest and lowest α-diversity, respectively. For various macroscale ecosystems, it is well established that high biodiversity increases the essential resource use efficiency of ecological communities and maintains the balance and stability of the ecosystem (Cardinale et al. 2012). Yibin enjoys a mid-subtropical humid monsoon climate (Zhao et al. 2013),

which is very suitable for the growth of liquor-making prokaryotes. This is probably why samples from Yibin show the highest prokaryotic α-diversity. In contrast, the relatively low α-diversity in Xiangyang might be closely related to the high relative abundance of *Lactobacillus* and *Bacillus*, which were found to be negatively correlated with that of other dominant genera.

The environment (oxygen, moisture, temperature, and pressure) within the pit mud at different depths in fermentation cellars is not the same, which may lead to differences in prokaryotic composition. Therefore, prokaryotic community structures of pit muds from different depths were collected and analyzed separately in this study. By calculating the influence of distilleries and sampling depth on prokaryotic community structure, we showed that the distillery had a greater influence than did the sampling depth. This may be related to the cellar age and brewing technology, and the oxygen content and mixing ratio of grain in different enterprises may be different (Xiang et al. 2015; Xu et al. 2017). Together, these factors could lead to vast differences in the prokaryotic community structure across different enterprises.

We also examined the genera that showed a significantly different abundance across the distilleries. Specifically, among the genera enriched in samples from Shihezi, *Sedimentibacter*, and *Aminobacterium* had similar functions, as both ferment amino acids into acetic and butyric acid (Imachi et al. 2016). In addition, *Petrimonas* produces acetic acid and H_2 as the main end products of carbohydrate fermentation, and *Fermentimonas* produces acetic acid, propionic acid, CO_2, traces of isovaleric acid, and H_2 (Grabowski et al. 2005; Hahnke et al. 2016). It is worth noting that several methanogenic genera, such as *Methanomicrobia* and *Methanobacteria*, were observed in samples from Yibin but were rarely found in samples from Shihezi and Xiangyang. Previous studies have found that specific members of *Clostridium*, *Bacteroidia*, *Methanobacteria*, and *Methanomicrobia* microorganisms have synergistic effects on anaerobic digestion (Barberan et al. 2012), and more caproate is produced when they are cocultured (Liu et al. 2003), which is beneficial for liquor quality.

In terms of the main metabolic functions of the prokaryotes in pit mud, the COG functional categories with the highest relative abundance across regions, including categories G, J, L, E, K, and R, were related to cell growth (Jeong et al. 2018). The pit mud environment is rich in various microbial species, and prokaryotes increase their relative abundance in the environment through continuous proliferation, to compete for available nutrients. In this process, functional metabolism related to cell growth and reproduction becomes more active in microbes.

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There was a close positive correlation between the abundance of *Lactobacillus* and *Bacillus*. This may be related to the strong ability of *Bacillus* to resist acidic conditions (Elshaghabee et al. 2017). It is also worth noting that the existence of *Lactobacillus* and *Bacillus* in pit mud is negatively correlated with the existence of almost all other dominant prokaryotes, which indicates growth competition between the two groups. The results of the correlation analysis between the microbial community and functional capacities showed that *Lactobacillus* and *Bacillus* had significant negative correlations with COG functional modules such as B, C, H, N, and U, which are necessary for the normal growth of microorganisms, and were significantly positively correlated with functional modules related to the biosynthesis and defense mechanism of microbial secondary metabolites. This suggests that the excessive presence of these two genera in pit mud may interfere with the normal metabolic activities of pit mud microbes and lead to intense competition between pit mud microbes. Procrustes analysis further revealed the close relationship between the taxonomic abundance and functional distribution of microbial communities in pit mud. This suggests that the directional regulation of pit mud prokaryotic community functions using specific strains might become an effective method of improving CSFB fermentation processes and lead to the production of uniformly high-quality products in the future.

Conclusions

In this study, the microbial community and functional capacities of pit mud from distilleries located in three different regions of China were analyzed by Illumina sequencing of 16S rRNA gene amplicons. *Clostridium, Lactobacillus, Aminobacterium, Petrimonas, Syntrophomonas, Methanoculleus, Syntrophaceticus, Sedimentibacter, Caloramator, Ruminococcus, Bacillus, Methanosarcina,* and *Garciella* were the dominant genera of pit muds. The relative abundance of those genera in pit mud from different distilleries were quite different. *Bacillus, Lactobacillus,* and *Croceifilum* were significantly enriched in pit muds collected in Xiangyang, and the relative abundance of methanogens, such as *Methanomicrobia* and *Methanobacteria*, was only significantly enriched in pit mud collected from Yibin. In addition, differences in microbial composition in pit mud will further lead to significant differences in various metabolic functions. Therefore, it may be an effective method to improve CSFB fermentation processes by directionally regulating the microbial community functions of pit mud using specific strains. It should be pointed out that CSFB are widely distributed in various regions of China. Because some trade secrets are involved, it was difficult for us to obtain pit mud samples from a large number of distilleries. Therefore, the results of this study cannot represent the microbial and functional composition of pit mud samples from all distilleries in the regions of Xiangyang, Shihezi, and Yibin.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-022-01671-x.

Additional file 1: Figure S1. Schematic diagram of pit mud sampling sites.

Additional file 2: Figure S2. Rarefaction curves and Shannon diversity index curves of different samples.

Additional file 3: Figure S3. Core operational taxonomic units (cOTUs) of bacteria in pit mud. The colors of the squares represent the mean relative abundance of each OTU.

Availability of data and materials
Raw sequence data are publicly available online through the MG-RAST project number mgp96217 (http://metagenomics.anl.gov/linkin.cgi?project=mgp96217).

Declarations

Ethics approval and consent to participate
Not applicable: no animals or human subjects were used in this project.

Consent for publication
All of the authors consent to the publication of this manuscript in Annals of Microbiology.

Competing interests
Author WL and SY were employed by the company Hubei Guxiangyang Liquor Industry Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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