



## Exploring the response patterns of strong-flavor baijiu brewing microecosystem to fortified *Daqu* under different pit ages

Yu Mu<sup>a</sup>, Jun Huang<sup>a</sup>, Rongqing Zhou<sup>a,b,\*</sup>, Fengjiao Mao<sup>a</sup>, Qianglin Pan<sup>a</sup>, Suqi Chen<sup>a</sup>, Zhongming Lu<sup>c</sup>, Liquan Du<sup>c</sup>, Fei Xie<sup>c</sup>

<sup>a</sup> College of Biomass Science and Engineering, Sichuan University, Chengdu 610065, China

<sup>b</sup> National Engineering Research Center of Solid-State Manufacturing, Luzhou 646000, China

<sup>c</sup> Forgood Distillery Co. Ltd., Mianyang 621000, China



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### ABSTRACT

The fermentation of strong-flavor baijiu depends largely on the evolution of the microbial community that originated from *Daqu* and pit mud (PM). Applying fortified *Daqu* (FD) has been proven to be an effective strategy to improve the quality and yield of baijiu. However, it is unclear what the effects of FD on the liquor brewing microecosystem under different pit ages because of the temporal heterogeneity of the PM community. Taking 2-year (new) and 40-year (aged) pits as the objects, the influence of FD on the metabolic profile, physicochemical parameters, and community diversity in *Zaopei* and PM was investigated by polyphase detecting approaches. Present results showed that the metabolic profiles of *Zaopei* were significantly improved by FD, whereas those of PM were mainly dependent on pit age. *Aspergillus*, *Caproiciproducens*, and *Methanoscincus* were more abundant in the aged pit, while *Kazachstanica*, *Lactobacillus*, and *Sphingomonas* dominated in the new pit, whether in *Zaopei* or in PM. The interaction relationships among the communities were also altered by FD, and the co-occurrence network, especially the increased links between archaea and bacteria in the new pit. Notably, this interaction in the aged pit distinctly affected the hexanoic acid content based on the Mantel test. The results of PICRUSt2 analysis inferred that FD perhaps improved the interspecies hydrogen transfer in the new pit and increased the carbon flow of hexanoic acid production during chain elongation in the aged pit. These results provide new insights into the production of high-quality strong-flavor baijiu and the aging of PM.

### 1. Introduction

Baijiu, a distinctive traditional fermented food, is closely associated with the Chinese nation's inheritance of culture and wisdom (Jin, Zhu, & Xu, 2017; Zheng & Han, 2016). The properties of the food fermentation process, as we know, are endowed by the natural succession and evolution of the microbial community in an open environment (Du, Wang, Zhang, & Xu, 2019; Zhang et al., 2021). As a result, the microbial population of fermented foods influences its quality, features, and safety (Jin et al., 2017; Wu, Zhu, Fang, & Wijffels, 2021).

Strong-flavor baijiu (SFB) is one of the most popular liquors in China, the SFB brewing microecosystem is primarily comprised of *Daqu* (starter), *Zaopei* (fermentation substrate), and mud pit (fermentation cellar) (Zou, Zhao, & Luo, 2018). *Daqu* is not only an indispensable crude enzyme and starter but also one of the special raw materials for *Zaopei* fermentation, and thus plays a critical role in the quality and yield

of baijiu (Wang, Du, Zhang, & Xu, 2017; Yang et al., 2021; Zheng, Tabrizi, Nout, & Han, 2011). As an anaerobic reactor, the mud pit provides a spatially linked environment for *Zaopei* and pit mud (PM), a unique clay inhabiting a variety of functional microbiota, and their interphase mass transfer has an impact on both material metabolism and microbial diversity (Ding, Wu, Huang, & Zhou, 2015; Li et al., 2016; Qian et al., 2021). Microorganisms in PM can migrate into *Zaopei* during fermentation, while those in *Zaopei* will transfer to PM under gravity via *huangshui* (liquid metabolites of *Zaopei* fermenting) (Gao, Liu, et al., 2021; Tan, Zhong, Zhao, Du, & Xu, 2019; Wang, Du, & Xu, 2017). However, *Daqu* is also produced in an open environment in which the microbial community is influenced by various factors such as the fermentation environment, the variety and origin of raw materials, and process parameters, which result in the difference in quality and microbiota of *Daqu* between batches (Du et al., 2019; Zheng et al., 2014). Meanwhile, the diversity and metabolic activity of PM

\* Corresponding author at: College of Biomass Science and Engineering, Sichuan University, Chengdu 610065, China.

E-mail address: [zhourqing@scu.edu.cn](mailto:zhourqing@scu.edu.cn) (R. Zhou).

microbiota are closely related to the using periods, usually becoming stable over 20–30 years (Liu et al., 2020; Tao et al., 2014; Wang et al., 2014). As a result, the yield and quality of SFB are influenced by the quality of *Daqu* and PM, as well as the interaction between their microbiota.

In order to optimize and intensify the process, the fortification technology based on inoculating functional strains or microbiota has been developed. For example, the content of tetramethyl-pyrazine in both *Zaopei* and baijiu increased by adding *Bacillus amyloliquefaciens* to *Zaopei* directly (Zhang, Si, et al., 2020). Besides, *Wickerhamomyces anomalus* was conducive to the synthesis of ester compounds in *Zaopei* (Wang et al., 2020), while *B. subtilis* and *B. amyloliquefaciens* could prevent the geosmin contamination during baijiu fermentation (Zhi, Wu, Du, & Xu, 2016). In fact, these technologies need to be further optimized for application on industrial scale, involving the culture time, inoculating rate, homogeneity and other factors. Recently, the FD technique based on directional bioturbation of functional strains has also proved an effective strategy for improving the quality of baijiu. For example, *Daqu* inoculated *Bacillus*, *Saccharomyces* and *Clavispora* significantly improved the enzyme activity and flavor profile (He, Dong et al., 2019; Li et al., 2019; Wang, Wu, et al., 2017). In addition, the directed evolution of functional microbiota was observed in the maturation process of artificial PM when FD used (Chen et al., 2020). In particular, the improved interspecies hydrogen transfer is beneficial to the reproduction of functional microbiota in PM and enhancing the content of ethyl hexanoate in baijiu (He, Huang, Wu, Jin, & Zhou, 2020; He, Huang, Zhou, Wu, & Jin, 2019). However, the microbiota and metabolites of PM vary with pit age due to the significant temporal heterogeneity (Chai et al., 2021; Ding et al., 2015; Li et al., 2016; Tao et al., 2014). It is so far unclear whether the effect of FD on PM activity is always positive.

In the present research, the differences in the responses of the community and the metabolites in the different microecosystems to FD were investigated with the help of polyphase detection and high-throughput sequencing approaches. These microecosystems were composed of *Zaopei* and PM in the mud pit, with the pit ages of 2 and 40 years, respectively. The co-occurrence network of dominant genera and the interaction between microorganisms and metabolites were explored by the Spearman correlation coefficient. Based on PICRUSt2, the potential mechanism of FD to regulate microbial communities and metabolites under different pit ages was also investigated. The aim is to know the characteristics of the regulating microbiota and metabolites by FD for different microecosystems. These results will provide a strategy for community evolution and metabolite regulation through FD bioturbation.

## 2. Materials and methods

### 2.1. *Daqu* production and baijiu fermentation

The production of fortified *Daqu* and the fermentation of SFB were conducted in a well-known distillery (Forgood Co., Ltd, Mianyang City, Sichuan province, China). The manufacturing process of fortified *Daqu* was described by He, Dong et al. (2019). Briefly, the activated strains of *B. velezensis* and *B. subtilis* were inoculated into beef extract peptone medium, respectively, and cultured at 37 °C with shaking at 120 rpm/min for 24 h. The suspension of these strains was used as the starter when the concentration of cells was up to  $> 10^9$  CFU/mL. Subsequently, the starters were inoculated into *Daqu* pei, brick shapes of crushed wheat with a moisture content of 34%~36% (30 cm × 20 cm × 7 cm), at an initial concentration of  $2.3 \times 10^6$  CFU/mL. Traditional *Daqu* produced from the same batch without inoculating was used as the control. According to the results obtained by our previous research, the abundance of major functional genera, such as *Bacillus*, *Lactobacillus*, *Candida*, and *Rhizomucor*, was higher in FD, the activity of hydrolase and some functional indexes were also increased via these two strains (He, Dong et al., 2019).

et al., 2019).

The fermentation process of SFB is as follows: (i) the *Zaopei* from the last batch of fermentation and fresh grain were mixed, steamed and cooled to about 40 °C; (ii) 20% of fortified or traditional *Daqu* was added to the cooled grains and mixed well; (iii) then the mixture was load into the mud pit with 2-year ages or 40-year ages, and fermented for two months anaerobically (Fig. S1A). The target samples were abbreviated as F2 and F40, while the corresponding control samples were called T2 and T40, respectively.

### 2.2. Sampling

From December 2019 to October 2020, three successive rounds of fermentation were carried out as described above. After the last round of fermentation, the *Zaopei* and PM samples from the corresponding pit were taken out according to the five-point sampling method as shown in Fig. S1B. Briefly, 100 g/each point of *Zaopei* was sampled from three different layers that involved upper, middle, and bottom layers, mixed evenly, and then taken out of 500 g. For PM, 100 g/each point were sampled from the five points of the pit's bottom, mixed evenly, and then taken out of 500 g. Each experiment group was carried out by triplicate biologically. The sample was divided into three parts for analyzing physicochemical parameters (stored at 4 °C), volatile compounds (4 °C), and microbial community ( $-80$  °C), respectively.

### 2.3. Detection of physicochemical parameters

The moisture content was determined using a gravimetric method after drying at 105 °C for 4 h. Samples were mixed with CO<sub>2</sub>-free water at a ratio of 1:5 (PM) or 1:10 (*Zaopei*) and extracted for 30 min at room temperature, then the pH was measured with a pH meter (pHS-3C, INESA, Shanghai, China) by inserting the electrode directly into the suspension. The acidity of *Zaopei* and the total acid of PM were measured by the alkali titration method with a standard NaOH (0.1 mol/L) solution. According to Chen et al. (2020), four primary organic acids (acetic acid, lactic acid, butanoic acid, and hexanoic acid) were quantified by the Agilent 1260 HPLC system equipped with an Alltech OA-1000 organic acid column (300 × 7.8 mm, Agilent, Santa Clara, USA). For *Zaopei* sample, the starch and reducing sugar were determined by 3,5-dinitrosalicylic acid (DNS) method, and the ethanol content was determined by alcoholmeter after distillation. For PM sample, the ammoniacal nitrogen (NH<sub>4</sub><sup>+</sup>-N) and available phosphorus (AP) were determined by Nessler's reagent colorimetric method and ammonium molybdate method, respectively, and the content of total ester was measured via the titrimetric method after saponification (Chen et al., 2020; Ding et al., 2015).

### 2.4. Determination of volatile compounds

HS-SPME/GC-MS approach was used to analysis volatile components. Briefly, 1.0 g of sample and 10 μL methyl octanoate (internal standard) were accurately added into a 20 mL headspace bottle, the volatile components were extracted using a 50/30 μm DVB/CAR/PDMS fiber (2 cm, Supelco, Bellefonte, PA, USA) at 60 °C for 50 min. Then, the extraction head was desorbed at 250 °C for 5 min. Volatile analysis was performed using a Trace 1300 gas chromatograph system coupled with a TSQ 9000 mass spectrometer (Thermo Scientific, Waltham, MA, USA), which is equipped with a VF-WAX-MS capillary column (30.0 m × 0.25 mm, 0.25 μm, Agilent, Santa Clara, USA). After comparing with the NIST2017 library, only volatile compounds with similarity (SI)  $> 800$  remained for further analysis (the highest value is 1000).

### 2.5. Microbial community analysis

Total genomic DNA was extracted from samples using the Fast DNA SPIN extraction kit (MP Biomedicals, Santa Ana, CA, USA) according to

the manufacturer's protocol. The DNA quality and quantity were assessed by 0.8% agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). PCR amplification procedures and primers were described in our previous study (He et al., 2020). After amplification, high-throughput sequencing of archaea (16S rRNA gene V5-V6 region), bacteria (16S rRNA gene V3-V4 region), and fungi (ITS1 region) was performed on the purified, pooled sample using the MiSeq Reagent Kit V3 platform ( $2 \times 300$  paired ends).

The original sequences were quality-controlled by Trimmomatic and merged by FLASH (Magoc & Salzberg, 2011). Briefly, the low-quality sequences with primers mismatches  $> 2$ , ambiguous bases  $> 0$ , mononucleotide repeats  $> 8$ , average quality score  $< 20$  and length  $< 150$  were removed. Then, the remaining effective sequences were further filtered by removing chimeras to obtain high-quality sequences and clustered into operational taxonomic units (OTUs) with 97% similarity. Finally, Silva (Release 132) and UNITE (Release 8.0) databases were applied to classify each sequence of 16S and ITS genes, respectively, with a confidence threshold of 80%.

## 2.6. Statistical analysis

All data in this study were presented as mean  $\pm$  standard deviation, and all statistical analysis were conducted in R software (v 4.1.1; <https://www.r-project.org/>) unless otherwise stated. The package "stats" was used to identify the statistical significance of the difference using the one- or two-way ANOVA followed by Duncan's test. To assess the  $\alpha$ -diversity of the microbial community, the Chao1 and Shannon indexes were calculated, while the microbial  $\beta$ -diversity was evaluated by principal coordinate analysis (PCoA) based on the Bray-Curtis distance. The flavor profiles were compared by principal component analysis (PCA) based on Euclidean distance, and the PERMANOVA test

was performed using the function "adonis" in the "vegan" package. The Venn plot of flavor compounds was conducted by "VennDiagram" package, and the orthogonal partial least squares discrimination analysis (OPLS-DA) model was established by Simca 14.0 (Umetrics AB, Umea, Västerbotten, Sweden). The Spearman correlation coefficient ( $\rho$ ) and significance ( $p$ ) between the dominant microbial genera (relative abundance  $> 1\%$ ) were calculated using the "Hmisc" package, and the co-occurrence network was constructed based on  $|\rho| > 0.6$  and  $p < 0.05$  and visualized in Cytoscape software (v. 3.60). Linear discriminant analysis effect size (LEfSe) was carried out in an open access pipeline (<http://huttenhower.sph.harvard.edu/galaxy/>) and the significant difference was determined by LDA  $> 4.0$  and  $p < 0.05$ . The transform-based redundancy analysis (tbRDA) was carried out using the "vegan" package, before the analysis the microbial dataset was transformed by method Hellinger. Further, the variance partitioning analysis (VPA) was applied to estimate the relative contributions of independent environmental factors to the total variance through the "rdacca.hp" package. The Mantel test between microbial communities and environmental factors was also performed and visualized to explore their correlation using the "ggcor" package. The functional composition of prokaryotic community in PM was analysed by PICRUSt2 (Douglas et al., 2019).

## 3. Results and discussion

### 3.1. Variation in the physicochemical properties of Zaopei and pit mud

Both *Daqu* type and pit age can significantly affect the physicochemical properties of Zaopei and PM (Table 1). Zaopei's alcohol concentration was positively correlated with the pit age, whereas the lactic acid content was the opposite (two-way ANOVA,  $p < 0.05$ ). Moreover, the acidity and starch consumption of Zaopei were increased after bioturbation, which might be related to the higher starch hydrolyzing

**Table 1**  
Effect of fortified *Daqu* on physicochemical properties of Zaopei and pit mud under different pit ages.

Zaopei	pH	Moisture (%)	Lactic acid (g/kg)	Acetic acid (g/kg)	Butanoic acid (g/kg)	Hexanoic acid (g/kg)	Acidity (mmol/10 g)	Reducing sugar (%)	$\Delta$ Starch (%)	Alcohol (%vol)
T2	3.47 $\pm$ 0.02	65.58 $\pm$ 1.21	9.25 $\pm$ 0.64a	2.39 $\pm$ 0.22	0.21 $\pm$ 0.03	0.11 $\pm$ 0.04	3.02 $\pm$ 0.29b	0.63 $\pm$ 0.17b	4.86 $\pm$ 0.34c	4.70 $\pm$ 0.26ab
F2	3.46 $\pm$ 0.08	65.12 $\pm$ 1.06	8.82 $\pm$ 0.40ab	2.13 $\pm$ 0.11	0.22 $\pm$ 0.02	0.18 $\pm$ 0.08	3.38 $\pm$ 0.13a	0.83 $\pm$ 0.30b	5.73 $\pm$ 0.32b	4.53 $\pm$ 0.12b
T40	3.48 $\pm$ 0.04	64.17 $\pm$ 0.82	8.23 $\pm$ 0.16b	2.34 $\pm$ 0.26	0.26 $\pm$ 0.05	0.13 $\pm$ 0.05	2.47 $\pm$ 0.10c	1.24 $\pm$ 0.16a	5.93 $\pm$ 0.34b	5.23 $\pm$ 0.38a
F40	3.56 $\pm$ 0.11	63.96 $\pm$ 1.03	8.70 $\pm$ 0.39ab	2.37 $\pm$ 0.21	0.23 $\pm$ 0.08	0.16 $\pm$ 0.08	2.90 $\pm$ 0.18b	0.74 $\pm$ 0.13b	6.89 $\pm$ 0.35a	6.00 $\pm$ 0.69a
Pit age	0.231	0.130	<b>0.045</b>	0.446	0.345	0.931	<b>0.001</b>	0.563	<b>0.000</b>	<b>0.003</b>
<i>Daqu</i> type	0.392	0.345	0.938	0.362	0.610	0.192	<b>0.006</b>	<b>0.002</b>	<b>0.002</b>	0.291
Pit $\times$ <i>Daqu</i>	0.319	0.813	0.108	0.259	0.610	0.551	0.788	0.815	0.815	0.251
Pit mud	pH	Moisture (%)	Lactic acid (g/kg)	Acetic acid (g/kg)	Butanoic acid (g/kg)	Hexanoic acid (g/kg)	Acidity (g/kg)	Total ester (g/kg)	$\text{NH}_4^+ \text{-N}$ (g/kg)	Available P (g/kg)
T2	3.89 $\pm$ 0.03B	32.63 $\pm$ 1.03B	4.19 $\pm$ 0.29A	0.66 $\pm$ 0.14B	0.16 $\pm$ 0.05B	0.41 $\pm$ 0.06D	1.04 $\pm$ 0.17A	0.58 $\pm$ 0.15A	0.73 $\pm$ 0.13B	0.02 $\pm$ 0.00B
F2	3.96 $\pm$ 0.15B	32.28 $\pm$ 2.71B	3.09 $\pm$ 0.14B	0.48 $\pm$ 0.10B	0.15 $\pm$ 0.02B	0.64 $\pm$ 0.04C	1.11 $\pm$ 0.15A	0.41 $\pm$ 0.02AB	0.73 $\pm$ 0.05B	0.02 $\pm$ 0.01B
T40	5.63 $\pm$ 0.03A	40.35 $\pm$ 1.82A	3.05 $\pm$ 0.71B	1.55 $\pm$ 0.33A	0.29 $\pm$ 0.01A	1.34 $\pm$ 0.04B	0.41 $\pm$ 0.03B	0.30 $\pm$ 0.22AB	2.79 $\pm$ 0.41A	0.16 $\pm$ 0.05A
F40	5.64 $\pm$ 0.05A	40.41 $\pm$ 0.45A	2.37 $\pm$ 0.50B	1.27 $\pm$ 0.19A	0.30 $\pm$ 0.03A	1.68 $\pm$ 0.06A	0.40 $\pm$ 0.05B	0.23 $\pm$ 0.14B	2.83 $\pm$ 0.34A	0.18 $\pm$ 0.02A
Pit age	<b>0.000</b>	<b>0.000</b>	<b>0.008</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.028</b>	<b>0.000</b>	<b>0.000</b>
<i>Daqu</i> type	0.442	0.889	<b>0.010</b>	0.101	0.777	<b>0.000</b>	0.651	0.201	0.886	0.540
Pit $\times$ <i>Daqu</i>	0.527	0.843	0.444	0.705	0.514	<b>0.001</b>	0.553	0.570	0.902	0.837

Note: Different letters in the same column indicate significant differences in one-way ANOVA ( $p < 0.05$ ), lowercase and uppercase letters indicate the physicochemical parameters of Zaopei and pit mud, respectively. Pit age, *Daqu* type, and their interactions on the physicochemical properties of Zaopei and pit mud were evaluated by two-way ANOVA, and the figures in bold represent significant differences ( $p < 0.05$ ).

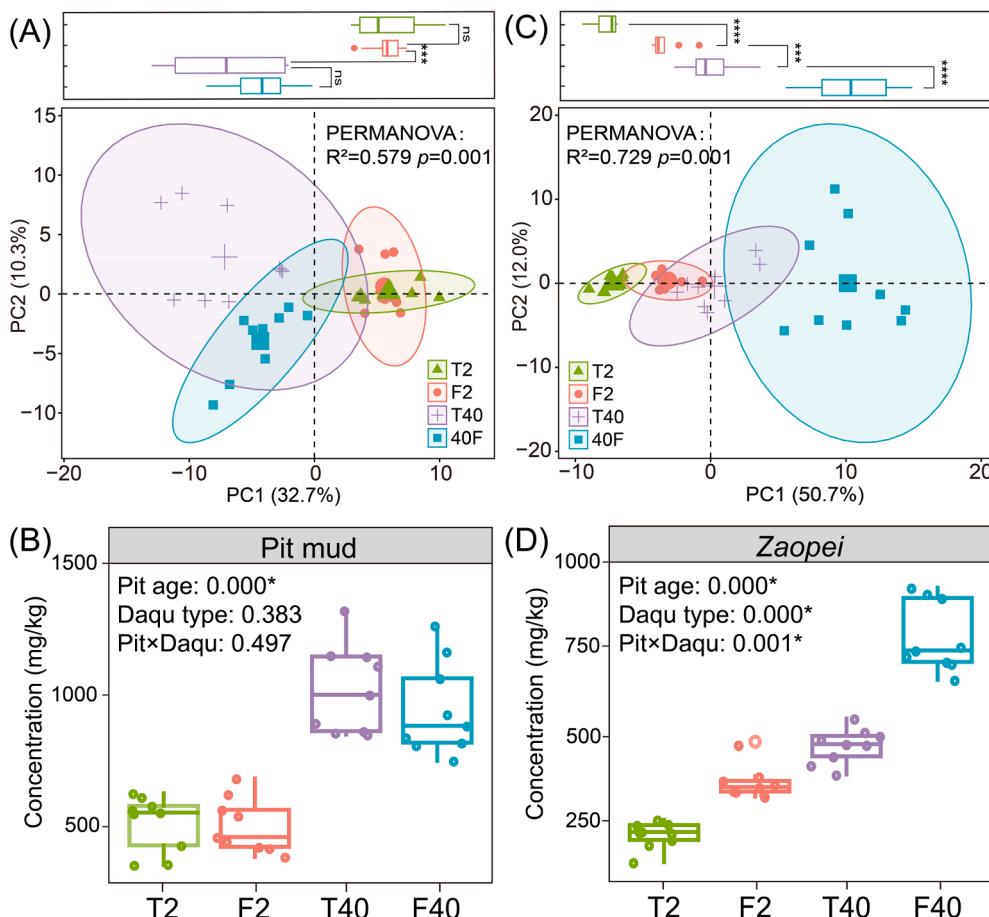
power of FD (He, Dong et al., 2019). In line with previous studies (Tao et al., 2014; Zhang, Wu, et al., 2020), the physicochemical properties of PM were closely related to pit age. Specifically, the contents of lactic acid, total acid, and total ester were higher in new pits, while pH and the contents of moisture, hexanoic acid,  $\text{NH}_4^+$ -N, and available phosphorus were higher in aged pits. After bioturbation, the concentration of lactic acid and acetic acid decreased, while that of hexanoic acid increased regardless of pit age. Functional bacteria in pit mud, such as *Caproci-producens* and *Clostridium*, can synthesize hexanoate through chain elongation using lactate and acetate as carbon sources (Candry et al., 2020; de Araújo, Leitão, Gehring, Angenent, & Santaella, 2017). Functional archaea in pit mud, such as *Methanosarcina*, can use acetate as a carbon source to produce methane, and have the potential to produce hexanoate (Fu et al., 2021). Moreover, hexanoate is an important precursor of ethyl hexanoate (one of the characteristic flavors of SFB). Therefore, these changes in physicochemical properties might imply the enhancement of microbial metabolic activity in PM and the improvement of flavor quality of Zaopei and baijiu. Furthermore, there were significant differences in physicochemical properties between Zaopei and PM, including pH, moisture, and organic acids, which was consistent with the results reported by Qian et al. (2021).

### 3.2. Changes in the flavor profile of pit mud and Zaopei

A total of 125 volatile compounds were identified from PM, including esters (82), acids (15), alcohols (12), ketones (4), aldehydes (3), phenols (2), and others (7) (Table S1). The Venn plot showed that T2, F2, T40, and F40 groups had 104, 106, 96, and 105 compounds,

respectively. Among them, 82 components were shared across all groups, and 19 and 15 unique components were identified in the new and aged PM, respectively (Fig. S2A). The principal component analysis (PCA) indicated that there was a significant difference in PC1 (32.7%) scores for the two kinds of PM ( $p < 0.001$ ) and this was also supported by the PERMANOVA test ( $R^2 = 0.579, p = 0.001$ ) (Fig. 1A). In addition, the content of volatile components in the aged PM was about twice as high as in the new PM (Fig. 1B), suggesting that the activity of the functional community will be enhanced to adapt to the microecological environment of PM during the long-term and uninterrupted repeated-batch process (Ding, Wu, Huang, & Zhou, 2016; Wang et al., 2014). To identify which metabolites were closely related to pit age, we constructed the OPLS-DA model and a total of 9 compounds were identified as significantly different metabolites based on VIP  $> 1.0$  (Fig. S3A & B). Consistent with the previous report (Zhang, Wu, et al., 2020), hexanoic acid, ethyl hexanoate, octanoic acid, and ethyl octoate were the dominant components in the aged PM.

We identified 90 volatile compounds from Zaopei, including esters (58), acids (11), alcohols (10), ketones (2), aldehydes (2), phenols (2), and others (5) (Table S2). Of them, 83, 82, 86, and 86 compounds were detected in the T2, F2, T40, and F40 groups, respectively, and 67 components were shared (Fig. S2B). Notably, the flavor profile of Zaopei was significantly affected by both Daqu type and pit age (Fig. 1C). Similarly, a recent study proved that the synergistic effects of microorganisms between Daqu and PM promoted the formation of diverse flavor compounds during SFB fermentation (Gao, Liu, et al., 2021). However, pit age played a more critical role in forming and accumulating flavor compounds in Zaopei (Fig. 1D) (Wang et al., 2014; Zhang, Wu, et al.,



**Fig. 1.** Effect of fortified Daqu on flavor profiles and contents of pit mud (A and B) and Zaopei (C and D) under different pit ages. T2, traditional Daqu used in the 2-year mud pit; F2, fortified Daqu used in the 2-year mud pit; T40, traditional Daqu used in the 40-year mud pit; F40, fortified Daqu used in the 40-year mud pit. Pit age, Daqu type, and their interactions on the flavor contents of Zaopei and pit mud were evaluated by two-way ANOVA.

2021). Furthermore, three OPLS-DA models were constructed to reveal the specific effects of pit age and fortified *Daqu* on the flavor quality of *Zaopei*. Totally, 10 volatile components were significantly related to pit age and fortified *Daqu* increased the contents of 8 and 12 components in *Zaopei* from new and aged pits, respectively, including six shared components (Fig. S3C-H). Among these components, hexanoic acid, ethyl hexanoate, butyl hexanoate, and ethyl octanoate have been identified as key aroma compounds in SFB because of their high odor activity value and aroma characteristics, including floral, fruity, sweet, and acidic (Fan & Qian, 2006; Zhao et al., 2018). These results reflected the contribution of pit age and fortified *Daqu* to flavor characteristics.

### 3.3. Temporal distribution and differential response patterns of pit mud and *Zaopei* microbiomes

After quality control, the effective sequences of archaea, bacteria, and fungi were 80,640–137,298, 70,035–116,136 and 70,242–130,016, respectively, and the high-quality sequences were 65,317–122,707, 48,537–103,739 and 69,551–127,104, respectively (Table S3). The rarefaction curve of each sample demonstrated that the sequencing data was enough for subsequent analysis (Fig. S4). Consistent with our previous studies (Ding et al., 2015; Li et al., 2016), the variations in  $\alpha$ - and  $\beta$ -diversity of *Zaopei* and PM communities were mainly dependent on pit age (two-way ANOVA,  $p < 0.05$ ). Moreover, fortified *Daqu* had a significant effect on those prokaryotic communities in PM that promoted the directional evolution of archaea in the new PM and improved the stability of the archaeal community in the aged PM (Fig. 2).

Ten dominant fungal genera belonged to Ascomycota and Basidiomycota, and their distribution patterns were similar in *Zaopei* and PM and mainly depended on pit age (Fig. 3A). *Aspergillus* was dominated in aged pits, whose relative abundance (RA) ranged from 41.89% to

83.83%, while *Kazachstania* and *Thermoascus* were prevalent in new pits, with an RA of 19.05%–44.35%. A similar distribution pattern of *Aspergillus* and *Thermoascus* has been observed in 5- and 100-year PM, while *Kazachstania* was not detected (Liu, Tang, Zhao, et al., 2017). Recently, *Kazachstania* has been found in *Daqu* (RA < 0.1%), *Zaopei* (>10%), and PM (>30%) (Qian et al., 2021; Xiao et al., 2021). According to Jood, Hoff, and Setati (2017), *Kazachstania* contributes some aroma characteristics to the wine, such as fruity, floral, and cheesy, whereas it inhibited the formation of 9 volatile compounds in the current study (Fig. S5A). In contrast, *Aspergillus* was positively correlated with 12 volatile compounds (Fig. S5A). Similarly, the aroma-production ability of *Aspergillus* has been confirmed in *Daqu* and other solid-state fermented foods, which can be attributed to its widely and highly expressed enzyme activities, including amylase, cellulase, and esterase (Li et al., 2018; Xu et al., 2017; Yang et al., 2021). Consistent with the previous study (He et al., 2020), fortified *Daqu* significantly increased the RA of *Aspergillus* (Fig. S6A), which may improve flavor quality.

The dominant bacteria consisted of 23 genera belonging to four phyla, and the interphase community composition differed greatly and varied by pit age. *Lactobacillus* was predominant in *Zaopei* regardless of pit age, with a higher RA in new pits (Fig. 3B). *Lactobacillus* is essential for cereal fermentation, its metabolite, lactic acid, can be used to produce hexanoic acid, the precursor of ethyl hexanoate (Chai et al., 2021; Gaglio et al., 2019). However, an excessive amount will inhibit the growth of acid-sensitive microorganisms and affect the balance of the microbial community in the *Zaopei*, thus decreasing the flavor quality of baijiu (Gao, Liu, et al., 2021; Zhao et al., 2019). In addition, *Sphingomonas* and *Acinetobacter* were prevalent in the new PM, while *Caproiciducens* was abundant in the aged PM (Fig. 3B). LEfSe analysis indicated that fortified *Daqu* had a weak effect on the bacterial community of new PM while significantly increasing the RA of

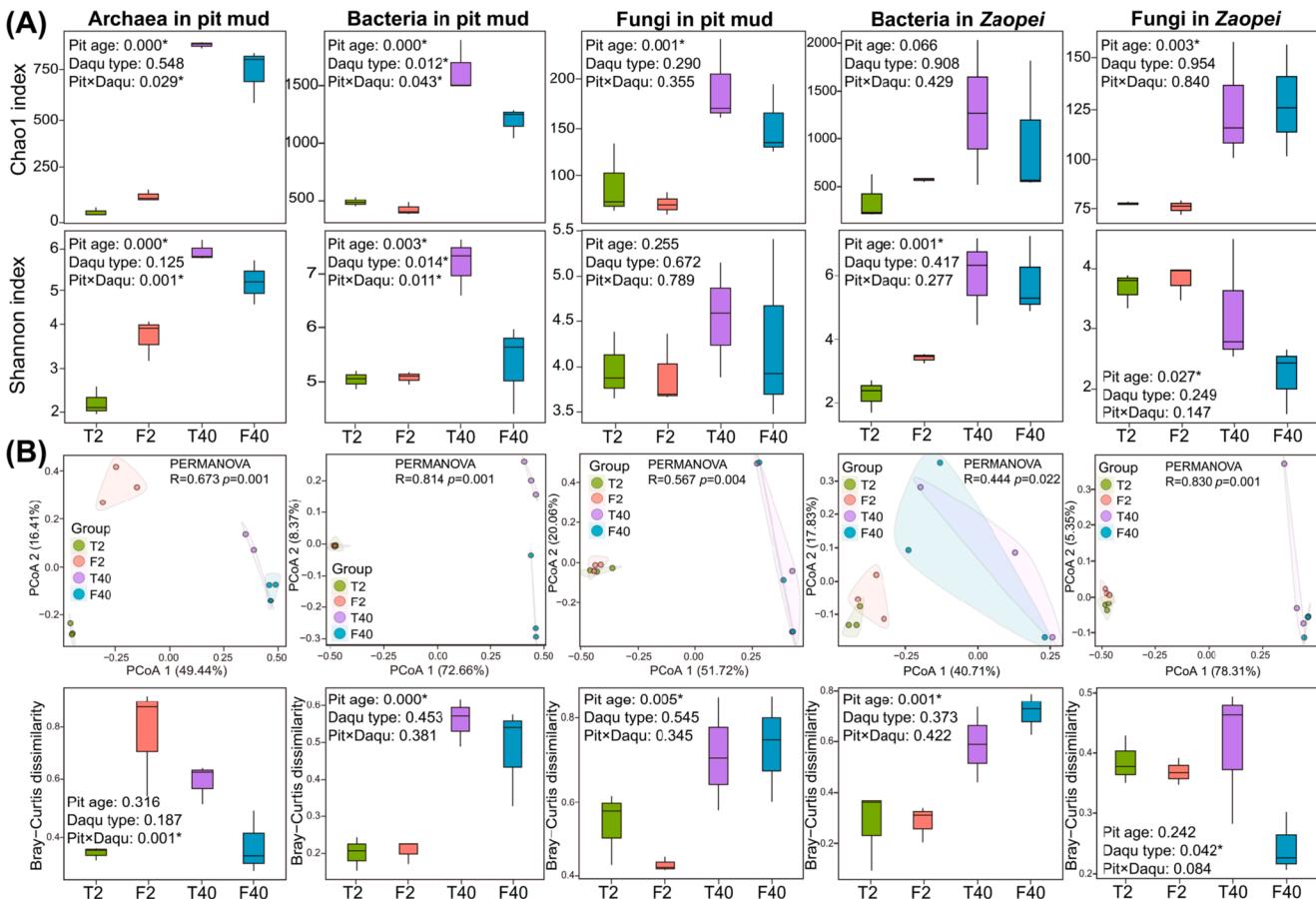


Fig. 2. Effects of fortified *Daqu* on  $\alpha$ -diversity (A) and  $\beta$ -diversity (B) of pit mud and *Zaopei* microbial communities under different pit ages.

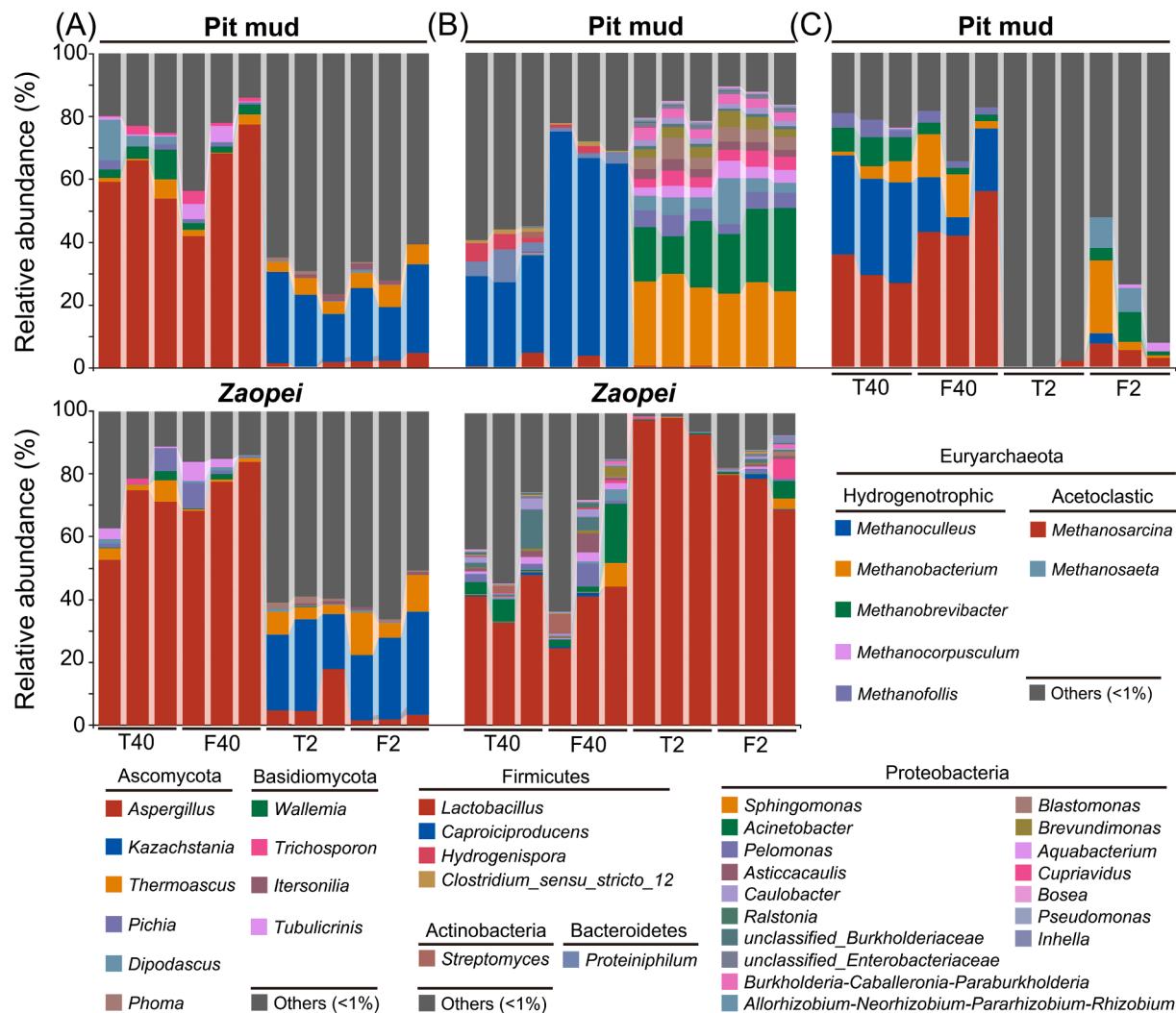


Fig. 3. Community composition patterns of pit mud and Zaopei at genus level. (A) Fungi; (B) Bacteria; (C) Archaea.

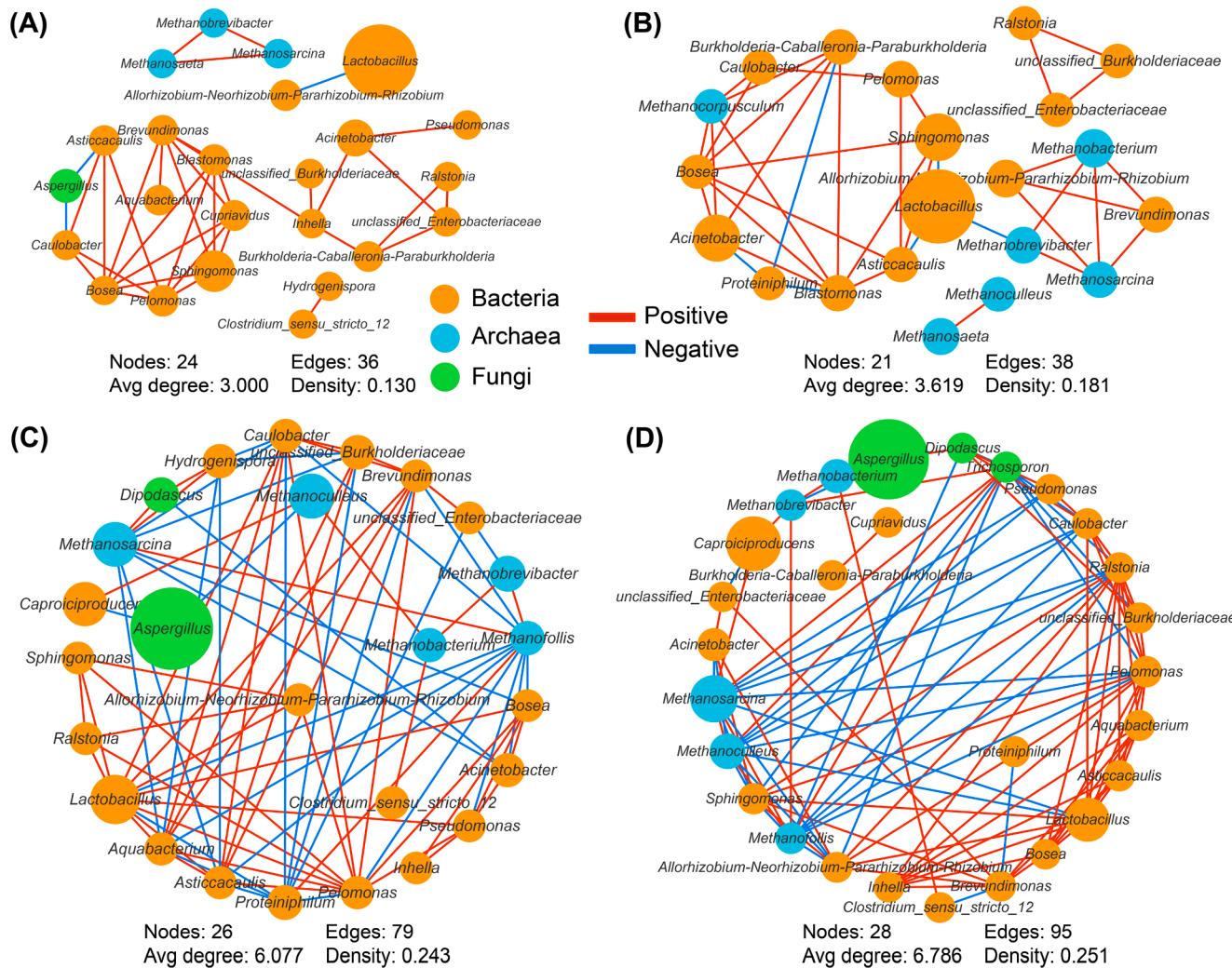
*Caproiciproducens* in the aged PM (Fig. S6B). Recent studies have proved that *Caproiciproducens* was positively correlated with the quality and age of PM, and it can not only convert lactic acid into hexanoic acid but also promote the formation of various volatile compounds (Chai et al., 2021; Gao, Lin, et al., 2021; Liu, Tang, Guo, et al., 2017), which was supported by the correlation analysis in this study (Fig. S5B). Therefore, the decrease of *Lactobacillus* and the increase of *Caproiciproducens* may be the reasons for the increased content of volatile compounds after bioturbation (Fig. 1D). Interestingly, the abundant *Sphingomonas* observed in the current study are usually rare microorganisms in the baijiu fermentation process, originating from the raw material and the air in the brewing environment (Ding, Wu, Huang, Li, & Zhou, 2014; Tan et al., 2019; Zhang, Wang, Wang, et al., 2020). Furthermore, *Sphingomonas* was positively correlated with 8 volatile compounds during the sauce-flavor baijiu fermentation (Zhang, Wang, Wang, et al., 2020); however, in the present study, it was negatively correlated with diverse flavor compounds, e.g., hexanoic acid, ethyl hexanoate, hexyl hexanoate, and butyl hexanoate (Fig. S5B). It is necessary to explore its prevalence and effect on baijiu fermentation in the future.

The archaeal community of PM was mainly composed of seven genera of Euryarchaeota, two and five of which were affiliated with acetoclastic and hydrogenotrophic methanogens, respectively (Fig. 3C). Consistent with Tao et al. (2014), the OTU number and abundance of archaea in the new PM were significantly lower than those in the aged PM. Fortified *Daqu* significantly promoted the enrichment of various

methanogens in the new PM while mainly increasing the RA of *Methanosarcina* in the aged PM (Fig. S6C & D). Previous studies have emphasized that the quality of PM is closely related to the composition and diversity of the archaeal community (Tao et al., 2014, 2017). In particular, *Methanosarcina* positively correlated with pit age and accounted for about 30%, 40%, and 60% of total archaea in 30-, 100-, and 300-year PMs, respectively (Chai et al., 2021). More importantly, both the previous reports (Chai et al., 2021) and the present study (Fig. S5B) indicated that *Methanosarcina* was significantly and positively correlated with hexanoic acid and a variety of volatile compounds because of its potential metabolic activity, including glycolysis and hexanoic acid production (Fu et al., 2021). Therefore, fortified *Daqu* may help to promote the formation of key flavors by driving the directional evolution of functional archaea, especially for the new PM.

#### 3.4. The interactions among dominant genera at the microecosystem level

Co-occurrence network analysis showed that both *Daqu* type and pit age affected the community interactions, and the latter played a decisive role (Fig. 4). The differences in the co-occurrence network caused by pit age were as follows: (i) the network structure of the new pit was simple and fragmented; (ii) the proportion of negative correlation in aged pits was significantly higher than in the new pit (Fig. 4A & C). In general, the community diversity and stability of the ecological network were markedly correlated with the proportion of negative interactions among

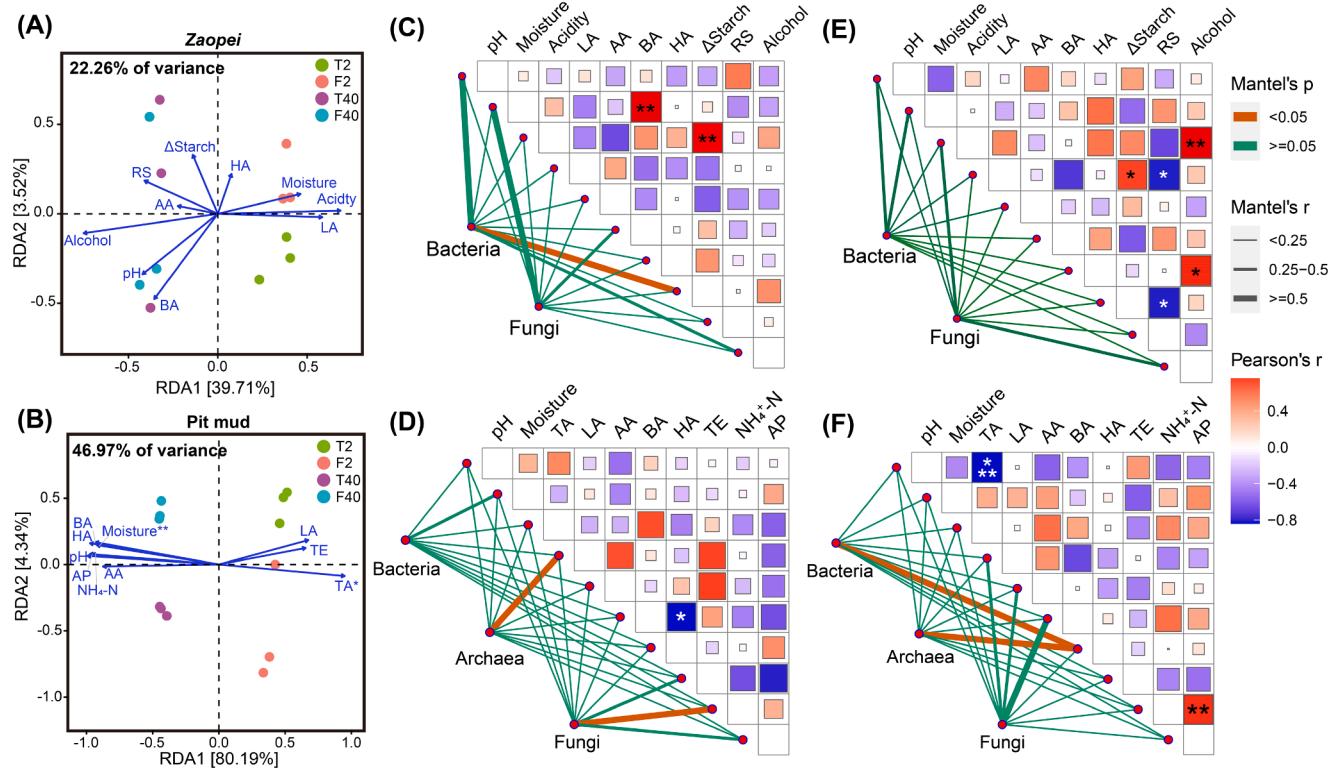


**Fig. 4.** Co-occurrence network of liquor brewing microecosystems with different pit ages and the effect of fortified *Dagu* on interspecies interaction. (A) T2; (B) F2; (C) T40; (D) F40. The network parameters, including number of nodes, number of edges, average degree, and density, are given below the specific networks.

species (Coyte, Schluter, & Foster, 2015; Oliverira, Niehus, & Foster, 2014). In the present study, the higher richness and diversity of the interphase community in aged pits drove the formation of a network with fierce competition (Figs. 2 and 4), indicating that traditional fermented foods can be valuable models for revealing the laws of the natural ecosystem evolving (Wolfe & Dutton, 2015). After bioturbation, the nodes and connections of archaea in the network of new pits were increased, and the interactions between hexanoic acid-producing bacteria and methanogens in the network of aged pits were enhanced (Fig. 4B & D), suggesting fortified *Dagu* might help to promote the stability of the ecological network. For example, *Caproiciproducens* was significantly positively correlated with *Methanoculleus* in the T40 network, but with *Methanosarcina* and *Methanobrevibacter* in the F40 network, consistent with the alteration of their abundance (Fig. 3). *Caproiciproducens*, *Methanosarcina* and *Methanobrevibacter* have been emphasized in previous studies because they are most highly represented in the PM samples, and their abundances increase with pit age (Chai et al., 2021; Liu, Tang, Guo, et al., 2017). Meanwhile, *Caproiciproducens* and *Methanosarcina* play a vital role in the formation of organic acid and volatile compounds in pit mud (Chai et al., 2021; Fu et al., 2021). Therefore, a possible explanation for the increasing flavor quality with pit age is the co-occurrence of *Caproiciproducens* and *Methanosarcina* with a high abundance in the aged PM.

### 3.5. Correlation between microbial communities and environmental variables

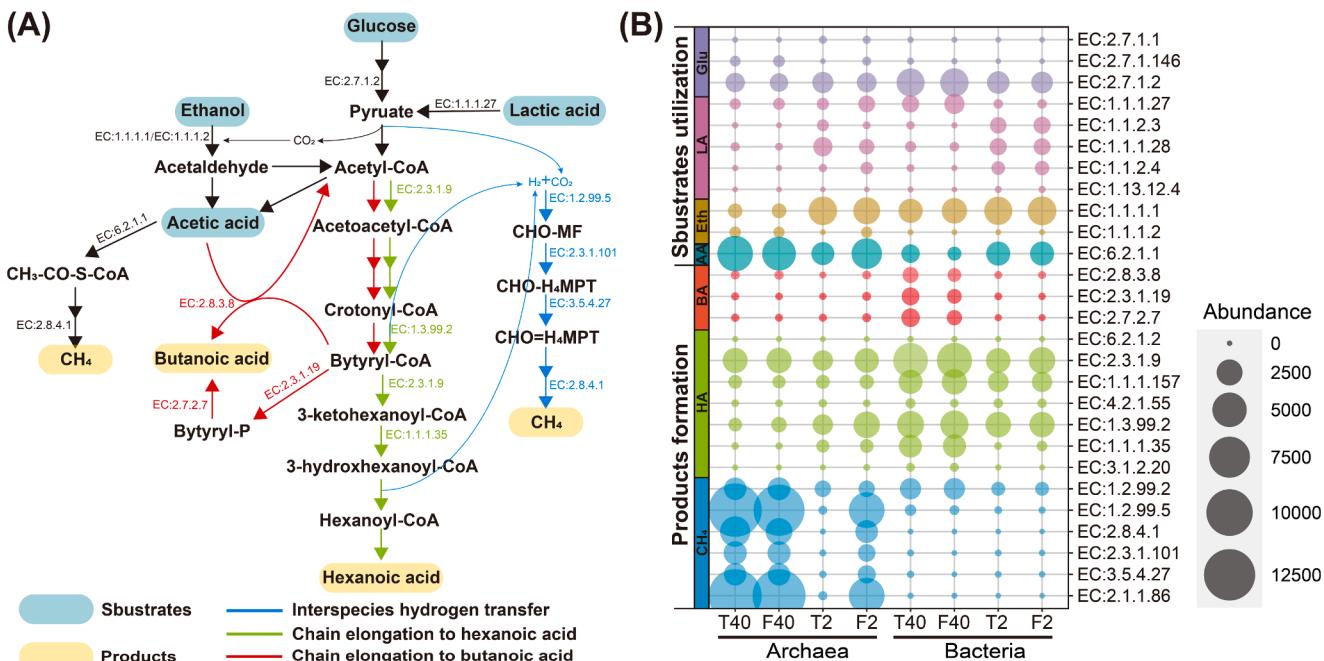
As shown in Fig. 5A & B, 22.26% and 46.97% of the total variation of the Zaopei and PM communities can be explained by the RDA model, suggesting a stronger correlation between PM community and environmental variables. However, samples with different pit ages were observably separated by RDA1 regardless of Zaopei or PM, demonstrating the temporal heterogeneity of the SFB brewing ecosystem (Ding et al., 2015; Li et al., 2016). Specifically, the community of new PM was related to lactic acid, total acid, and total ester, while that of aged PM was positively correlated with pH, hexanoic acid, NH<sub>4</sub><sup>+</sup>-N, and moisture, which was in line with previous studies (Tao et al., 2014; Zhang, Wang, Meng, et al., 2020). In addition, the Monte Carlo test indicated that moisture content ( $p = 0.001$ ) and total acid ( $p = 0.027$ ) were significantly associated with the PM community, and variance partitioning analysis (VPA) indicated that 11.8% and 10.0% of the total variation can be independently explained by moisture content and acidity, respectively (Table S4). Similarly, significant correlations between moisture content and pit age or pit quality were reported by Hu et al. (2021) and Chai et al. (2021). Furthermore, the overall correlation between microbial communities and environmental variables was analyzed by the Mantel test. The acidity and alcohol content of Zaopei increased continuously during baijiu fermentation, while the starch content was opposite (Tan et al., 2019). Therefore, a positive correlation



**Fig. 5.** Redundancy analysis of microbial community and environmental variables (A: Zaopei; B: pit mud). Mantel test of overall community and environmental variables under different pit ages (C-D: Zaopei and pit mud from 2-year pits; E-F: Zaopei and pit mud from 40-year pits). LA, lactic acid; AA, acetic acid; BA, butanoic acid; HA, hexanoic acid; RS, reducing sugar; TA, total acid; TE, total ester; AP, available phosphorus.

between starch consumption, acidity, and alcohol content in Zaopei can be observed (Fig. 5C & E). Moreover, the starch consumption was significantly associated with the bacterial community in the new pit, which was consistent with the results reported by Guan, Lin, Chen, Ou, and Zhang (2020). For pit mud, AP was negatively correlated with hexanoic acid in the new pit while positively correlated with NH<sub>4</sub><sup>+</sup>-N in

the aged pit (Fig. 5D & F). AP and NH<sub>4</sub><sup>+</sup>-N are the nutrient sources for the microbial community, the synthesis of NH<sub>4</sub><sup>+</sup>-N may increase pH and indirectly promote the growth of hexanoic acid-producing bacteria and methanogens (Tao et al., 2014; Zhang, Meng, et al., 2020). Correspondingly, the hexanoic acid content was closely related to the synergistic effects of bacterial and archaeal communities in the aged PM



**Fig. 6.** Schematic overview of pathways for production of hexanoic acid from common substrates in pit mud (A), and the abundance variation of functional genes encoding for key enzymes involved in hexanoic acid synthesis in prokaryotic communities of pit mud with different pit ages (B).

(Fig. 5F).

### 3.6. Functional composition analysis of prokaryotic microbial community in pit mud

Based on the above results, we speculate that the effect of interspecies hydrogen transfer between hexanoic acid-producing bacteria and methanogen is more robust in the aged pit, and it will be improved by fortified *Daqu*, especially for the new pit. Therefore, PICRUSt2 was applied to predict the abundance variation of the functional genes encoding for key enzymes involved in hexanoic acid synthesis in PM based on the 16S rRNA gene sequencing dataset according to previous studies (Candry et al., 2020; de Araújo et al., 2017; Gao, Lin, et al., 2021; Tao et al., 2017), including the chain elongation and interspecies hydrogen transfer (Fig. 6).

Similar to Qian et al. (2021), glucokinase (EC:2.7.1.2) was the key enzyme involved in glucose utilization, and it was enriched in the bacterial community of the aged PM. In contrast, the abundance of enzymes related to lactic acid utilization was higher in the new PM except for lactic acid dehydrogenase (EC:1.1.1.27), which may be correlated with its abundant lactic acid (Table 1). However, EC:1.1.1.27 is the key enzyme that catalyzes L-lactic acid to acetyl-CoA and then produces hexanoic acid through the chain elongation pathway (Chai et al., 2021; de Araújo et al., 2017; Tao et al., 2017). After bioturbation, its abundance was enhanced in the aged (bacteria) and new (archaea) PMs, corresponding to the decrease in lactic acid and the increase in hexanoic acid (Table 1). Moreover, the abundance of ethanol dehydrogenase complex (EC:1.1.1.1/ EC:1.1.1.2) was also increased, probably because CO<sub>2</sub> released through the oxidization of lactic acid to acetyl-CoA was conducive to ethanol metabolism (Gao, Lin, et al., 2021). Among them, EC:1.1.1.1 and EC:1.1.1.2 were enriched in bacterial and archaeal communities, respectively, consistent with metagenomic sequencing (Fu et al., 2021). Compared with the advantage of the bacterial community in the utilization of glucose, ethanol, and lactic acid, the archaeal community, especially in the aged PM, preferred to use acetic acid through acetic acid-CoA ligase (EC:6.2.1.1). Fortified *Daqu* elevated the abundance of EC:6.2.1.1 in the archaeal community of new PM and formed the advantage of methane metabolism based on acetic acid utilization, following the enrichment of *Methanosarcina* and *Methanoanaeta* (Fig. 3C).

Additionally, the production of butanoic acid and hexanoic acid was synergistically regulated by bacterial and archaeal communities, while the methane metabolism was mainly dependent on the archaeal community (Fu et al., 2021; Tao et al., 2017). The microbial community in the aged PM showed tremendous potential for synthesizing butanoic acid, hexanoic acid, and methane, consistent with the content of corresponding metabolites (Table 1). It is worth noting that the effect of fortified *Daqu* on the abundance of these key enzymes varied by the pit age (Fig. 6). More specifically, the abundance of enzymes involved in the production of hexanoic acid (EC:2.3.1.9, acetyl-CoA acetyltransferase; EC:1.3.99.2, butyryl-CoA dehydrogenase) and methane (EC:1.2.99.5, formylmethanofuran dehydrogenase; EC:2.1.1.86, tetrahydromethanopterin S-methyltransferase) were increased in the new PM after bioturbation, indicating improved interspecies hydrogen transfer (He et al., 2020; Tao et al., 2017). However, fortified *Daqu* inhibited the phosphorylation of butyryl-CoA (phosphate butyryl-transferase, EC:2.3.1.19) by decreasing both the *buk* (butyrate kinase, EC:2.7.2.7) and *but* (butyryl-CoA: acetate CoA-transferase, EC:2.8.3.8) pathways in the aged PM, consistent with the reduction of *Proteiniphilum* (Fu et al., 2021) and *Hydrogenispora* (Chai et al., 2019) (Fig. 3B). This change might allow more carbon flow to be distributed to hexanoyl-CoA synthesis in the chain elongation process, leading to enhanced hexanoic acid production (Candry et al., 2020; de Araújo et al., 2017). Therefore, although fortified *Daqu* improved the concentration of hexanoic acid and its derivatives regardless of pit age, the potential mechanism may be different in the new and aged pits.

## 4. Conclusion

This study highlights that fortified *Daqu* contributed to improving the flavor quality of SFB regardless of pit age, but the potential mechanism might be varied by pit age. In the new pit, the decreased *Lactobacillus* in *Zaopei* and the enhanced interspecies hydrogen transfer between archaea and bacteria in PM drove the changes in metabolic functions. In the aged pit, fortified *Daqu* promoted the formation of hexanoic acid and other flavor compounds by increasing the abundance of functional microorganisms, namely *Caproiciproducens*, *Methanosarcina* and *Aspergillus*, and diverging the carbon flow from butanoic acid production. Additionally, the current study also showed that pit age played a critical role in forming and developing the SFB flavor profile. These results are beneficial to construct a controllable and directional community evolution process.

## CRediT authorship contribution statement

**Yu Mu:** Investigation, Data curation, Visualization, Writing – original draft. **Jun Huang:** Conceptualization, Supervision, Investigation. **Rongqing Zhou:** Conceptualization, Resources, Formal analysis, Supervision, Writing – review & editing. **Fengjiao Mao:** Investigation, Data curation, Formal analysis. **Qianglin Pan:** Investigation, Data curation, Formal analysis. **Suqi Chen:** Investigation, Data curation. **Zhongming Lu:** Investigation, Project administration, Resources. **Liquan Du:** Project administration, Supervision. **Fei Xie:** Investigation, Resources.

## 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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## Date availability statement

The raw sequence data of *Zaopei* and pit mud generated in this study can be accessed using the NCBI SRA database with the Accession ID PRJNA806387 and PRJNA806388.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2022.111062>.

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