Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Characterizing the regulation mechanism of microbial community and metabolites in multi-layer vinegar starter (*Cuqu*) *via Muqu* fortification strategy

Qiuxiang Tang[a], Nan Zhou[a], Jun Huang[a], Jian Guo[b], Xuemei Kang[b], Wen Luo[b], Rongqing Zhou[a,c,*]

[a] *College of Biomass Science and Engineering, Sichuan University, Chengdu 610065, China*
[b] *Qianhe Condiment and Food Co., Ltd., Meishan 620010, China*
[c] *National Engineering Research Centre of Solid-State Brewing, Luzhou 646000, China*

ARTICLE INFO

Article history:
Received 29 January 2024
Received in revised form 19 March 2024
Accepted 26 April 2024

Keywords:
Microbial communities
Metabolites pattern
Spatial distributions
Pyrazine content

ABSTRACT

Improving the quality of *Cuqu* is one of the necessary strategies increasing the quality and yield of fresh vinegar. Enhancing the consistency and characteristics of *Cuqu* is currently a prominent area of research. Here, we investigated the impact of fortified *Muqu* (FM) consisting of *Bacillus velezensis* and *Bacillus subtilis* on enhancing the quality of *Cuqu* compared to conventional *Muqu* (CM). Our results demonstrated that FM significantly increased the abundance of *Bacillus* by 1.85–8.19 times, as well as substantially elevated *Kroppenstedtia* abundance in *Cuqu*. Source Tracker analysis revealed a higher proportion of bacteria originating from FM in *Cuqu* compared to those derived from CM, indicating superior environmental adaptability for bacteria in the FM. Additionally, upregulation in expression levels of pyrazine-related key enzymes within *Cuqu* microbial communities was observed due to FM, resulting in a remarkable 39.70-fold increase in pyrazine compounds production. Multiphase detection technology confirmed that FM improved similarity in microbial community structure and function at different spatial positions within *Cuqu*. These results elucidated the regulatory function of FM in forming microbial communities and metabolic mechanisms within *Cuqu*, providing valuable insights for optimizing and predicting traditional fermentation processes in industrial production.

1. Introduction

The vinegar starter (*Cuqu*) is an essential fermentation starter for the production of Chinese grain vinegar. Generally, *Cuqu* is manufactured from wheat, corn, bran, pea, etc., through solid-state spontaneous fermentation in an open environment, and can be categorized into 2 types: low-temperature *Cuqu* (40–50 ℃) and medium-temperature *Cuqu* (50–60 ℃)[1]. The former is mainly used for the brewing of Shanxi aged vinegar (SAV), Tianjin Duliu mature vinegar, etc., whereas the latter is employed in the production of Sichuan bran vinegar (SBV). However, the microbiota structure in *Cuqu* varies with the spatiotemporal characteristics even though the technique is the same. For example, *Weissella*, *Lactobacillus*, *Streptomyces*, *Saccharomycopsis fibuligera*, and *Pichia kudriavzevii* dominate in both of *Cuqu* for brewing SAV and Tianjin Duliu mature vinegar, respectively. Yet, *Lactococcus* and *Aspergillus niger* dominate in the former's, whereas *Eurotium intermedium* and *Monascus ruber* dominate in the latter's[2-3]. The difference of microbiota structure caused by microenvironments (culturing *Cuqu* room) were further revealed by combining metagenomic sequencing and quantitative polymerase chain reaction[4]. *Staphylococcus*, *Saccharopolyspora*, *Mucor*, *Rhizomucor*, and *Hyphopichia dominate* in *Cuqu* used for SBV, except for *Aspergillus* and *Streptomyces*[5-6].

The dynamics of microbial community structure in *Cuqu* are mainly driven by abiotic factors, such as temperature, humidity, and

* Corresponding author at: College of Biomass Science and Engineering, Sichuan University, Chengdu 610065, China
E-mail address: zhourqing@scu.edu.cn (R.Q. Zhou)
Peer review under responsibility of Beijing Academy of Food Sciences.

SciOpen Publishing services by Tsinghua University Press

http://doi.org/10.26599/FSHW.2025.9250464

pH, resulting in the differences between cross-sections and fluctuations during the process in physicochemical properties, community composition and metabolic components[4,7]. In fact, certain strains from the raw material and microenvironment, including the production facility and indoor premises, colonize into the initial starter[8]. These results not only suggest the spatiotemporal characteristics of *Cuqu*, but also lay the theoretical foundation for improving its quality. Therefore, the bioturbation by inoculating function strains or microbiota is one of the important strategies for improving the quality of *Cupei*[9]. For example, inoculating *Candida ethanolica* Y18, an isolate originating from SAV Cupei, into a simulated fermentation system noticeably enhanced the contents of acids and esters, thereby improving their flavor profiles[10]. Similarly, the content of ethylidene succinate was increased by inoculating *Lactobacillus* sp. and *Acetobacter pasteurianus* into Zhenjiang aromatic vinegar[11]. When Zhenjiang aromatic vinegar was fortified with *Komagataeibacter europaeus*, not only was the bacterial microbiota structure reshaped, but also the robustness of the symbiotic network and the abundance of genes associated with sugar and alcohol metabolism were improved[12]. The metabolic profile of Baoning vinegar *Cupei*, including the rate of starch degradation, acidity, organic acid content, etc., was evidently improved by biofortification of *A. niger*, and the community structure remained relatively stable even during multiple batches of fermentation[6,13]. By adding *Monascus purpureus* in the starter, the abundance of *Lactobacillus* and *Acetobacter* was enhanced in SBV *Cupei*, and the proliferation of foodborne pathogens like *Erwinia*, *Proteus*, and *Ignatzschineria* was inhibited, resulting in significantly increased contents of organic acids, aromatic esters, and alcohols[5]. Besides, *Cuqu* fortified with *Bacillus amyloliquefaciens* improved the contents of acetoin and tetramethylpyrazine contents by 191.84% and 123.17% in SBV *Cupei*[14], respectively.

These results suggested that biofortification was an effective strategy for evolving the community assembly and regulating their metabolic pathways[9]. However, these results mainly focused on the contribution of endogenous isolates, with little attention paid to the impact of the microenvironment spatiotemporal characteristics on the attributes of *Cuqu*. *Muqu*, a high quality matured *Daqu* produced in the last year, was often appended into *Qupei* (circa 6%–8% of raw materials dried weight) for the stability of high-temperature *Daqu* attributes. Furthermore, the multi-layer shelf type *Daqu* manufacture pattern has been used in order to apply intelligent manufacturing technology for medium-temperature *Daqu*[15]. Nevertheless, there have been no reports on the manufacture of SBV *Cuqu* using both models mentioned above simultaneously.

In the present research, the multi-layer shelf type *Cuqu* manufacture pattern as the object, the contribution of fortified *Muqu* to *Cuqu* attributes was investigated by multi omics technology. Additionally, the differences in interspecific interactions were also explored, and metabolic functions were predicted based on gene sequence abundance to elucidate the difference in the regulating mechanisms. These findings establish a theoretical foundation for optimizing process parameters of *Cuqu* with new techniques.

2. Materials and methods

2.1 Experiment procedure

The *Cuqu* manufacturing process was conducted according to the procedure described by Pan et al.[15], with an inoculated *Muqu* ratio of 1% (*m*/*m*, dried weight). The internal temperature of *Qupei* was recorded at fixed positions every day during the process. The brief process description is as follows: raw wheat after being moistened with hot water (< 60 °C) and crushed (humidity: 15%–17%), and then mixed with warm water and mechanically pressed into *Qu* bricks (initial *Qupei*, humidity circa 34%). These *Qupei* were placed on the shelves, with 12 pieces per layer across a total of 6 layers within the fermentation room. The base layer of the shelves is positioned 15 cm above the ground, maintaining a 27 cm gap between each layer. The process parameters, such as temperature and humidity, were regulated according to the operating standard DB 5105/T36–2020[16], matured for 6 months at environmental temperature. To produce fortified *Muqu* (FM), the process followed the procedure outlined by Pan et al.[15]. The inoculated suspension is a synthetic microbiota composed of *Bacillus subtilis* and *Bacillus velezensis* in 1:1 ratio, and the initial concentration in *Qupei* was 4.3×10^6 CFU/g, whereas uninoculated *Qupei* was used as the conventional *Muqu* (CM). The resulting *Cuqu*, post-inoculation with FM and CM were designated as FQ and CQ, respectively. Samples from both types of matured *Cuqu* samples were randomly collected from various spatial positions within *Qufang*, specifically from the upper, middle, and lower layers of the shelf, and directly from the ground. These samples were categorized into 8 groups: FQ-S (upper), FQ-Z (middle), FQ-X (lower), FQ-P (ground), CQ-S (upper), CQ-Z (middle), CQ-X (lower), and CQ-P (ground), respectively. Each group of samples consisted of 5 bricks of *Cuqu*, which were crushed, mixed, and piled into a cone. From this conical arrangement, samples of 100 g each were gathered using the five-point sampling method, including an additional 100 g from the cone's center, totaling 500 g. This composite sample was then mixed thoroughly and divided into two parts, with 450 g stored at 4 °C for the detection of physicochemical parameters, and the remaining 50 g stored at −80 °C for the analysis of microbial community structure.

2.2 Determining metabolic composition

The physiochemical properties of *Cuqu*, including humidity, acidity, liquefying ability, saccharifying ability, fermenting ability and esterifying ability, were determined according to the general methods of analysis for *Daqu* QB/T 4257–2011[17].

The analysis of volatile metabolites in *Cuqu* was conducted utilizing headspace solid-phase microextraction paired with gas chromatography-mass spectrometry (HS-SPME-GC-MS). A 50/30 μm divinybenzene/carboxen/polydimethylsiloxane fiber, procured from Supelco (Bellefonte, PA, USA) facilitated the extraction of volatiles. For each extraction, 0.50 g of *Cuqu* powder, along with 10 μL internal standard solution of methyl octanoate (0.007 9 g/100 mL) were introduced into a 15-mL headspace vial. The vial was subsequently placed in a thermostatic block stirrer to undergo equilibration at 60 °C for 15 min, followed by a 45 min extraction period under stirring at 500 r/min. Upon completion of extraction, the SPME fiber was retracted into the needle and immediately inserted into the injection port of the GC-MS system (Gas Chromatography Coupled System TSQ 9000 Mass Spectrometer, Thermo trace 1300, Waltham, MA, USA). Here, the volatiles were desorbed thermally at 250 °C for 5 min and transferred directly into the analytical column (HPINNO WAX capillary column, 30.0 m × 0.25 mm, 0.25 μm, Agilent Technologies Inc., Electron Co., Waltham, MA, USA). The protocol for detection and the procedure for data acquisition adhered to previously described methodologies[18].

2.3 Detecting microbial communities

Total genomic DNA was extracted directly from these samples using the E.Z.N.A.® Soil DNA kit (Omega Bio-tek, Norcross, GA, USA), and then obtained 16S rRNA reads and ITS regions using Illumina Novaseq6000/Hiseq Xten (Illumina, USA) platform at the Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China)[18]. The sequences were analyzed with QIIME2 (version 2019.4), whose processing included removal, quality control, denoising, splicing, and chimaera removal. Each de-weighted sequence resulting from quality control using DADA2 is called amplicon sequence variants (ASVs).

2.4 Data analysis

Analyses of sequence data applied the one-way analysis of variance (One-way ANOVA) with Duncan's *post hoc* test using SPSS 24.0 software (SPSS Inc. Chicago, IL, USA) for assessing significance ($P < 0.05$, $n = 3$). Spearman correlation coefficients were calculated and heatmaps were generated using R software (version 4.2.1). The data visualization was performed using Cytoscape (version 3.8.2.). Graphs were created using Origin (version 2022). The website (http://huttenhower.sph.harvard.edu/galaxy/) was utilized for evaluating the differences in volatile components among samples based on linear discriminant analysis effect size (LEfSe). Microbial functions were predicted using PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States)[19], based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/pathway.html), and microbial network prediction[20-22]. Bayesian source tracking was performed using the Source Tracker algorithm in R software to identify putative sources of *Cuqu* microbes[23].

3. Results and discussion

3.1 Difference in the microbial diversity of Cuqu

The microbial diversity between FQ and CQ exhibited a notable disparity (Table 1). In the corresponding spatial positions, the Chao1/Observed index and Shannon/Simpson index of bacteria were higher in FQ compared to CQ. Additionally, both the richness and diversity of bacterial communities showed a decreasing trend from top to bottom in spatial locations. Similarly, the richness and diversity of the fungal community showed a correlation with spatial locations. The Chao1/Observed species and Shannon/Simpson index of fungi in CQ-S were slightly lower than those in FQ-S, whereas the opposite trend was observed in CQ-Z, CQ-X, and CQ-P. The results indicated that the disturbance of FM affected the α-diversity of bacteria, enhancing the richness and diversity of bacteria but slightly reducing the α-diversity of fungi. Variations in microbial community diversity were observed due to gradient differences in environmental conditions[24], such as temperature and humidity between different layers of *Cuqu*. The bacterial community α-diversity of both types of *Cuqu* decreases from top to bottom, while the fungal community α-diversity of the middle and lower layers of *Cuqu* was higher than that of the upper layer.

The results of principal coordinate analysis (PCoA) for the community (Figs. 1A and B) revealed that the bacterial communities of FQ clustered together, while the spatial positions significantly influenced the bacterial community structure of CQ. The bacterial communities of CQ-S, CQ-Z, CQ-P, and CQ-X were clustered in the second and third quadrants, displaying varied spatial patterns compared to the FQ's. The biotic disturbance caused by FM resolved the impact of spatial positions on the β-diversity of bacterial communities, leading them towards directional succession. However, the influence on the β-diversity of fungal communities was relatively modest. For instance, FQ-Z, FQ-X, and FQ-P were situated in the first quadrant, while FQ-S, CQ-Z, and CQ-S occupied the fourth quadrant. CQ-P and CQ-X were positioned in the third quadrant, yet the fungal community structure of FQ exhibited higher similarity than that of CQ. These results underscored the notable impact of FM on bacterial β-diversity, while demonstrating a relatively minor influence on fungal β-diversity.

Table 1
α-Diversity indexes of microbial community.

Sample	Bacteria				Fungi			
	Chao1	Observed species	Shannon	Simpson	Chao1	Observed species	Shannon	Simpson
CQ-S	549.32	511.00	5.19	0.94	41.53	40.00	1.55	0.55
CQ-Z	519.99	476.50	4.97	0.93	57.93	57.80	2.56	0.71
CQ-X	370.51	353.00	4.02	0.85	66.00	66.00	3.72	0.89
CQ-P	326.24	297.00	3.67	0.84	77.54	76.30	3.19	0.80
FQ-S	1 074.19	1 049.20	6.13	0.96	48.72	48.40	1.33	0.44
FQ-Z	979.76	960.30	5.67	0.93	55.21	54.60	2.53	0.74
FQ-X	912.37	833.50	5.40	0.91	54.33	54.00	2.39	0.72
FQ-P	858.78	798.00	5.35	0.91	51.60	51.30	2.20	0.71

3.2 Impact on the dominant microbiota composition

The dominant bacterial constituents in both FQ and CQ were Bacillales and Lactobacillales (Figs. 1C and D). The abundance of Bacillales accounted for 87.42%–95.42% and 0.60%–22.25%, while that of Lactobacillales ranged 2.48%–4.91% and 33.71%–56.97%, respectively, in FQ and CQ. The community composition in *Cuqu* varied with the spatial location. For example, Bacillales, Lactobacillales and Pseudonocardiales were dominant in both CQ-S and CQ-Z, in which their abundances being 21.29%, 22.25% and 33.71% for the former, and 33.03%, 32.19% and 22.82% for the latter, respectively. However, Streptomycetales was also dominant with abundances of 36.65% and 36.49%, respectively, except for Lactobacillales, which accounted for 36.65% and 36.49%, in CQ-X and CQ-P. Bacillales became the dominant microbes in FQ with abundances of 87.42% and 88.62% in FQ-S and FQ-Z, while reaching 94.91% and 95.42% in FQ-X and FQ-P. But the abundance of Lactobacillales was notably reduced in FQ, ranging from 2.48% to 4.91% in the samples collected from different spatial locations.

Hierarchical clustering analysis showed distinct disparities between CQ and FQ (ASVs > 0.50%) for bacterial genus-level (Fig. 1E). For instance, the abundance of five dominant bacterial genera was slightly higher in different layer samples in CQ compared to the corresponding samples in FQ. These dominant bacteria included *Pediococcus*, *Leuconostoc*, *Weissella*, *Lactobacillus*, and *Lactococcus*, whereas, *Bacillus*, *Kroppenstedtia*, and *Thermoactinomyces* were predominant in FQ. The abundance of dominant bacteria was also impacted by spatial positions and abiotic factors, such as temperature, humidity, and CO_2 concentration[25]. The abundance of *Bacillus* in the sample from different positions in FQ was notably higher than that of the corresponding positions in CQ. Besides its abundance in the lower

and ground layers of FQ was higher than that of the upper and middle layers, in which one in the middle layer was least, whereas was opposite for CQ. The abundance of *Streptomyces*, a dominant microbe, was almost same (36.64% and 36.44%) in both CQ-X and CQ-P. Except for *Streptomyces*, the abundances of *Pediococcus* and *Lactobacillus* were also declined orderly from top to bottom layers for CQ. The abundance of *Thermoactinomyces* was 10.05% and 3.10% in FQ-S and FQ-Z, respectively, which were slightly higher than those in the samples in corresponding spatial location of CQ (7.13% and 2.49%). Nevertheless, the abundance of *Thermoactinomyces* in the lower two layers (FQ-X and FQ-P) was evidently higher compared to that in CQ. Both *Bacillus* and *Thermoactinomyces* were pivotal species with a high ability to produce hydrolase and various aromatic substance or their precursor[26]. *Kroppenstedtia* was one of dominant bacteria in FQ, especially the abundance in the middle-layer sample was up to 33.89%, and was positively correlated with functional compounds and volatile components like pyrazines and aldehydes[27-28]. Conversely, *Saccharopolyspora* was one of the dominant microbes in CQ, and the abundances was also declined orderly from top to bottom layers.

Eurotiales and Mucorales dominated in both FQ and CQ, ranging from 76.81% to 92.32% and from 4.90% to 17.58%, respectively, and Saccharomycetales dominated in CQ-X (19.72%) and CQ-P (39.55%). The variation in the fungal community structure was illustrated in Fig. 1F, with heat-tolerant fungi becoming dominant in the later stages due to an increase in bioheat during the early and middle stages[29]. *Thermoascus* and *Thermomyces* dominated in both FQ and CQ, although their abundances differed significantly. In CQ, the abundance of *Thermoascus* ranged from 3.46% to 29.91%, while that of *Thermomyces* ranged from 20.54% to 30.11%. Whereas, the abundance of *Thermoascus* and *Thermomyces* spanned from 0.67% to 60.28% and 38.29% to 71.70%, and also declined orderly from top to bottom layers in FQ, might be related to the heat- and arid-tolerance. The high activity of heat-tolerance hydrolase, antimicrobial agents, and antiviral components endow them with self-purification capabilities and functional roles[28]. *Rhizomucor*, often examined in *Daqu* or *Cuqu*, prevails due to the combination of elevated temperature and evaporation ratio in the upper layer in CQ, and the abundance ranged from 6.32% to 26.49%. Conversely, *Rasamsonia* abundance increased from top to bottom in FQ (ranging from 0.03% to 25.61%), as it thrived in high humidity and warmth. Moreover, *Rhizopus*, known for thriving in humid and warm conditions, demonstrated higher abundances in the lower layer of *Cuqu*.

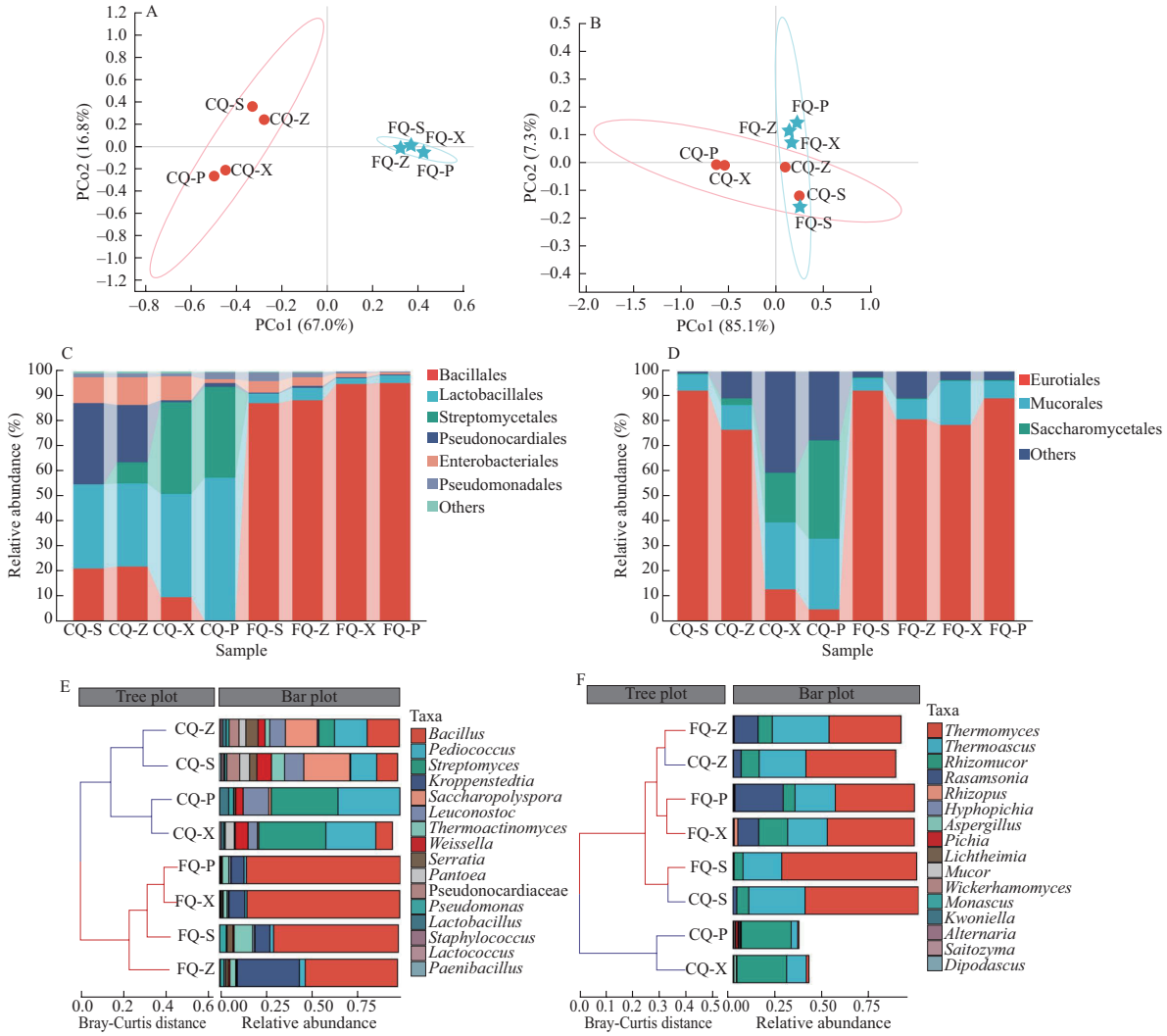

Fig. 1 Analysis of (A) bacterial community and (B) fungal community β-diversity based on PCoA. Relative abundance microbial community at order level (C) bacteria and (D) fungi. Relative abundance microbial community at genus level and hierarchical clustering analysis for (E) bacteria and (F) fungi. Random forest analysis of key (G) bacteria and (H) fungi.

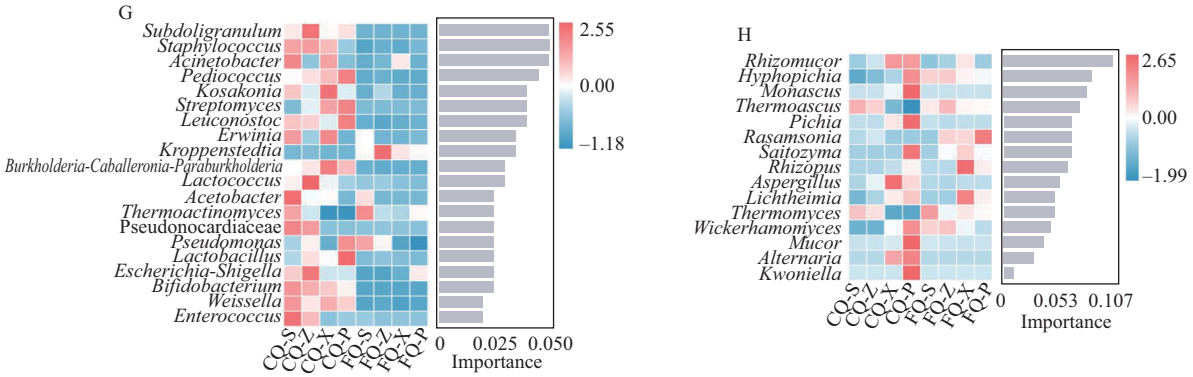

Fig. 1 (*Continued*)

3.3 *Difference in the microbiota structure of Cuqu*

The bacterial communities in CQ and FQ were distributed into different clusters (Fig. 1E). The bacterial microbiota structure in FQ-Z differed from that in other *Cuqu* as it was located in a separate subcluster. The structure of FQ-S showed a slight divergence compared to that of FQ-X and FQ-P since it was located in a different group within the same subcluster, while the structures of FQ-X and FQ-P were quite similar despite being located in different groups within the same subcluster. For CQ, the bacterial microbiota structure exhibited remarkable similarity between CQ-Z and CQ-S as they were classified within the same subcluster group, and so did between CQ-P and CQ-X, while there was a slight diversity between the groups. The fungal microbiota structure of CQ-X and CQ-P were slightly similar which were located in different subcluster within the same cluster (Fig. 1F). Among another 6 kinds of *Cuqu*, the structure of FQ-Z and CQ-Z, as well as FQ-P and FQ-X were quite similar as they were located in different group within the same subcluster, while that of FQ-S and CQ-S was slightly similar since they were located in different subcluster within the same cluster. These results revealed that the contribution of *Muqu* significantly influenced the bacterial community structure, resulting in notable differences in the community structure between the upper and middle layers of *Cuqu*, with a relatively minor impact on the fungal community.

According to the random forest analysis[30], key microbes of three bacterial species and three fungal species were identified in FQ (Figs. 1G and H). They were *Bacillus*, *Kroppenstedtia*, *Scopulibacillus*, *Oidiodendron*, *Rhizopus*, and *Rasamsonia*, respectively. In addition, there were key microbes of four bacterial genera and 3 fungal genera in CQ, including *Burkholderia-Caballeronia-Paraburkholderia*, *Staphylococcus*, *Kosakonia*, *Pediococcus*, *Aspergillus*, *Rhizomucor*, and *Mucor*.

The results of Source Tracker analysis revealed that the proportion of bacteria originating from FM in FQ was significantly higher than that originating from CM in CQ, except for that in the upper layer (Figs. 2A and B). Especially, the proportions of bacteria from *Muqu* in FQ-X and FQ-P were 34.82% and 32.29%, while those in CQ-X and CQ-P were only 9.35% and 7.83%, respectively. The ratio of fungi originated from Muqu in both FQ and CQ of their corresponding upper and middle layer were similar. However, the proportion of fungi originating from *Muqu* in CQ was much higher than that in FQ for both the lower and ground layers (Figs. 2C and D). For the former, the ratios accounted for 26.68% and 18.99%, while they were 2.08% and 2.62% for the latter, respectively, in the corresponding lower and ground layers. These differences might be closely related to the changes of liquefying and saccharifying abilities in both types of *Cuqu*.

The functional prediction analysis was performed based on sequencing data (Figs. 2E and F). The bacterial functional unit samples from 2 distinct types of *Cuqu* demonstrated significant differentiation, exhibiting no overlap between them. In the FQ, bacterial functionalities across four spatial positions were remarkably consistent, with the closest similarity observed between FQ-X and FQ-P, where the samples nearly overlapped. In contrast, the CQ type showed a more dispersed distribution of bacterial functions across its spatial positions, with CQ-P and CQ-X, being relatively closer, akin to the proximity between CQ-Z and CQ-S. This variation suggests that biofortification may mitigate spatial discrepancies in bacterial functionalities within *Cuqu*. Furthermore, the aggregation of fungal functions within FQ surpassed that in CQ. With the exception of FQ-S, fungal functions across three spatial positions in FQ displayed substantial overlap. Conversely, in CQ, despite the observed similarity between CQ-Z and CQ-S, the gap between CQ-P and CQ-X, as well as their separation from the

Table 2
Differences on physicochemical properties of different *Cuqu*.

Sample	Moisture (g/100 g)	Acidity (mmol/10 g)	Saccharifying ability (mg/(g·h))	Liquefying ability (g/(g·h))	Esterifying ability (mg/(50 g·7 days))	Fermenting ability (g/(0.5 g·72 h))
CQ-0	36.81 ± 0.38^{j}	0.02 ± 0.00^{a}	$1\,243.99 \pm 1.91^{c}$	ND	ND	ND
CQ-S	20.65 ± 0.14^{d}	0.06 ± 0.01^{c}	$1\,259.84 \pm 2.73^{d}$	1.41 ± 0.00^{ab}	$1\,079.41 \pm 5.31^{e}$	1.38 ± 0.00^{e}
CQ-Z	24.31 ± 0.06^{f}	0.07 ± 0.00^{c}	$1\,303.00 \pm 0.37^{f}$	1.61 ± 0.02^{d}	$1\,534.21 \pm 3.73^{f}$	1.33 ± 0.09^{cde}
CQ-X	27.56 ± 0.18^{g}	0.11 ± 0.00^{f}	$1\,388.40 \pm 1.67^{g}$	1.79 ± 0.00^{e}	$1\,537.76 \pm 5.39^{f}$	1.33 ± 0.04^{cde}
CQ-P	29.70 ± 0.11^{h}	0.14 ± 0.00^{g}	$1\,400.85 \pm 2.84^{h}$	1.63 ± 0.00^{d}	$1\,582.70 \pm 4.82^{g}$	1.34 ± 0.01^{de}
FQ-0	35.08 ± 0.19^{i}	0.02 ± 0.00^{a}	$1\,246.09 \pm 1.54^{c}$	ND	ND	ND
FQ-S	14.13 ± 0.11^{a}	0.03 ± 0.00^{b}	$1\,096.35 \pm 3.08^{a}$	1.39 ± 0.02^{a}	917.91 ± 4.61^{a}	1.27 ± 0.01^{bc}
FQ-Z	17.60 ± 0.18^{b}	0.03 ± 0.00^{b}	$1\,137.86 \pm 0.67^{b}$	1.56 ± 0.02^{c}	$1\,056.03 \pm 1.71^{b}$	1.29 ± 0.02^{bcd}
FQ-X	19.78 ± 0.18^{c}	0.05 ± 0.00^{c}	$1\,299.83 \pm 3.61^{f}$	1.83 ± 0.03^{f}	$1\,339.50 \pm 4.08^{c}$	1.19 ± 0.09^{a}
FQ-P	21.73 ± 0.28^{e}	0.06 ± 0.00^{d}	$1\,282.13 \pm 1.54^{e}$	1.42 ± 0.01^{b}	$1\,132.90 \pm 3.43^{d}$	1.26 ± 0.20^{b}

Note: Different letters in the same column represent significant differences (Duncan's test, $P < 0.05$). CQ-0 and FQ-0 respectively refer to the initial *Qupei* of FQ and CQ on day 0.

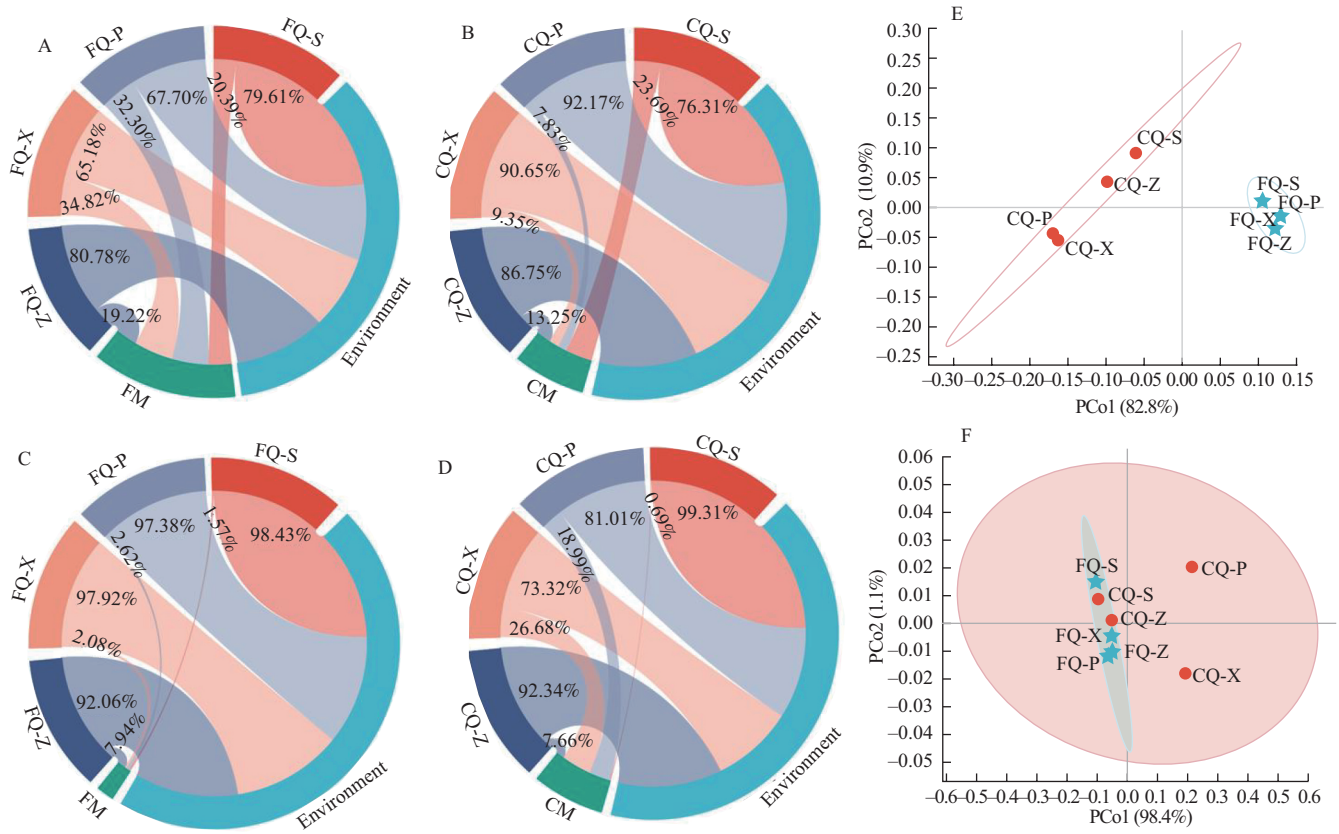

Fig. 2 Source Tracker analysis of microbes. Bacterial source of (A) FQ and (B) CQ. Fungal source of (C) FQ and (D) CQ. Analysis of (E) bacterial community and (F) fungal community functional units based on PCoA.

aforementioned samples and functional units across various spatial positions in FQ indicates that biofortification potentially minimizes the spatial heterogeneity of *Cuqu*, thereby, enhancing the predictability and control of the fermentation process.

3.4 Difference of physicochemical properties among Cuqu

The change of the temperature can be divided into three main stages during the process (Fig. 3A), which were the initial stage (day 0 and day 3), the middle stage (from day 3 to day 6), and the later stage (from day 7 to day 10). The temperature was elevated due to the bioheat produced by the propagation and metabolism of microbial communities in the initial stages when environmental conditions were highly suitable. The temperature slightly increased to its peak value on day 6 and then decreased monotonically in the later stages. Compared to CQ, the temperature amplification of FQ was higher and might be related to *Muqu*, where the abundance of *Bacillus* spp., which are high amylase-producing, was greater and improved the ability of starch conversion. According to Xiao et al.[31], disparities in abiotic factor such as humidity, thermal conditions, fluid flow and spatial positions among shelves, caused the differences in the community propagation rate, which gradually intensified in the initial- and middle-stages, especially in FQ. Even though the attributes among the initial *Qupei* were almost same, the moisture in FQ and CQ ranged from 14.13 to 21.73 g/100 g and 20.65 to 29.70 g/100 g when fermented (Table 2). For similar reasons, the moisture and acidity in the samples placed on the ground surface were higher than those in the corresponding middle- and upper-layers. The ability to hydrolyze starch in both types of the initial *Qupei* was contributed by endogenous hydrolytic enzymes in wheat. However, the liquefaction ability and saccharification ability in FQ was slightly lower than that of CQ at corresponding spatial positions, except for liquefaction ability in FQ-X. The ratios of the liquefaction ability to saccharification ability in FQ, except for FQ-P, were higher than those in CQ. This ratio not only determined the conversion efficiency of starch but also affected the rate of multiple metabolic pathways and their synergies relationship, resulting in the difference of the yield and quality of vinegar[18].

3.5 Metabolic profiles of different vinegar starters

In CQ and FQ, a total of 57 and 48 volatile components were identified, respectively, with concentrations ranging from 10.43 to 15.57 μg/g and from 7.18 to 14.33 μg/g. The former comprised esters (38), alcohols (9), ketones (4), and pyrazines (1), while the latter consisted of esters (31), alcohols (3), ketones (1), and pyrazines (6). In CQ and FQ, esters dominated, accounting for 86.47%–90.24% and 86.23%–89.98% (Fig. 3B), respectively. The proportion of alcohols in CQ (ranging from 7.28% to 10.59%) was higher than that in FQ, while the proportion of pyrazines (ranging from 3.59% to 6.69%) was higher in FQ. Meanwhile, the volatiles content of both *Cuqu* groups appeared an increasing trend from bottom to top position in shelves. Specifically, the ester contents in CQ-S and CQ-Z were 1.43 and 1.71 times higher, respectively, than that of FQ at the corresponding positions. These esters were primarily comprised of methyl esters and ethyl esters, accounting for approximately 94.98%–98.80% and 1.20% –14.06%,

severally. The methyl esters mainly involved the format derivatives of palmitic acid, linoleic acid, oleic acid, etc., while the ethyl esters were the acetate derivatives of palmitic acid, caprylic acid, and phenylacetic acid. The ethyl ester content in CQ was significantly higher than in FQ, especially in CQ-P, where it reached 1.75 μg/g. It might be related with the microenvironment conditions, such as high moisture (29.70 g/100 g), variation amplitude of temperature, etc., which were suitable for propagating of acetic acid-producing microbes and promote ester synthesis.

The pyrazine content in FQ, fortified by *Muqu* with *Bacillus* that has high pyrazines production capabilities, was significantly higher than that in CQ, ranging from 0.26 to 0.83 μg/g. Pyrazines were composed of 2,5-dimethylpyrazine, tetramethylpyrazine, 2,6-dimethylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2-ethyl-3,5-dimethylpyrazine, in which the contents of the first two constituents were higher, ranging from 0.085 to 0.502 μg/g and 0.152 to 0.326 μg/g, respectively. Only 2,5-dimethylpyrazine was detected in CQ-S and CQ-Z, were 0.463 and 0.013 μg/g, respectively. The alcohol content in CQ and FQ ranged from 0.759 to 1.53 μg/g and 0.224 to 0.788 μg/g, respectively, with the former exhibiting significantly higher levels than the latter. Moreover, the alcohol content in CQ was found to be 1.94–5.99 times higher than that in FQ within the same layer. The concentration of phenylethanol in CQ was observed to be 2.11–6.01 times higher compared to FQ. Isobutanol was only detected in CQ, with concentrations ranging from 0.118 to 0.377 μg/g. Conversely, the content of 2,3-butanediol in FQ was found to be 1.45–6.13 times higher than that in CQ, and this component can be interconverted with ethylidene succinate through enzymatic reactions.

LEfSe was applied to identify distinctive substances with statistically significant differences between the FQ and CQ (Figs. 3C and D). A total of 23 distinctive substances, including tetramethylpyrazine, 2,5-dimethylpyrazine, methyl palmitate, phenylethanol, isobutanol, glyceryl linoleate, etc., exhibited significant differences between CQ and FQ. The contents of isoamylol, phenylethanol, isobutanol,

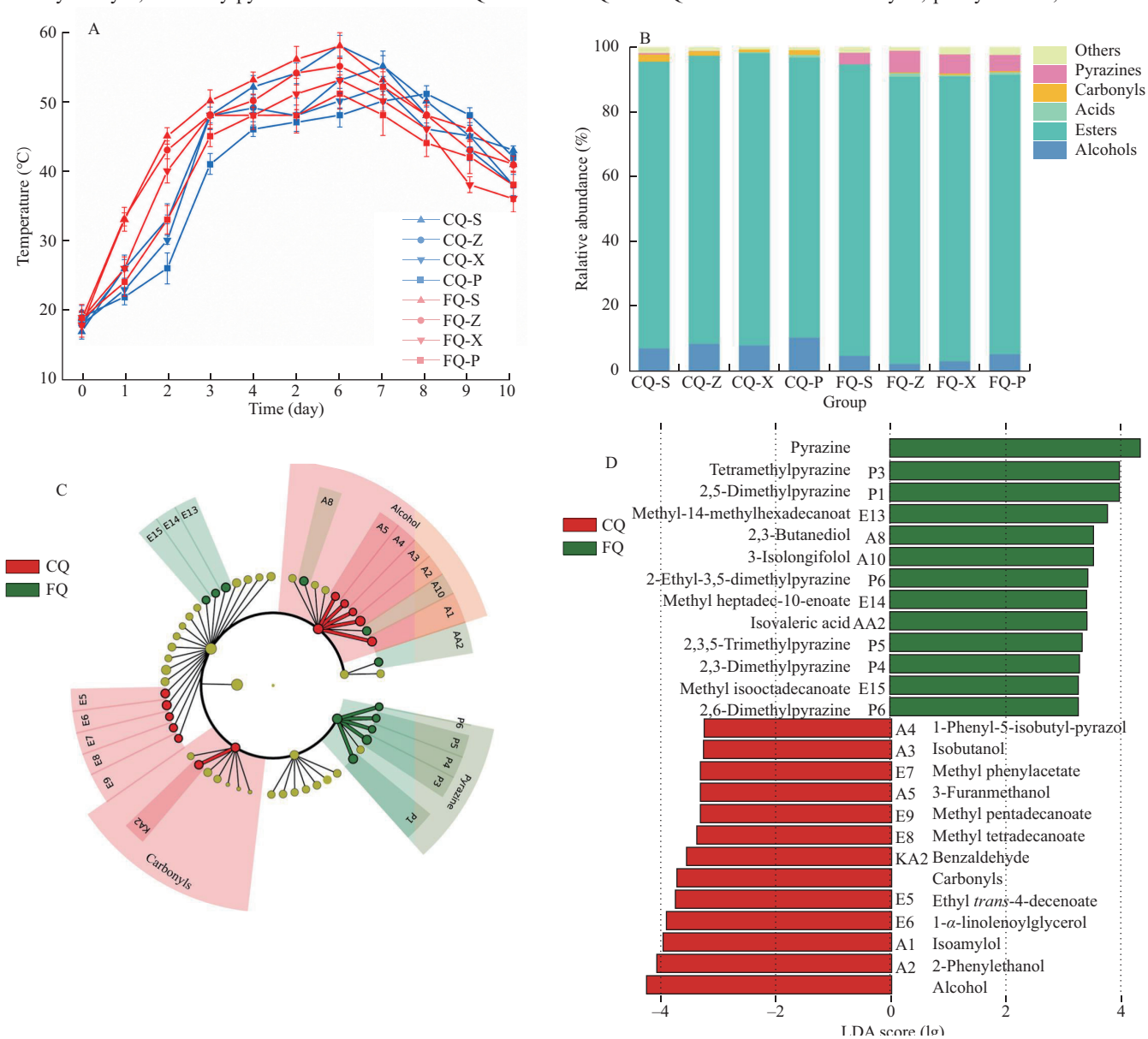

Fig. 3 (A) Variation of the temperature in center of *Cuqu* during the fermentation. (B) Relative abundance of *Cuqu* volatiles. (C) Plot cladogram and (D) bar graph are the analysis of differences between the volatile components of CQ and FQ based on LEfSe (LDA > 2, $P < 0.05$). (E) Correlation analysis between volatiles and microbes ($P < 0.05$). (F) Interaction among microbial community ($P < 0.05$). Red line and blue line showed positive and negative correlation, respectively.

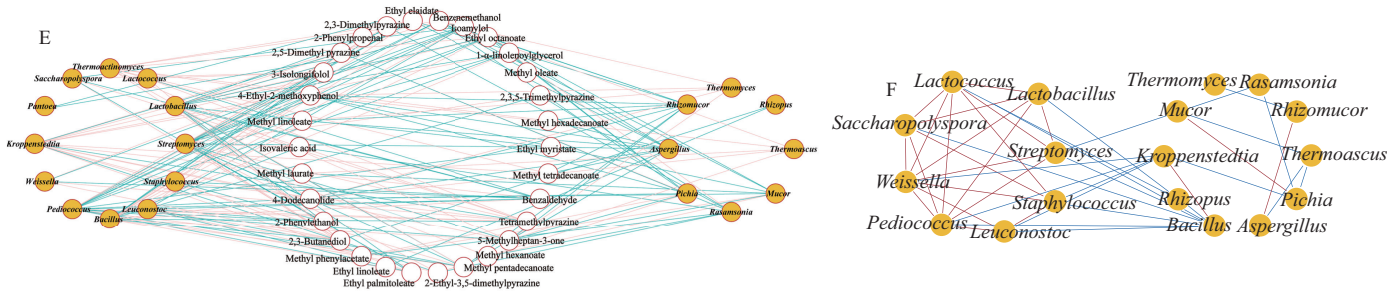

Fig. 3 (*Continued*)

and 3-octanol in CQ were significantly higher compared to those in FQ. Except for 2,6-dimethylpyrazine, the remaining pyrazines, 2,3-butanediol, and isoheptanol, were characteristic components of FQ. Components such as ethyl decenoate, glyceryl linoleate, methyl benzoate, and methyl myristate were characteristic components in CQ, while methyl hexadecanoate, isoarachidic acid methyl ester, and others were characteristic components in FQ. Pyrazine components were distinctive in FQ, especially tetramethylpyrazine, which contributed delightful flavors to FQ[32].

3.6 Correlating dominant microbes with flavour constituents

As depicted in Fig. 3E, the correlation analysis was performed to investigate the relationship between dominant microbes and flavor constituents ($P < 0.05$). As reported by Zhang et al.[28], *Kroppenstedtia* and *Bacillus* were closely associated with pyrazines. *Bacillus* exhibited positive correlations with tetramethylpyrazine, 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2,5-dimethylpyrazine. Additionally, *Bacillus* displayed a positive correlation with one of the precursors of pyrazines, 2,3-butanediol. *Kroppenstedtia* exhibited a positive correlation with 2,5-dimethylpyrazine, tetramethylpyrazine, and 2-ethyl-3,5-dimethylpyrazine. Several esters were positively correlated with *Pediococcus*, *Streptomyces*, *Lactobacillus*, *Rhizomucor*, *Pichia*, *Aspergillus*, including ethyl palmitate, ethyl caprylate, ethyl myristate, ethyl oleate. *Pichia* showed a positive correlation with ethyl caprylate, lauric acid methyl ester, and *δ*-dodecalactone. Phenethyl alcohol exhibited a positive correlation with bacterial genera, such as *Pediococcus*, *Leuconostoc*, and *Lactobacillus*. 4-Ethylguaiacol was positively correlated with *Pichia*, *Pediococcus*, *Streptomyces*, and other genera similar to reported in previous studies[33-34].

Interspecific interactions among the communities revealed a positive correlation between *Bacillus* and *Kroppenstedtia* in FQ (Fig. 3F), and both were negatively correlated with lactic acid bacteria such as *Pediococcus*, *Leuconostoc*, and *Weissella*. The negative correlations of *Bacillus* with *Lactobacillus* and *Lactococcus* were observed, and it suggested potential competition for carbon sources with lactic acid bacteria, resulting in *Bacillus* dominated in FQ. Additionally, *Bacillus* secreted various hydrolase, thereby improving substrate hydrolysis rates and the growth of microbiota, contributing to rise the temperature of *Qupei* and hinder the growth of lactic acid bacteria. Positive correlations form modular relationships among genera such as *Pediococcus*, *Streptomyces*, *Saccharopolyspora*, *Weissella*, *Lactococcus*, and *Lactobacillus*. Lactic acid bacteria with strong acid and ethanol tolerance can produce lactic acid, balancing the stimulating acidity and taste of acetic acid[35]. The interspecific interactions among fungi were relatively simple compared to that of bacteria. *Aspergillus*, *Pichia*, *Mucor*, and *Thermoascus* showed negative correlations, while *Rhizomucor* was negatively correlated with *Thermomyces*. However, *Aspergillus* exhibited a positive correlation with *Rhizomucor*. Additionally, 2 thermophilic microbes, *Thermoactinomyces* and *Thermomyces*, showed a positive correlation.

3.7 Metabolic pathway analysis

The metabolic pathways of the community were predicted based on the MetaCyc metabolic database and PICRUSt2 (Figs. 4A and B). The bacteria and fungi involved in *Cuqu* fermentation primarily participate in 4 key metabolic pathways, including biosynthesis, degradation/utilization/assimilation, generation of precursor metabolite and energy, and metabolic clusters, covering 46 and 27 secondary metabolic pathways, respectively. The metabolic pathways associated with the biosynthesis of nucleosides, nucleotides, cofactors, coenzymes, electron carriers, vitamins, and amino acids exhibited activity that was intricately linked to microbial growth and vital activities. Subsequently, the abundance of metabolic pathways associated with fatty acids, carbohydrates, cell structure, lipids, and secondary metabolite biosynthesis was slightly lower but still significant. In addition, bacterial metabolic pathways linked to the aromatic compounds and amino acids, critical flavor components in vinegar, were slightly higher in FQ compared to CQ. The former were annotated to range from 2 375.00 to 2 506.46, and 31 420.02 to 34 418.34, respectively, while the latter ranged from 1 657.84 to 2 023.94 and 22 547.16 to 30 798.18. In the degradation/utilization/assimilation category, the primary pathways involved the degradation of nucleosides, nucleotides, carbohydrates, and carboxylic acids. Fungi and bacteria exhibited differential levels of activity in secondary metabolic pathways related to precursor metabolite generation and energy production. Fungi primarily utilized respiration, the pentose phosphate pathway, and electron transfer. In contrast, bacteria exhibited more active metabolic pathways related to fermentation, the tricarboxylic acid (TCA) cycle, and glycolysis. These processes generated various intermediate metabolites that could enter sugar metabolism pathways. Although the overall abundance of bacterial fermentation metabolic pathways in CQ was higher than in FQ, the latter had a higher abundance of the TCA cycle pathway. This process linked the metabolism of sugars, lipids, and amino acids, playing a crucial role in the production of flavor compounds. Furthermore, perturbation of *Muqu* resulted in a slight enhancement of metabolic pathways related to *L*-aspartate biosynthesis in FQ.

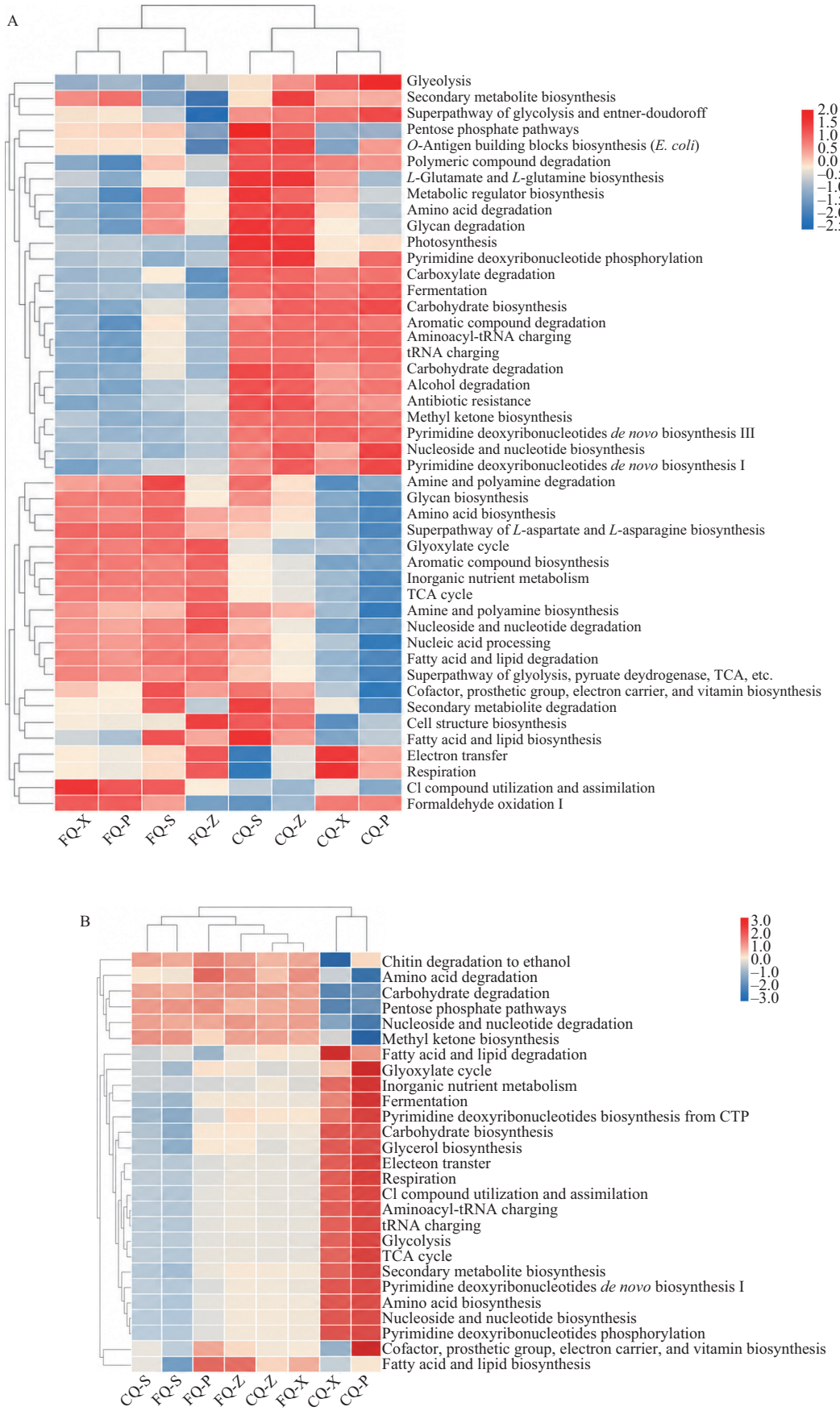

Fig. 4 Heat map of metabolic pathway in (A) bacterial community and (B) fungal community. Main metabolic pathways and the expression of related (C) enzymes, (D) bacteria, and (E) fungi.

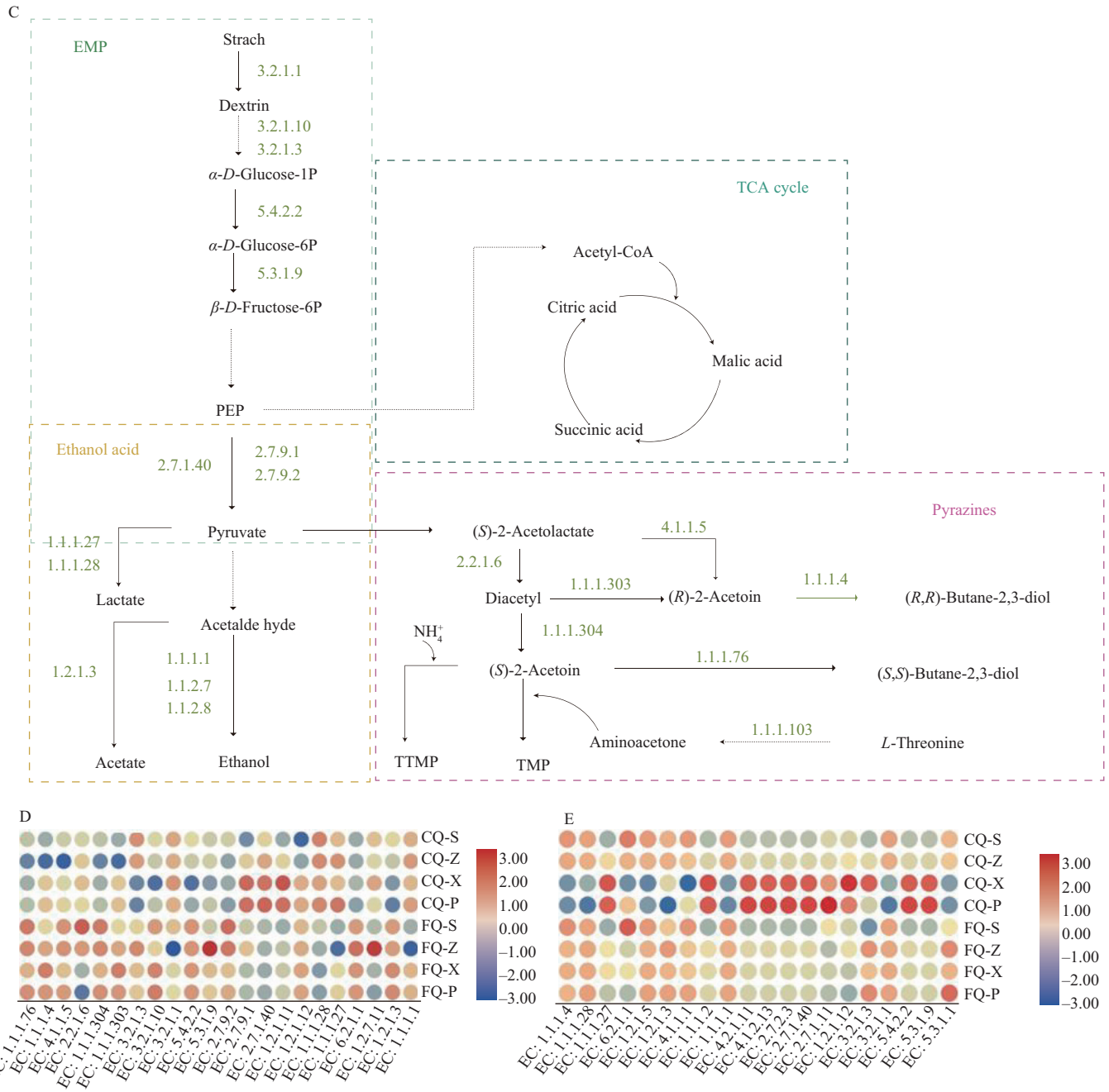

Fig. 4 (*Continued*)

The predicted results of key enzyme expression related to bacterial metabolic pathways were shown in Figs. 4C, D and F. Microbiota fortification upregulated the expression of bacterial enzymes such as amylase (EC: 3.2.1.3), isomaltase (EC: 3.2.1.10), phosphoglucomutase (EC: 5.4.2.2), and glucose-6-phosphate isomerase (EC: 5.3.1.9) in FQ. However, it slightly decreased the expression abundance of alpha-amylase (EC: 3.2.1.1). These results indicated an enhancement in the degradation of carbohydrates such as starch, cellulose, etc., an increased in the flux of essential intermediate metabolites such as pyruvic acid and acetyl-CoA, and the synthesis of beneficial flavor components. Furthermore, FQ biofortification resulted in an upregulation of fungal enzyme expression including pyruvate decarboxylase (EC: 4.1.1.1), alcohol dehydrogenase (EC: 1.1.1.1), and aldehyde dehydrogenase (NAD^+). This upregulation enhanced the synthesis capacity of ethanol and acetic acid. Similarly, the expressions of key enzymes involved in pyrazine synthesis, including diacetyl reductases (EC: 1.1.1.303 and EC: 1.1.1.304) responsible for synthesizing precursors 2,3-butanediol and ethylmalonic acid, pyruvate decarboxylase (EC: 4.1.1.5), and 2,3-butanediol dehydrogenases (EC: 1.1.1.4 and EC: 1.1.1.76), were found to be upregulated. In contrast, the expression of lactate dehydrogenase (EC: 1.1.1.27 and EC: 1.1.1.28) involved in lactate synthesis was downregulated, explaining the correlation between increased pyrazine levels and changes in lactic acid bacteria abundance. Biofortification strategies have been identified as effective in enhancing the pyrazine content *Cuqu*, a critical component in

the flavor profile of fermented products like vinegar and wine. Pyrazine, characterized by their roasted, hazelnut, peanut, and cocoa aroma, play a pivotal role in defining the sensory attributes of these products. Tetramethylpyrazine, in particular, stands out for its unique physiological benefits, especially its positive effects on cardiovascular health[36]. The augmentation of pyrazine levels through biofortification not only enriches the flavor complexity of *Cuqu*, but also promises to improve the overall quality of SBV, suggesting a multifaceted advantage encompassing both sensory and health-related aspects.

This research investigated the impact of biofortified *Muqu* on the microbial community and quality of *Cuqu* in multi-layer shelves. The results demonstrated a cascading effect of fortified *Muqu*, which significantly enhanced the directional succession of the bacterial community structure in *Cuqu*, resulting in a reduction of spatial variations within the community structure of FQ. It resulted in a 1.85- to 8.19-fold increase in the abundance of *Bacillus* and a significant increase in the abundance of *Kroppenstedtia* while reducing the abundance of lactic acid bacteria. This approach not only substantially increased pyrazine diversity and content, but also improved the proportional relationships among major physicochemical parameters. The spearman correlation analysis revealed negative correlations of *Bacillus* and *Kroppenstedtia* with *Pediococcus*, *Leuconostoc*, and *Weissella*. Positive correlations in a modular pattern were observed among *Pediococcus*, *Streptomyces*, *Saccharopolyspora*, *Weissella*, *Lactococcus*, and *Lactobacillus*. These results provided a theoretical foundation and feasible strategies for effectively improving the community and metabolic functions during the fermentation process of traditional fermented foods in open environments.

This study employed advanced detection methodologies, including high-throughput sequencing and HS-SPME-GC-MS, to reveal the microbial community structure and metabolite profiles of biofortified *Cuqu*. While these techniques provided valuable insight, they exhibited limitations in thoroughly deciphering microbial interaction and their association with flavor compounds. To overcome these limitations, further research will leverage metagenomic and metaproteomic approaches to acquire a more comprehensive understanding of the microbiota and metabolic patterns, aiming for a holistic analysis of the microbial community while identifying key microbes and genes within *Cuqu*. Furthermore, integrating metabolomic technologies, such as liquid chromatograph-mass spectrometer, will enhance our comprehension of specific metabolic pathways contributing to the synthesis of flavor compound, thereby advancing the field of flavor science.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

[1] H. Tang, H. Liang, J. Song, et al., Comparison of microbial community and metabolites in spontaneous fermentation of two types *Daqu* starter for traditional Chinese vinegar production, J. Biosci. Bioeng. 128 (2019) 307-315. https://doi.org/10.1016/j.jbiosc.2019.03.011.

[2] Z. Nie, Y. Zheng, M. Wang, et al., Exploring microbial succession and diversity during solid-state fermentation of Tianjin duliu mature vinegar, Bioresour. Technol. 148 (2013) 325-333. https://doi.org/10.1016/j.biortech.2013.08.152.

[3] Z.Q. Nie, Y. Zheng, H.F. Du, et al., Dynamics and diversity of microbial community succession in traditional fermentation of Shanxi aged vinegar, Food Microbiol. 47 (2015) 62-68. https://doi.org/10.1016/j.fm.2014.11.006.

[4] X. Gan, H. Tang, D. Ye, et al., Diversity and dynamics stability of bacterial community in traditional solid-state fermentation of Qishan vinegar, Ann. Microbiol. 67 (2017) 703-713. https://doi.org/10.1007/s13213-017-1299-6.

[5] M. Ai, X. Qiu, J. Huang, et al., Characterizing the microbial diversity and major metabolites of Sichuan bran vinegar augmented by *Monascus purpureus*, Int. J. Food Microbiol. 292 (2019) 83-90. https://doi.org/10.1016/j.ijfoodmicro.2018.12.008.

[6] A.P. Liu, Y. Peng, X.L. Ao, et al., Effects of *Aspergillus niger* biofortification on the microbial community and quality of Baoning vinegar, LWT-Food Sci. Technol. 131 (2020) 109728. https://doi.org/10.1016/j.lwt.2020.109728.

[7] Q. Zhang, C. Fu, C. Zhao, et al., Monitoring microbial succession and metabolic activity during manual and mechanical solid-state fermentation of Chinese cereal vinegar, LWT-Food Sci. Technol. 133 (2020) 109868. https://doi.org/10.1016/j.lwt.2020.109868.

[8] H. Du, X. Wang, Y. Zhang, et al., Exploring the impacts of raw materials and environments on the microbiota in Chinese *Daqu* starter, Int. J. Food Microbiol. 297 (2019) 32-40. https://doi.org/10.1016/j.ijfoodmicro.2019.02.020.

[9] Z.M. Lu, Z.M. Wang, X.J. Zhang, et al., Microbial ecology of cereal vinegar fermentation: insights for driving the ecosystem function, Curr. Opin. Biotechnol. 49 (2018) 88-93. https://doi.org/10.1016/j.copbio.2017.07.006.

[10] X. Xing, Y. Wang, N. Huo, et al., *Candida ethanolica* strain Y18 enhances aroma of Shanxi aged-vinegar, Food Sci. Technol. Res. 24 (2018) 1069-1081. https://doi.org/10.3136/fstr.24.1069.

[11] L.J. Chai, T. Qiu, Z.M. Lu, et al., Modulating microbiota metabolism *via* bioaugmentation with *Lactobacillus casei* and *Acetobacter pasteurianus* to enhance acetoin accumulation during cereal vinegar fermentation, Food Res. Int. 138 (2020) 109737. https://doi.org/10.1016/j.foodres.2020.109737.

[12] M.Y. Peng, X.J. Zhang, T. Huang, et al., *Komagataeibacter europaeus* improves community stability and function in solid-state cereal vinegar fermentation ecosystem: non-abundant species plays important role, Food Res. Int. 150 (2021) 110815. https://doi.org/10.1016/j.foodres.2021.110815.

[13] A. Liu, R. Wang, J. Li, et al., Multiple rounds of *Aspergillus niger* biofortification confer relatively stable quality with minor changes of microbial community during industrial-scale Baoning vinegar production, Food Res. Int. 150 (2021) 110768. https://doi.org/10.1016/j.foodres.2021.110768.

[14] L. Zhang, J. Huang, R. Zhou, et al., Evaluating the feasibility of fermentation starter inoculated with *Bacillus amyloliquefaciens* for improving acetoin and tetramethylpyrazine in Baoning bran vinegar, Int. J. Food Microbiol. 255 (2017) 42-50. https://doi.org/10.1016/j.ijfoodmicro.2017.05.021.

[15] Q. Pan, J. Huang, S. Zhang, et al., Response of microbiota to exogenous inoculation improved the enzymatic activities of medium-temperature *Daqu*, Front. Microbiol. 13 (2022) 1047041. https://doi.org/10.3389/fmicb.2022.1047041.

[16] Luzhou City Market Supervision Administration. 2020. Geographical indication product-technical specifications for the production of Luzhou Laojiao Series *Daqu* (Jiuxiang Brand) (DB5105/T36–2020). Sichuan Province: Luzhou City Market Supervision Administration.

[17] Ministry of Light Industry of China. 2011. General methods of analysis for *Daqu* (QB/T 4257–2011). Beijing: China Light Industry Press.

[18] Q. Tang, G. He, J. Huang, et al., Characterizing relationship of microbial diversity and metabolite in Sichuan *Xiaoqu*, Front. Microbiol. 10 (2019) 696. https://doi.org/10.3389/fmicb.2019.00696.

[19] M.G.I. Langille, J. Zaneveld, J.G. Caporaso, et al., Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences, Nat. Biotechnol. 31 (2013) 814-821. https://doi.org/10.1038/nbt.2676.

[20] D.H. Huson, S. Mitra, H.J. Ruscheweyh, et al., Integrative analysis of environmental sequences using MEGAN4, Genome Res. 9 (2011) 1552-1560. https://doi.org/10.1101/gr.120618.111.

[21] A. Ramette, Multivariate analyses in microbial ecology, FEMS Microbiol. Ecol. 62 (2007) 142-160. https://doi.org/10.1111/j.1574-6941.2007.00375.x.

[22] P. Shannon, A. Markiel, O. Ozier, et al., Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498-2504. https://doi.org/10.1101/gr.1239303.

[23] D. Knights, J. Kuczynski, E.S. Charlson, et al., Bayesian community-wide culture-independent microbial source tracking. Nat. Methods. 8 (2011) 761-U107. https://doi.org/10.1038/nmeth.1650.

[24] K.C. Dannemiller, C.J. Weschler, J. Peccia, Fungal and bacterial growth in floor dust at elevated relative humidity levels, Indoor Air, 27 (2017) 354-363. https://doi.org/10.1111/ina.12313.

[25] L. Wang, Y. Shen, X. Wang, et al., The spatial differences of the Chinese sauce aroma liquor *Daqu* and identification by FT-MIR spectroscopy, Food Control, 154 (2023) 109990. https://doi.org/10.1016/j.foodcont.2023.109990.

[26] P. Wang, Q. Wu, X. Jiang, et al., *Bacillus licheniformis* affects the microbial community and metabolic profile in the spontaneous fermentation of *Daqu* starter for Chinese liquor making, Int. J. Food Microbiol. 250 (2017) 59-67. https://doi.org/10.1016/j.ijfoodmicro.2017.03.010.

[27] L. Deng, X. Mao, D. Liu, et al., Comparative analysis of physicochemical properties and microbial composition in high-temperature *Daqu* with different colors, Front. Microbiol. 11 (2020) 588117. https://doi.org/10.3389/fmicb.2020.588117.

[28] Y. Zhang, Y. Shen, W. Cheng, et al., Understanding the shifts of microbial community and metabolite profile from wheat to mature *Daqu*, Front. Microbiol. 12 (2021) 714726. https://doi.org/10.3389/fmicb.2021.714726.

[29] H.Y. Wang, Y. Xu, Effect of temperature on microbial composition of starter culture for Chinese light aroma style liquor fermentation, Lett. Appl. Microbiol. 60 (2015) 85-91. https://doi.org/10.1111/lam.12344.

[30] T. Yatsunenko, F.E. Rey, M.J. Manary, et al., Human gut microbiome viewed across age and geography, Nature 486 (2012) 222-227. https://doi.org/10.1038/nature11053.

[31] C. Xiao, Z.M. Lu, X.J. Zhang, et al., Bio-heat is a key environmental driver shaping the microbial community of medium-temperature *Daqu*, Appl. Environ. Microbiol. 83 (2017) e01550-17. https://doi.org/10.1128/AEM.01550-17.

[32] M. Guo, Y. Liu, D.Z. Shi, Cardiovascular actions and therapeutic potential of tetramethylpyrazine (active component isolated from rhizoma chuanxiong): roles and mechanisms, Biomed. Res. Int. 2016 (2016) 2430329. https://doi.org/10.1155/2016/2430329.

[33] Y. Xiao, S. Zhang, Z. Liu, et al., Effect of inoculating *Pichia* spp. starters on flavor formation of fermented chili pepper: metabolomics and genomics approaches, Food Res. Int. 173 (2023) 113397. https://doi.org/10.1016/j.foodres.2023.113397.

[34] Y. Zhao, W. Wei, L. Tang, et al., Characterization of aroma and bacteria profiles of Sichuan industrial paocai by HS-SPME-GC-O-MS and 16S rRNA amplicon sequencing, Food Res. Int. 149 (2021) 110667. https://doi.org/10.1016/j.foodres.2021.110667.

[35] Q.S. Zhao, J.G. Yang, K.Z. Zhang, et al., Lactic acid bacteria in the brewing of traditional Daqu liquor, J. Inst Brewing. 126 (2020) 14-23. https://doi.org/10.1002/jib.593.

[36] R. Yuan, Q.Q. Xin, W.L. Shi, et al. Tetramethylpyrazine and paeoniflorin combination (TMP-PF) alleviates atherosclerosis progress by reducing hyperlipemia and inhibiting plaque angiogenesis *via* the NR4A1/VEGFR2 pathway, Food Sci. Hum. Wellness 13(5) (2024) 2642-2652. https://doi.org/10.26599/FSHW.2022.9250212