



Domination of pit mud microbes in the formation of diverse flavour compounds during Chinese strong aroma-type Baijiu fermentation

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ABSTRACT

The pit mud microbes are tightly correlated with the quality of Chinese strong aroma-type *Baijiu*. However, it is still unclear the explicit contributions of pit mud microbes to flavour formation. Through down-scaled fermentation with or without pit mud, we found that 85 genera from pit mud, dominating by *Caproiciporducens*, *Caloramator*, *Sedimentibacter* and *Caldicoprobacter*, migrated into fermented grains. These microbes increased the microbial diversity in fermented grains and resulted in an improved flavour profiles: 13 volatiles acids, 6 linear-chain alcohols and 37 esters were more abundant in the pit mud group. Most of these compounds were positively correlated with anaerobes derived from pit mud by network correlation analysis. Moreover, a synergistic effect of diverse esters formation was found between pit mud microbes and starter (*Daqu*). The pit mud microbes contributed to abundant volatile acids and linear-chain alcohols that were selectively esterified by *Daqu* (starter), and the metabolism of pit mud microbes elevated the pH of fermented grains, which further elevated the esterification capability of *Daqu*. These findings improve the understanding of the diverse flavour compounds formation during strong aroma-type *Baijiu* fermentation.

Chemical compounds studied in this article: Ethyl hexanoate (PubChem CID: 31265), Ethyl butyrate (PubChem CID: 7762), Ethyl octanoate (PubChem CID: 7799), Ethyl lactate (PubChem CID: 7344), Hexanoic acid (PbuChem CID: 8892), Butanoic acid (PbuChem CID: 264), Octanoic acid (PbuChem CID: 379), Lactic acid (PbuChem CID: 612), Butyl hexanoate (PbuChem CID: 12294), 1-hexanol (PbuChem CID: 8103).

1. Introduction

Chinese liquors (also called *Baijiu*) are traditional fermented distilled spirits. Based on flavor characters, Chinese liquors are classified into four basic aroma types, including strong aroma-type, light aroma-type, soy sauce aroma-type, and rice aroma-type. Among these, the strong aroma-type *Baijiu* dominates the market because of its rich aroma and sweet taste (Liu & Sun, 2018). Previous studies have demonstrated that the most important odor characteristics in strong aroma-type *Baijiu* are fruity, sweet, and floral, and these aromas are mainly contributed by esters, including ethyl hexanoate, ethyl butyrate, ethyl pentaenoate, ethyl octanoate, isopentyl hexanoate, and butyl hexanoate (Fan & Qian, 2005,

2006a, 2006b; Wang, Fan, & Xu, 2014). Moreover, the concentration of these flavour compounds in strong aroma-type *Baijiu* are much higher than other distilled liquor in the world, especially ethyl hexanoate with the concentration as high as 0.6–2.8 g/L (Fan, Xu, & Qian, 2019; Fang, Du, Jia, & Xu, 2018). These flavour compounds are essential to maintain the aroma characteristics of the product. Therefore, it is very important to elucidate the source of flavour compounds and the flavour formation mechanism to improve the production quality.

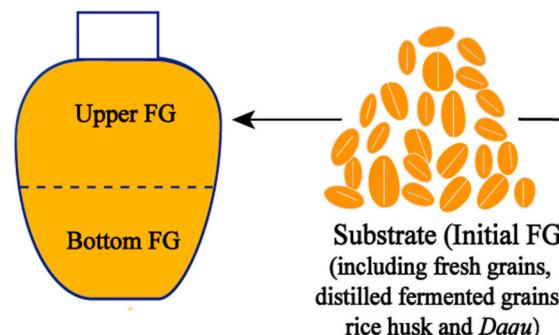
The production of strong aroma-type *Baijiu* is a spontaneous solid-state fermentation using cereals with *Daqu* (a sort of Koji) as a starter (Jin, Zhu, & Xu, 2017). *Daqu* contains various microbes and enzymes to initiate the alcoholic fermentation process and the microbes from *Daqu* also produce various flavour compounds during fermentation (Zheng, Tabrizi, Nout, & Han, 2011). More importantly, the characteristic of strong aroma-type *Baijiu* production is using a special fermentation pit (about 3.4 m long, 1.8 m wide, and 2.0 m deep) as a fermentation vessel. The inside walls and bottom of the fermentation pit are covered with pit mud, a fermented clay with diverse anaerobic microbes. It is generally believed that the synthesis of many important flavour compounds is related to pit mud (Hu, Du, Ren, & Xu, 2016; Tao et al., 2014; Wang, Du,

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The control group



The pit mud group

Fig. 1. The diagram of the experimental design. In the pit mud group, the bottoms of the jars were covered with pit mud. In the control group, pit mud was absent. Then the jars were filled with the same substrate (initial FG) at the beginning of fermentation. After a 56-d's fermentation, the fermented grains of each jar were divided into two layers, i.e., the upper layer and the bottom layer, these fermented grains were separately collected, and labeled as upper FG and bottom FG, respectively. FG, fermented grains.

& Xu, 2017). However, we don't exactly know the formation of these flavor compounds in the fermentation pit.

So far, microbes in pit mud have been studied through culture-dependent and culture-independent approaches. The microbial community in pit mud is mainly composed of bacteria and archaea (Tao et al., 2017; Zou, Zhao, & Luo, 2018). The dominant prokaryotic phyla are Firmicutes, Bacteroidetes, Euryarchaeota, including *Clostridium*, *Caproiciproducens*, *Ruminococcus*, *Caloramator*, *Sedimentibacter*, *Syntrophomonas*, *Lactobacillus*, *Petrimonas*, *Methanobrevibacter*, *Methanobacterium* and *Methanoculleus* genera (Hu et al., 2016; Liu et al., 2017; Tao et al., 2014, 2017; Zou, Zhao, & Luo, 2018). Among them, the class Clostridia is believed to be one of the key microbe groups contributing to the synthesis of short- and medium-chain fatty acids, such as butanoic acid and hexanoic acid. Several species belonging to Clostridia have been isolated from pit mud, e.g. *Clostridium kluveri* (Hu et al., 2016), *Ruminococcaceae bacterium* CPB6 (Zhu et al., 2017) and *Clostridium liquoris* (Yin et al., 2016). In addition, previous studies indicated that the microbial diversity in pit mud increases with the pit age (Tao et al., 2014), and high quality pit mud has a stable and robust community structure (Hu et al., 2016). These studies mainly focused on deciphering the community structure of the pit mud itself. However, it is still unclear which flavour compounds are contributed by pit mud microbes and how the pit mud microbes affect these flavour compounds formation.

To explore the role of pit mud microbes in flavour formation, down-scaled fermentation was performed by setting strict controls. When the fermentation finished, we compared flavour compounds in the fermented grains via multiphasic metabolite target analysis. Meanwhile, high-throughput sequencing was applied to examine the bacterial and fungal community structures. The correlation between the flavour compounds and the microbial communities was also analyzed. Finally, the effect of pit mud microbiota on flavour formation was elucidated.

2. Materials and methods

2.1. Experimental design and sample collection

To determine the flavour compounds produced by pit mud microbes during liquor fermentation, we used 4-L glass jars as laboratory reactors to simulate the industrial fermentation process. We set two groups: the pit mud group and the control group, each group has two parallel jars. In the pit mud group, the bottom of the glass jar was covered with pit mud; and in the control group, pit mud was absent. The same substrates (initial raw materials) for fermentation, i.e., a mixture of distilled fermented grains, rice husk, *Daqu* (starter) and fresh grains including sorghum, rice, wheat, and corn, were filled into the jars of the two groups, then sealed and fermented at indoor temperature (23–30 °C) for 56 days. The substrates and pit mud were obtained from a distillery, located in Bozhou, Anhui province, China. After a 56-d fermentation (the end of

fermentation), the fermented grains were divided into the upper layer and the bottom layer (Fig. 1), these samples were separately collected in each jar. Finally, all 10 samples (8 fermented grains after 56-d fermentation, substrate and pit mud) were stored at -20 °C for further analysis.

2.2. Chemicals

All of chemical standards and internal standards for quantification were GC grade (>97% purity) purchased from Sigma-Aldrich Co., Ltd. (Shanghai, China). A standard mixture of 17 amino acids and n-alkane mixture (C₇–C₄₀) were purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China). Others reagents were purchased from China National Pharmaceutical Group Corporation (Shanghai, China).

2.3. Chemical analysis of fermented grains

The water content in fermented grains was determined with a gravimetric method by drying 10-g samples at 105 °C for 3 h. The pH of fermented grains was measured by a pH meter (FE20, Mettler Toledo, Shanghai, China) after mixing with ultrapure water at a 1:2 (w/v) ratio. Starch content was measured by the method described by Wu, Chen, and Xu (2013). To determine the contents of ethanol, lactic acid and amino acids, 5 g of sample was added into 10 mL of ultrapure water, vortexed and soaked for 30 min, and then centrifuged at 4 °C and 9000×g for 5 min. The supernatant was filtered through a 0.22 µm filter, then the filtrate was applied to the following analysis. Ethanol content was measured by high-performance liquid chromatography (1200, Agilent Technologies, Santa Clara, CA) based on the method described by Wu et al. (2013). Lactic acid content was determined on a Waters Acuity ultra performance liquid chromatography (UPLC) H-Class system based on the method described by Tang et al. (2015). Amino acids were measured on UPLC system based on the method described by Boogers, Plugge, Stokkermans, and Duchateau (2008).

2.4. Volatile flavour compounds analysis of fermented grains

Fermented grains (5 g) were mixed with 25 ml of 10% ethanol solution, vortexed for 30 min and then centrifuged at 4 °C, 9000×g for 5 min. The supernatant (8 ml) and 40 µL of internal standard (2,6-dimethyl phenol) were mixed in a 20-ml headspace vial. The volatile flavour compounds were analyzed by HS-SPME-GC-MS (Headspace-Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry, GC 7890-MS 5975C, Agilent Technologies, Santa Clara, CA) based on the method described by Gao, Fan, and Xu (2014). Identification of flavour compounds was carried out by matching mass spectra with NIST11 spectrum database and verified by comparison of retention indices (RIs) with the RI reported in literatures, the RIs of compounds were calculated from the retention times of n-alkanes (C₇–C₄₀).

To determine the absolute concentration of major volatile

compounds in fermented grains, GC (Agilent 6890A, Santa Clara, CA) equipped with a flame ionization detector (FID) was employed, modified method from Wang et al. (2014). A total of 28 volatile compounds (including 10 esters, 9 acids and 9 alcohols) were selected for quantification depending on their high concentrations in strong aroma-type liquor (Wang et al., 2014; Zhao et al., 2018). The volatile compounds in fermented grains were extracted with 60% (v/v) ethanol solution by vortexing for 30 min, followed by centrifugation and filtration. A total of 10 mL supernatant was transferred into a clean tube, spiked with 100 μ L of a mixed internal standard (tert-amyl alcohol, n-pentyl acetate, 2-ethyl butanoic acid). After vortex mixing, 1 μ L of liquid was injected into GC. The GC separations were performed using a CP-Wax column (50 m length, 0.25 mm i.d., 0.20 μ m film thickness; J&W Scientific, Folsom, CA) with an oven temperature program of 35 °C, ramped at 2 °C/min to 150 °C (4 min), then heated at 6 °C/min to 195 °C for 20 min. The carrier gas was N₂ with a velocity of 1 mL/min. The split ratio was 30:1. The injector and detector temperatures were 230 °C. Individual standard stock was mixed and then diluted with 60% ethanol solution to a serial concentration of solution. These standard solutions were analyzed by GC-FID, then the calibration curves were set up (Table S3) and used for the calculation of volatile compounds in the sample.

2.5. Assessment of esters synthesis catalyzed by starter (*Daqu*)

To examine the ethyl esters synthesis capability of the starter (*Daqu*), eight acids (acetic acid, propionic acid, lactic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid) were added together into 100 mL of 20% (v/v) ethanol solution in a 100 mL-serum bottle. The molar concentration of each acid was 0.05 mol/L. Then *Daqu* powder (10% w/v) was added into the above mixed solution. The control group replaced the *Daqu* powder with autoclaved *Daqu* powder, in which the enzymes were inactivated by autoclaving at 121 °C for 30 min. The pH of the solution was separately adjusted to 3.5, 4.0, 4.5 and 5.0 by using HCl or NaOH, and then followed by incubation at 30 °C for 7 d. All experiments were done in triplicate.

To evaluate the butanoate esters and hexanoate esters synthesis capability of *Daqu*, two acids (butanoic acid, hexanoic acid) and four alcohols (butanol, hexanol, isopentanol, phenethyl alcohol) were added together into 100 mL of 20% ethanol solution in a serum bottle. The molar concentration of each acid and alcohol was 0.1 mol/L. Then *Daqu* and autoclaved *Daqu* were added and other procedures were the same as mentioned above.

To determine the concentration of esters in solutions, the esters were extracted via distillation. The esterification solution together with *Daqu* powder residue in the serum bottle was transferred into a distillation flask, and 50 mL of 30% (v/v) ethanol solution was used to rinse the serum bottle and then merged into the distillation flask. The distillation was conducted at 100 °C till 50 mL of distillate was collected, then the esters in the distillate were quantified by GC-FID as mentioned above. Due to ethyl propionate cannot be quantified by GC-FID, GC combined with selective ion monitoring (SIM) mass spectrometry was used to quantify the ethyl propionate. The monitored ions of the ethyl propionate and n-pentyl acetate (internal standard) were m/z 57, 74, 75, 102 and m/z 55, 61, 70, 73, respectively, and the quantification ion was m/z 57, m/z 70, respectively. The separation was performed with a DB-wax column (60 m length, 0.25 mm i.d., 0.25 μ m film thickness; J&W Scientific, Folsom, CA) and an oven temperature program of 35 °C, ramped at 2 °C/min to 60 °C (4 min), then heated at 6 °C/min to 150 °C, 10 °C/min to 200 °C for 10 min. The carrier gas was helium with a velocity of 1.6 mL/min. One microliter of liquid was injected into the GC. The split ratio was 30:1. The standard curves of the 16 esters were established (Table S4).

2.6. DNA extraction, PCR amplification, and sequencing

The total genomic DNA was extracted using DNeasy UltraClean

Table 1

Chemical and physical properties of the fermented grains in the down-scaled simulating fermentation.

Group	Sample	Water content (% w/w)	Residual starch (% w/w)	Ethanol (g/kg)	Lactic acid (g/kg)	pH
Control group	Initial FG	58.90	20.71	3.08	9.23	4.59
	Upper FG	68.35 ± 1.48 ^b	9.63 ± 0.10 ^a	42.92 ± 6.96 ^a	31.35 ± 1.69 ^a	3.52 ± 0.01 ^d
	Bottom FG	78.25 ± 1.06 ^a	7.03 ± 0.40 ^b	54.76 ± 5.83 ^a	29.09 ± 0.17 ^a	3.59 ± 0.00 ^c
	Bottom FG	68.15 ± 0.35 ^b	9.95 ± 0.38 ^a	17.68 ± 1.81 ^b	31.33 ± 2.06 ^a	3.64 ± 0.01 ^b
Pit mud group	Upper FG	77.70 ± 0.28 ^a	7.43 ± 1.44 ^b	17.64 ± 2.32 ^b	18.91 ± 2.15 ^b	4.64 ± 0.06 ^a
	Bottom FG					

Note: Initial FG represents the fermented grains at the start of fermentation (0 day). Upper FG and Bottom FG represent the upper layer's and the bottom layer's fermented grains at the end of fermentation, respectively. Results are the mean of two biological replicates. Values in the same column with different superscript letters are significantly different statistically ($p < 0.05$).

Microbial Kit, following manufacturer's instruction, and stored at -20 °C prior to further analysis. The concentration and integrity of the extracted DNA were determined using a NanoDrop ND-8000 spectrophotometer and agarose gel electrophoresis, respectively. For fungi, the internal transcribed spacer (ITS2) region was amplified with the primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGTTATTGATATGC-3'). The purified amplicons were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA) at Beijing Auwogene Tech. Ltd. (Beijing, China). For bacteria, the V3-V4 hypervariable regions of the 16S rRNA gene were amplified using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAA-3'). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The raw sequences were processed by QIIME pipeline (v 1.9.1). The low-quality sequences were filtered, and the chimera sequences were identified and removed using UCHIME. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE. A single representative sequence from each clustered OTU was used to align to the Silva database (v 138) and the Unite fungal ITS database (v 7.2). The Shannon diversity index and Chao 1 richness index were calculated based on resulted OTU numbers by the mothur (v 1.30.1). All the sequence data were deposited in the NCBI Sequence Read Archive (SRA) BioProject PRNJA561573 under the accession number SRP219163.

2.7. Statistical analysis

Statistical significances of the differences on the physicochemical properties, flavour compounds and diversity indexes were examined by one-way analysis of variance (ANOVA). The heatmap and cluster analysis were performed using the R software (v 3.6.2) with pheatmap package. The correlation coefficient between microbes and flavour compounds was visualized by cytoscape (v 3.7.2).

3. Results

3.1. The chemical properties of the down-scaled fermentation with- and without-pit mud

To investigate the exact effects of pit mud on liquor fermentation,

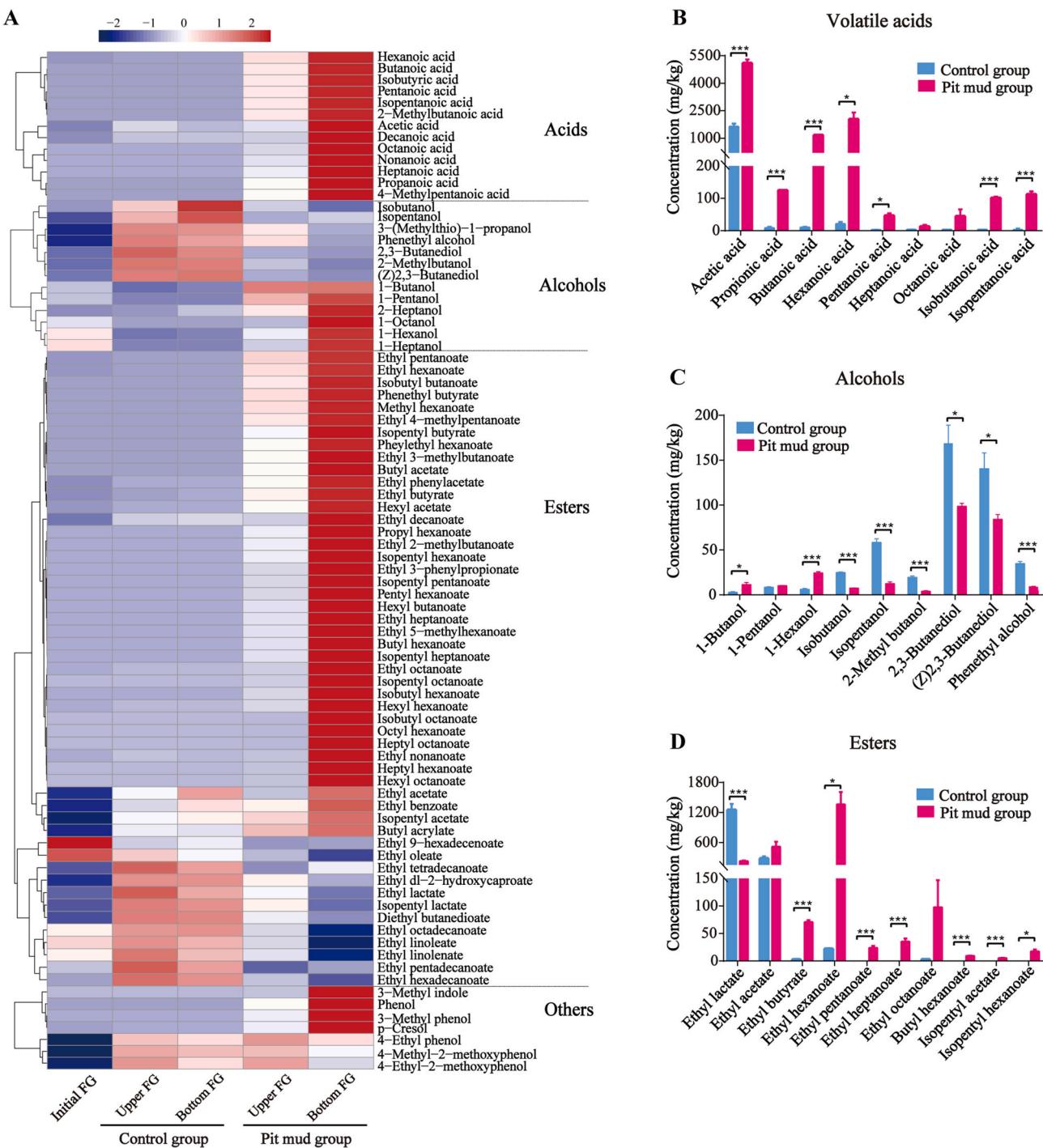


Fig. 2. The comparison of flavour compounds in fermented grains between the control group and the pit mud group. (A) Heatmap of flavour compounds in the fermented grains. The relative values for each compound in different samples were calculated based on the normalized peak areas from HS-SPME-GC-MS analysis. A total of 84 flavour compounds (including 13 acids, 13 alcohols, 51 esters and 7 others) were clustered separately. The major flavour compounds in the bottom layer's fermented grains, including volatile acids (B), alcohols (C) and esters (D) were quantified by GC-FID. The data are presented as mean \pm standard deviation of two independent replicates. The sign *, **, *** indicate significance at $p < 0.05$, $P < 0.01$, $P < 0.001$, respectively. FG, fermented grains.

down-scaled fermentation with- and without-pit mud was performed. After a 56-d's fermentation, the starch content in fermented grains decreased, whereas the contents of water, ethanol and lactic acid increased in the pit mud group as well as the control group when comparing with their initial states (Table 1). The water produced by microbial metabolism gradually accumulated and sank to the bottom layer's fermented grains during the fermentation, resulting in a higher water content in the bottom layer's fermented grains than that in the

upper layer (Table 1, Fig. S1). The changes of these key fermentation indicators in the down-scaled fermentation were similar to the changes of that in the industrial-scale fermentation (Song, Du, Zhang, & Xu, 2017; Tan, Zhong, Zhao, Du, & Xu, 2019), suggesting that the down-scaled fermentation system can be used to determine the exact roles of pit mud on flavour production of Chinese strong aroma-type *Baijiu*. During the fermentation process, the dominant products were ethanol and lactic acid. Although the starch consumptions were similar,

Table 2

Richness and diversity indexes of microbial community in fermented grains.

Group	Sample	Bacterial community			Fungal community		
		No. of representative OTUs	Chao 1	Shannon	No. of representative OTUs	Chao1	Shannon
Control group	Initial FG	15	227.64	2.96	131	186.85	2.11
	Upper FG	18 ± 4 ^b	26.00 ± 8.49 ^b	0.02 ± 0.01 ^c	148 ± 84 ^{bc}	242.30 ± 100.17 ^{ab}	1.15 ± 0.77 ^a
	Bottom FG	23 ± 3 ^b	59.80 ± 46.95 ^b	0.06 ± 0.01 ^b	108 ± 0 ^c	174.31 ± 35.04 ^b	1.14 ± 0.30 ^b
Pit mud group	Upper FG	26 ± 7 ^b	39.63 ± 5.83 ^b	0.02 ± 0.01 ^{bc}	262 ± 21 ^a	295.66 ± 14.72 ^a	2.22 ± 0.28 ^a
	Bottom FG	190 ± 21 ^a	239.75 ± 18.98 ^a	0.75 ± 0.23 ^a	150 ± 9 ^b	204.60 ± 6.22 ^b	2.71 ± 0.11 ^a

Note: Initial FG represents the fermented grains at the start of fermentation (0 day). Upper FG and Bottom FG represent the upper layer's and bottom layer's fermented grains at the end of fermentation (56 day), respectively. Results are the mean of two biological replicates. Values in the same column with different superscript letters are significantly different statistically ($p < 0.05$).

significant differences were observed on the production of ethanol and lactic acid (Table 1). At the end of fermentation, the concentration of ethanol in the pit mud group was significantly lower than that in the control group. Moreover, the lower concentration of lactic acid and higher pH were only observed in the bottom layer's fermented grains in the pit mud group.

3.2. Comparison of volatile compounds in fermented grains between the pit mud group and the control group

We identified 84 volatile compounds from all fermented grains, including 13 alcohols, 13 acids, 51 esters and 7 others compounds by HS-SPME-GC-MS analysis (Fig. 2A, Table S1). Overall, the contents of volatile compounds were significantly increased in the pit mud group, especially in the bottom layer's fermented grains (Fig. S2), suggesting

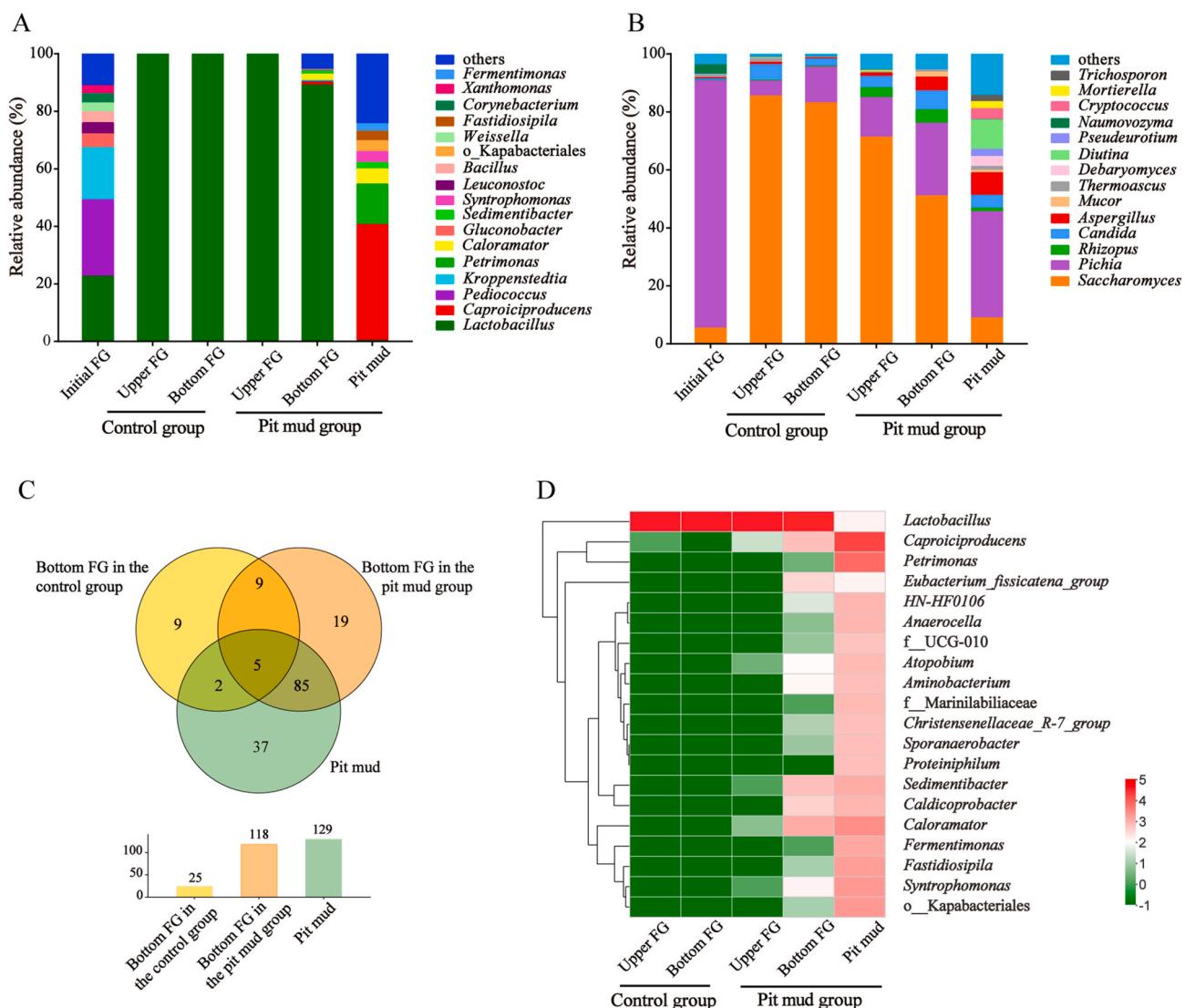
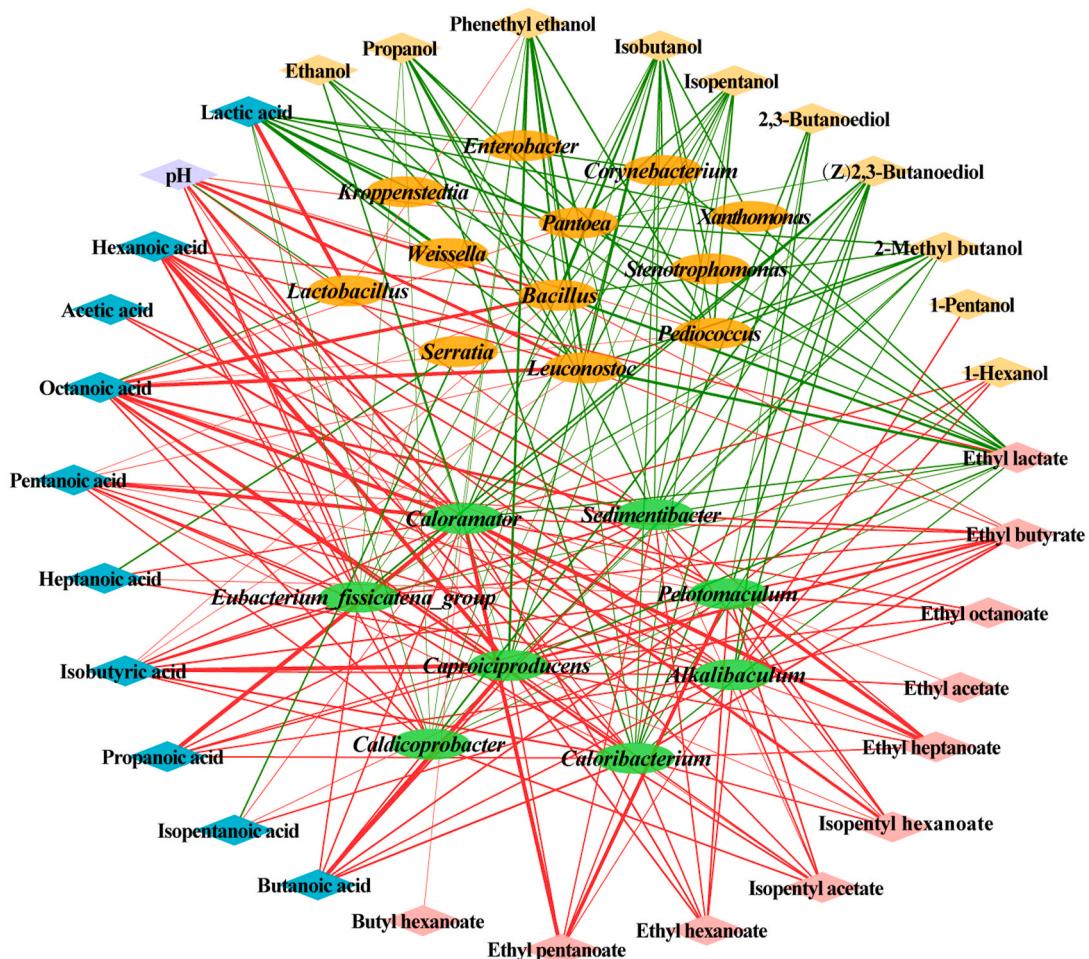


Fig. 3. The relative abundance of bacterial (A) and fungi (B) communities in the fermented grains and pit mud at the genus level. The Venn diagram (C) shows the distribution of bacterial communities in the bottom layer's fermented grains and pit mud at the genus level. The heatmap (D) shows that bacteria from pit mud strongly affected the bacterial community composition in fermented grains. FG, fermented grains.

A



B

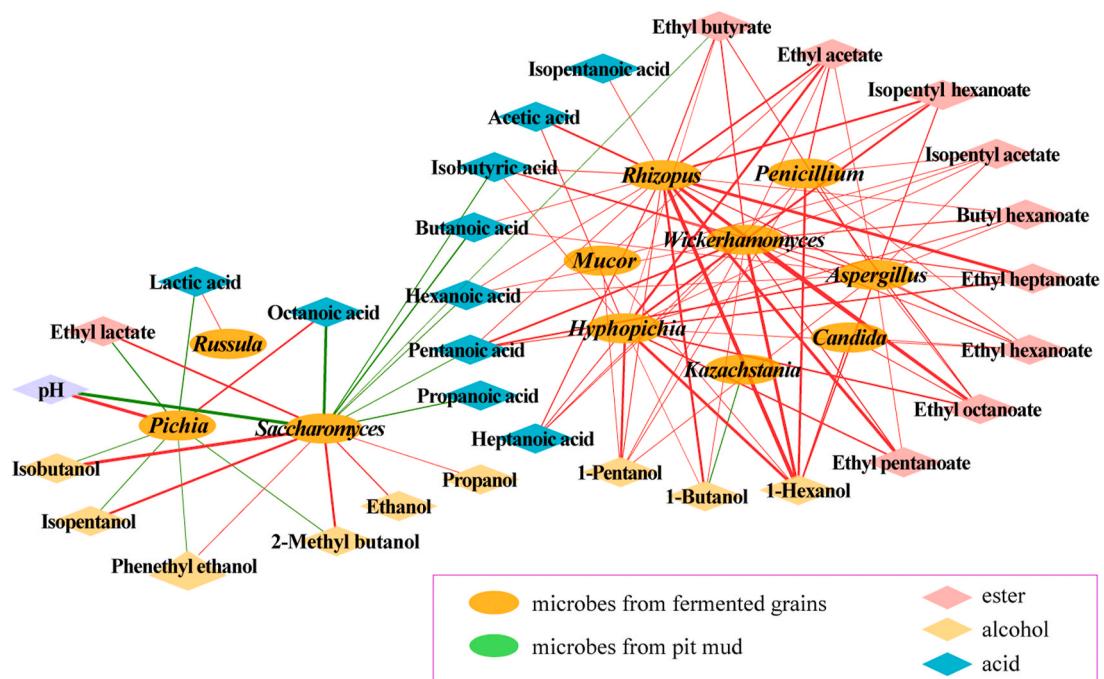


Fig. 4. The correlation of bacterial (A) and fungi (B) communities with flavour compounds by network correlation analysis. The color of the lines corresponds to a positive (red) or negative (green) correlations. The thickness of lines is proportional to the value of Spearman's correlation coefficient. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

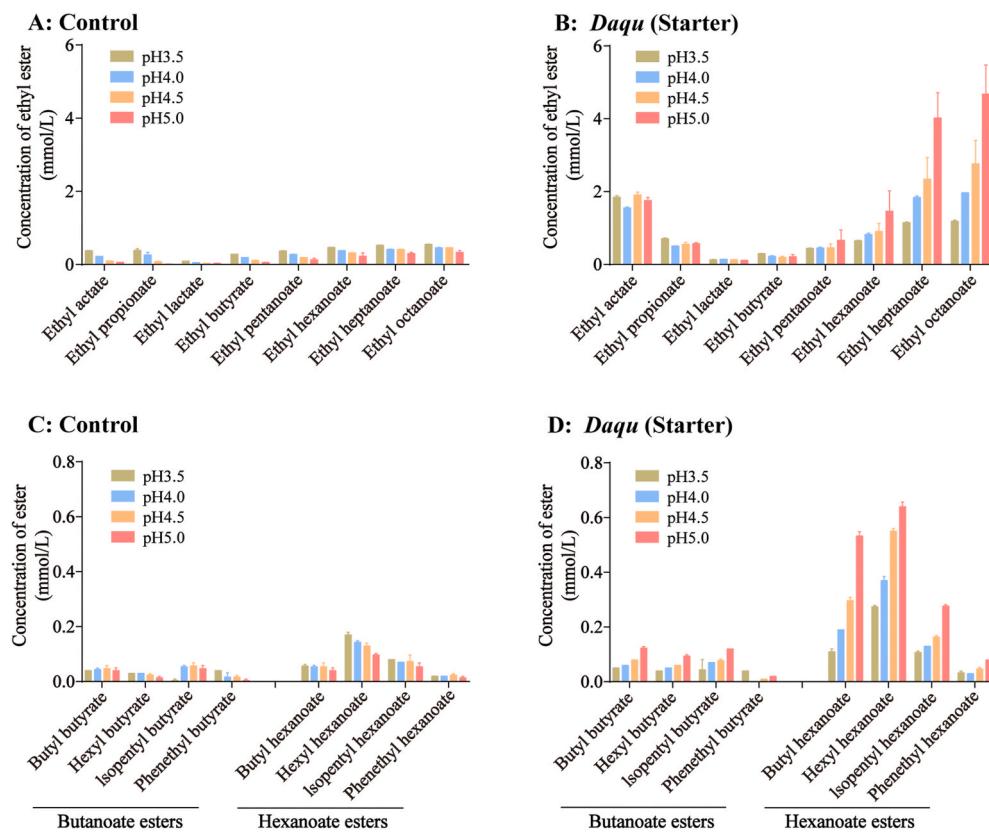


Fig. 5. The effects of starters (*Daqu*) on esterification. Comparison of ethyl esters under different pH conditions with autoclaved *Daqu* (A) or with native *Daqu* (B). Comparison of hexanoate esters and butanoate esters under different pH conditions with autoclaved *Daqu* (C) or with native *Daqu* (D). The acids and alcohols had the same molar concentration in the initial 20% (v/v) ethanol solution. All experiments were done in triplicate.

that pit mud greatly affected the flavour production in the bottom layer's fermented grains.

Fig. 2A shows that pit mud affected the distribution of volatile acids, linear- or branched-chain alcohols, and esters in fermented grains. The control group was characterized by branched-chain alcohols, lactate esters and long-chain fatty acid ethyl esters, e.g. isobutanol, 2,3-butandiol, isopentanol, ethyl lactate, isopentyl lactate, ethyl hexadecanoate and ethyl oleate. However, the pit mud group was rich in volatile acids, linear-chain alcohols and their related esters. Among these 84 detected compounds, the contents of 13 volatile acids, 6 linear-chain alcohols and 37 esters were more abundant in the pit mud group, and in particular, 19 esters were only detected in the pit mud group (Table S1). Moreover, when pit mud was present, most of the flavour compounds in the bottom layer's fermented grains were higher than that in the upper layer. Then absolute concentration of the volatile compounds in the bottom layer's fermented grains were further quantified by GC-FID (Fig. 2B through 2D). In the control group, extremely low contents of volatile acids were detected except for acetic acid (Fig. 2B), and ethyl acetate and ethyl lactate were the dominant esters (Fig. 2D). By contrast, all of the detected volatile acids and majority of the detected esters in the pit mud group were much higher than that in the control group. Specifically, acetic acid, hexanoic acid and butanoic acid were the predominate volatile acids and the concentration of these acids were all above 1000 mg/kg (Fig. 2B). Ethyl hexanoate was the most abundant ester with a concentration exceeding 1000 mg/kg (Fig. 2D), which corresponded to the fact that ethyl hexanoate has the highest concentration in strong aroma-type *Baijiu*. However, branched-chain alcohols including isobutanol, isopentanol, 2,3-butanediol and phenethyl alcohol in the pit mud group were lower than that in the control group (Fig. 2C).

3.3. The influence of pit mud on bacteria community and fungi community in fermented grains

High-throughput sequencing was applied to characterize the microbial community structures in fermented grains and pit mud. Compared to the start state of the fermentation, the bacterial diversity in fermented grains decreased at the end of the fermentation. Meanwhile, the fungal diversity decreased in the control group, whereas slightly increased in the pit mud group (Table 2). For the pit mud group, the richness indicated by Chao 1 index and the diversity indicated by Shannon index of the bacterial community in the bottom layer's fermented grains were higher than that in the upper layer (Table 2), suggesting that the microbial richness and diversity were strongly elevated by pit mud.

A total of 221 bacterial genera and 127 fungal genera were identified from all samples. For bacterial communities, ten genera (*Pediococcus*, *Lactobacillus*, *Kroppenstedtia*, *Glucoronobacter*, *Leuconostoc*, *Bacillus*, *Corynebacterium*, *Weissella*, *Xanthomonas* and *Enterobacter*) over 1% abundance were dominant at the beginning of fermentation (0 day). However, at the end of fermentation, *Lactobacillus* became the most dominant genus in the pit mud group as well as the control group (Fig. 3A). For the control group, the relative abundance of *Lactobacillus* in the fermented grains exceeded above 99%. For the pit mud group, the relative abundance of *Lactobacillus* in the upper layer's fermented grains also reached 99%, but decreased to around 90% in the bottom layer's fermented grains. Venn diagram shows that 85 genera were shared by pit mud and the bottom layer's fermented grains in the pit mud group, whereas only 2 genera were shared by pit mud and the bottom layer's fermented grains in the control group (Fig. 3C, Table S2). Therefore, these 85 genera may originate from pit mud. More importantly, four genera (*Caproiciproducens*, *Caloramator*, *Sedimentibacter* and *Caldicoprobacter*) that were abundant in pit mud were also observed in the bottom

layer's fermented grains in the pit mud group, and the total relative abundance of these genera accounted for 5.26% (Fig. 3A and D). Interestingly, the relative abundance of *Lactobacillus* in pit mud was less than 1%, although *Lactobacillus* was the most dominant bacterial genus in fermented grains (Fig. 3A).

For the fungi community, *Pichia* was the most abundant genus with a relative abundance of 85.34% in the start state of the fermentation, whereas *Saccharomyces* became the most dominant genus in fermented grains at the end of the fermentation. Compared with the control group, lower *Saccharomyces* was observed in the pit mud group, especially in the bottom layer's fermented grains. By contrast, *Pichia*, *Rhizopus*, *Aspergillus* and *Mucor* were more abundant in the pit mud group (Fig. 3B).

Network correlation analysis was applied to investigate the correlation between microbes and flavour compounds. We calculated the Spearman's correlation coefficient between the dominate genera (relative abundance >0.5%) and the quantified flavour compounds, and chose the coefficient ($|ρ|>0.7$ and significance (P) < 0.05 as strongly correlated nodes to construct correlation network. Fig. 4A shows that the dominate microbes from pit mud including *Caproicacidobacter*, *Caloramator*, *Sedimentibacter* and *Caloribacterium* were positively correlated with all of the volatile acids and majority of the esters except for ethyl lactate, and these microbes were negatively correlated with alcohols except for 1-pentanol and 1-hexanol. Moreover, the pH was positively correlated with six genera from pit mud, i.e. *Caproicacidobacter*, *Caloramator*, *Sedimentibacter*, *Caloribacterium*, *Eubacterium fissionum* group and *Caldicoprobacter*.

For ester formation, most of esters was strongly positively correlated with *Aspergillus*, *Rhizopus*, *Hyphopichia*, and *Wickerhamomyces*. In addition, *Saccharomyces* was positively correlated with ethanol, isobutanol, isopentanol, 2-methyl butanol and phenethyl alcohol, suggesting that these compounds might be mainly produced by *Saccharomyces*. However, *Saccharomyces* was negatively correlated with six volatile acids including hexanoic acid, octanoic acid, butanoic acid, propanoic acid, pentanoic acid and isobutanoic acid (Fig. 4B).

3.4. Volatile acids produced by pit mud microbes are selectively esterified by enzymes in starters

Abundant esters were the remarkable characteristics of the fermented grains contacted with pit mud. However, it is unclear that how these diverse esters are synthesized. It has been reported that pit mud anaerobes are deficient to synthesize esters (Zou, Ye, & Zhang, 2018). *Daqu*, a sort of starter, not only provides microbes for alcohol production but also contains abundant enzymes, including amylases, proteases, lipases and esterases (Liu, Chen, Fan, Huang, & Han, 2018). To confirm whether volatile acids and alcohols in fermented grains can be used to synthesize esters by the esterase from *Daqu*, *in vitro* esterification verification was performed.

Fig. 5 shows that much fewer esters were synthesized in the group with autoclaved *Daqu* than that in the group with native *Daqu*. When equal molar amounts of eight acids were added together to ethanol solution supplemented with *Daqu*, the concentration of synthesized ethyl esters showed a concave trend as the increase of carbon number in the fatty acid molecule (Fig. 5B), suggesting that *Daqu* had a selective esterification capacity. More specifically, *Daqu* hardly catalyzed lactic acid and butanoic acid to form corresponding ethyl esters. However, for medium-chain fatty acids with carbon number ≥ 5 , the esterification capacity of *Daqu* was increased with the increase of carbon number in the fatty acid molecule and the concentration of corresponding ethyl esters was increased at higher pH (Fig. 5B). The esterification pattern found in verification was consistent with the concentration ratio of esters in the bottom layer's fermented grains in the pit mud group. The concentration of ethyl lactate in fermented grains was 5 times lower than ethyl hexanoate, although the concentration of lactic acid was 10 times higher than hexanoic acid (Fig. S3). Similarly, although the

concentration of hexanoic acid in fermented grains was 1.5 times higher than butanoic acid, the concentration of ethyl hexanoate was surprisingly 19 times higher than ethyl butyrate (Fig. 2B and D), indicating that the ethyl hexanoate in fermented grains was mainly esterified by enzymes from *Daqu*, while the ethyl lactate and ethyl butyrate may be mainly esterified by non-enzymatic catalysis. In addition, when equal molar amounts of two acids and four alcohols were added to ethanol solution supplemented with *Daqu*, we found that *Daqu* was more prone to prompt hexanoate esters synthesis rather than butanoate esters, and hexanoate esters were also increased as pH was elevated (Fig. 5D).

4. Discussion

As an essential anaerobe source to strong aroma-type *Baijiu* fermentation, pit mud contributes to the special flavour of strong aroma-type *Baijiu*. However, it is still unclear that the explicit contribution of pit mud to flavour compounds as well as the anaerobes involved in flavour production. Through down-scaled simulating fermentation with strict controls, we clarified the effects of pit mud microbes on the flavour formation as well as the microbial community structure in fermented grains.

The previous studies focused on the acid-producing function of pit mud microbes, especially butanoic acid (Chai et al., 2019) and hexanoic acid (Hu, Du, & Xu, 2015; Zhu et al., 2017). Besides these two dominant volatile fatty acids, we proved here that other volatile acids such as pentanoic acid, octanoic acid and isopentanoic acid, and volatile linear-chain alcohols such as 1-butanol, 1-hexanol and 1-octanol are derived from pit mud microbes. Although the concentrations of these volatile fatty acids and linear-chain alcohols in fermented grains were low, they are essential flavour compounds and also the vital precursors for diverse esters synthesis (Fan et al., 2019). The versatile volatile fatty acids and linear-chain alcohols produced by pit mud microbes shapes the qualities of strong aroma-type *Baijiu*. For instance, high concentration of ethyl hexanoate (>1.2 g/L) is one of the indicators of high-qualified strong aroma-type *Baijiu*.

Our previous study applying microbial source tracking analysis indicated that pit mud is a sustained-release source for anaerobic bacteria which continuously migrate into fermented grains (Wang et al., 2017). In this study, we found that pit mud not only affected the bacteria community, but also affected the fungi community in fermented grains, especially decreasing the relative abundance of *Saccharomyces*, the dominant ethanol producer. Network analysis shows that *Saccharomyces* was negatively correlated with some volatile acids, e.g., butanoic acid, hexanoic acid and octanoic acid (Fig. 4B), indicating that these acids could inhibit the growth of *Saccharomyces*. A recent study proves that the growth of *Saccharomyces* is seriously inhibited by hexanoic acid exceeding 6 mg/L (Pinu, Villas-Boas, & Martin, 2019). In this study, the concentration of hexanoic acid in the pit mud group reached as high as above 1000 mg/kg, which may strongly inhibit the growth of *Saccharomyces*. This explains that the lower content of ethanol was found in the pit mud group. Thus, improving the acid tolerance of *Saccharomyces* to hexanoic acid may help to enhance ethanol production in strong aroma-type *Baijiu* fermentation.

Except for the lower content of ethanol, the lower content of lactic acid in the pit mud group was also observed in the bottom layer's fermented grains. We speculate that the reduction of lactic acid in the bottom layer's fermented grains is mainly utilized by anaerobic microbes derived from pit mud. Recent studies have proved that specific *Caproicacidobacter* spp. can convert lactate into hexanoate (Zhu et al., 2017), and several *Clostridium* species can convert lactate into butyrate (Tao et al., 2016). These lactate-conversion anaerobes were detected in the fermented grains of the pit mud group. However, the consumption of lactate was rarely observed in the bulk industrial fermentation, may be result of mass transfer issue (Ding, Wu, Huang, & Zhou, 2016). Furthermore, the conversion of lactate to butyrate and hexanoate consumes H^+ (Zhu et al., 2017), which may contribute to the rising of pH in

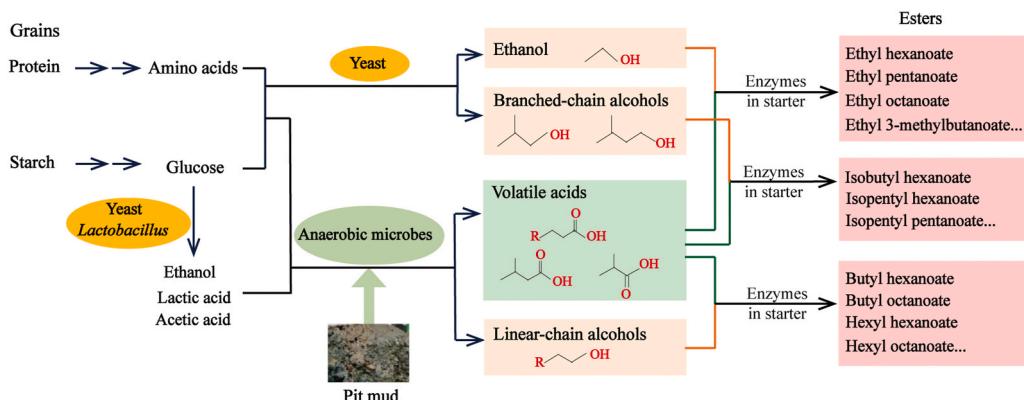


Fig. 6. A schematic representation of the flavour compounds formation during the solid-state fermentation of strong-aroma type *Baijiu*. The microbes marked in green originated from pit mud, while the microbes marked in orange originated from starters (*Daqu*). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fermented grains.

Moreover, the lower contents of amino acids were also observed in the bottom layer's fermented grains in the pit mud group (Fig. S4). Previous researches showed that amino acids can be degraded to short- and branched-chain volatile acids by some anaerobic microbes, such as *Sedimentibacter* (Imachi et al., 2016), *Caloramator* (Tarlera & Stams, 1999), and *Aminobacterium* (Baena et al., 2000; Liu, Huang, & Zhang, 2016). The presence of these anaerobic microbes in the bottom layer's fermented grains may result in the higher contents of volatile acids, especially branched-chain acids. The deamination reactions release ammonium, which could also promote the rise of pH in fermented grains. Taken together, the lactate utilization and deamination of amino acids by pit mud microbes elevated the pH in fermented grains. Moreover, the esterification capacity of starter was increased with the rise of pH, implying that the metabolism of pit mud microbes could promote the formation of esters.

The ester formation can be classified to enzymatic esterification and chemical esterification (Sumby, Grbin, & Jiranek, 2010). The esters in fermented foods such as wine and cheese are mainly formed by microbial enzymatic reactions, and their synthesis mechanism has been widely investigated (Holland et al., 2005; Sumby et al., 2010). However, the synthetic characteristics of diverse esters has not been well examined in solid-state fermentation of *Baijiu*. In this study, the substrate specificity of *Daqu* on ester synthesis was similar to the enzymatic properties of the lipase derived from fungi, which is influenced by the carbon chain length of the substrate (Yan, Zhang, & Wang, 2014; Oliveira et al., 2017). The majority of lipases show high capability to synthesize ethyl butyrate (Martins et al., 2014), differently, the low selectivity of butyrate for esterification found in fermented grains may indicate that certain unidentified lipase may be present in *Daqu*. The optimal pH of lipase is usually neutral or alkaline (Cong et al., 2019), corresponding to the higher esterification capability of *Daqu* at elevated pH (Fig. 5). Although yeasts can produce various esters, their capability to synthesize medium-chain fatty acid ethyl esters are relatively weak, usually with around 1 mg/L in liquid fermentation (Hu, Jin, Mei, Li, & Tao, 2018; Saerens, Delvaux, Verstrepen, & Thevelein, 2010). In this study, we found that >1000 mg/kg ethyl hexanoate were produced when hexanoate was sufficient. Therefore, we speculate that the key esters of medium-chain fatty acids, e.g. ethyl hexanoate and ethyl octanoate in fermented grains, were mainly synthesized by the lipase from *Daqu* rather than yeasts.

In summary, pit mud provided anaerobic bacteria to produce abundant volatile acids and linear-chain alcohols, together with ethanol and branched-chain alcohols contributed by microbes from starters, a wide range of esters were finally synthesized under the esterification of starters. The unique synthesis of the diverse flavour compounds in the fermentation pit was illustrated in Fig. 6. These flavour compounds

formation pattern, especially diverse esters production pattern displayed a synergistic effect between pit mud microbes and starter (*Daqu*) during fermentation. Expect for producing acids and linear-chain alcohols, the pit mud microbes determine the esterification capability of *Daqu* by regulating pH, and the selectively esterification of *Daqu* determines the type of ester produced, which endows the unique flavour character to strong aroma-type *Baijiu*.

This study provided a comprehensive insight into the sources of diverse volatile flavour compounds in the liquor fermentation using mud pits as fermentation vessels, and especially illuminated the contribution of pit mud microbes to diverse flavour formation. Our findings may help to design effective strategies to regulate flavour formation in liquor or other food fermented system. Future research needs to focus on the isolation of key species responsible for flavour formation, and elucidate the metabolic characteristics and microbial interactions, to pave the way to control the flavour formation and to improve the product quality.

CRediT authorship contribution statement

Jiangjing Gao: Investigation, Data curation, Writing - original draft. **Guoying Liu:** Resources, Data curation. **Anjun Li:** Resources, Project administration. **Chenchen Liang:** Investigation, Validation. **Cong Ren:** Conceptualization, Writing - review & editing, Visualization. **Yan Xu:** Conceptualization, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2020.110442>.

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