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Environment microorganism and mature *daqu* powder shaped microbial community formation in mechanically strong-flavor *daqu*

Shuangping Liu [a,c,d], Yu Zhou [a], Dongna Ma [a], Suyi Zhang [b,**], Yi Dong [b], Xiu Zhang [b], Jian Mao [a,c,d,*]

[a] National Engineering Research Center for Cereal Fermentation and Food Biomanufacturing, State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, 214122, China
[b] Luzhou Laojiao Group Co. Ltd, Luzhou, 646000, China
[c] Shaoxing Key Laboratory of Traditional Fermentation Food and Human Health, Jiangnan University (Shaoxing) Industrial Technology Research Institute, Shaoxing, Zhejiang, 312000, China
[d] National Engineering Research Center of Huangjiu, Zhejiang Guyuelongshan Shaoxing Wine Co., Ltd, Shaoxing, Zhejiang, 312000, China

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ABSTRACT

Daqu is the fermenting starter in Chinese Baijiu brewing, contributing functional microorganisms, enzymes and flavor substances to the grain fermentation. Traditional *daqu* is produced in open environment where environmental factors and microbiota are critical to *daqu* and Baijiu quality. Mechanization is the direction of Baijiu production development. In this study, we investigated the mechanism of the effect of mechanized environment and manufacturing process on *daqu* production. Many functional microorganisms (e.g. *Acetobacter*, lactic acid bacteria and yeast) were distributed in the process environment, including indoor air, tube and equipment surfaces, which contributed to the initial bacterial/fungal composition in *daqu* (71.62%/28.47%) along with mature *daqu* powder (21.1%/61.65%). Our results also showed *daqu* microbiota formation was influenced by both stochastic and deterministic processes. The initial microorganisms produced large amounts of organic acids (19.24 g/kg) and bioheat (58.3 °C) during early fermentation stage, leading to a rapid temperature rise and water loss under the stacking process. Mature *daqu* powder mainly provided thermophilic microorganisms for fermentation stages during and after temperature rise, including *Thermoactinomyces*, *Thermoascus*, *Thermomyces*, *Rhizomucor* and *Rhizopus*, which produced enzymes and amino nitrogen for Baijiu fermentation, also ensuring stable microbiota among process batches. This work revealed the origin of the microbial community of strong-flavor *daqu* and showed that ecological environment suitable for *daqu* fermentation was created during the long-term continuous production process by adding mature *daqu* powder during *daqu* brick making and sprinkling it in the workshop, by which product quality was guaranteed.

1. Introduction

Chinese Baijiu is an ancient distilled alcoholic beverage and the backbone of Chinese fermented products (Zheng & Han, 2016). Strong-flavor Baijiu, also known as Luzhou style Baijiu, is widely welcomed by consumers for its unique flavor (Xu et al., 2022). Strong-flavor *daqu* (medium-temperature *daqu*) is the fermenting starter for strong-flavor Baijiu brewing, providing raw materials, microorganisms, enzymes and flavor substances for grain fermentation (Zhao et al., 2019; Zheng et al., 2011). Traditional *daqu* is made from wheat, and the production steps include crushing of raw materials, pressing of brick, fermentation, stacking and storage, cultivating various microorganisms through natural inoculation (Guan et al., 2021). Nowadays, automation technology has been used in mechanically strong-flavor *daqu* production, which effectively improves efficiency and reduces labor. Mechanical strong-flavor *daqu* is produced under the same process as traditional one (e.g. raw material, process parameter, fermentation period), but adapting automated facility for raw material pretreatment and brick production (Fig. S1), thus changing the quality and characteristics (Zhu, Cheng, et al., 2022).

* Corresponding author. National Engineering Research Center for Cereal Fermentation and Food Biomanufacturing, Jiangnan University, Wuxi, Jiangsu, 214122, China.
** Corresponding author.
E-mail addresses: zhangsy@lzlj.com (S. Zhang), maojian@jiangnan.edu.cn (J. Mao).

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Microorganism is an important part of fermented foods, for its metabolic activity key to food safety, flavor, texture and aroma (Macori & Cotter, 2018). Microbial community is affected by a variety of abiotic factors (e.g. temperature, salinity, moisture, nutrients, metabolites, etc.) and biotic factors (intervention of exogenous microorganisms and interaction between endogenous microorganisms), resulting in the quality and taste changes in various products (Chen et al., 2022; Gao et al., 2020a, 2023; Zhang et al., 2022). *Daqu* is produced in an open environment, whose quality and function are influenced by raw materials and process parameters (Hu, Yang, et al., 2021; Zhu, Zheng, et al., 2022). Therefore, a comprehensive understanding of the influence of environmental microbiota and physicochemical parameters on strong-flavor *daqu* fermentation is essential.

Microbial composition, functional strain screening and flavor composition of strong-flavor *daqu* have been studied extensively (He, Huang, Zhou, Wu, & Jin, 2019). Microorganism in traditional strong-flavor *daqu* is intensively driven by temperature, moisture and acidity (Xiao et al., 2017). In addition, raw materials and process environment are important sources of microorganism, and bacteria may be the drivers of microbial succession in *daqu* manufacturing process (Du et al., 2019). However, the mechanisms by which highly variable environmental parameters and microbial composition affect microorganism in mechanically strong-flavor *daqu* are still unclear. Defining the factors influencing microbial succession may assist to produce *daqu* with stable quality.

In this study, SMRT sequencing was applied to investigate microbial structure in *daqu* fermentation process as well as manufacturing environment. The origin of the microbiota in *daqu* manufacturing process was analyzed by SourceTracker tool. The interactions among *daqu* microorganisms and effects of environmental parameters were further analyzed. This study aimed to provide new insights into microbiota sources and formation process in mechanically strong-flavor *daqu* product, serving as references for microbial control during manufacture.

2. Material and methods

2.1. Sample collection

All Samples were collected in one strong-flavor Baijiu enterprise, located in Luzhou, Sichuan Province, China (28°51′N, 105°34′E). The workshop for mechanical *daqu* has been used for 2 years. The samples were collected in December 2021, including *daqu*, raw materials and environmental samples. Sample illustration is shown in Table S1, each containing 3 parallels. All samples were transported to the laboratory via cold chain and stored at −80 °C.

The process flow and sampling points of *daqu* are shown in Fig. 1. Raw wheat (YL) was shipped by truck and dumped into wheat inlet (JL) at workshop gate, then transported by bucket elevator (DT) through the silo pipe (TC) to the silo for storage. At the beginning of production, wheat stored in silo (TM) was moistened with water for 4–24 h, then crushed into milled wheat (XM). Milled wheat was mixed thoroughly with 37–40% water and 0.3% mature *daqu* powder (QF) then. The mixture (LX) passed through the grain rinsing conveyor (RL), then pressed and shaped by crimping machine (YQ), finally placed neatly on the shelves by robotic arm (QJ). *Daqu* would be cultivated in fermentation room for 10–15 d after 2–3 h in *daqu* brick processing room for drying, then flipped before being placed in storage room for 3 months. Finally, the finished products (finished *daqu*) were crushed and put into steamed grains for Baijiu fermentation.

Daqu samples during fermentation process (0, 1, 2, 4, 6, 8, 10, 17, 24, 32, 62 d and finished *daqu*) were collected in the *daqu* brick processing room, fermentation room and storage room. All samples were taken in three parallels, collected on the 1st, 3rd, and 5th floors of the shelves, each weighing approximately 1000 g. Raw wheat, silo wheat and mature *daqu* powder were randomly selected and mixed at multiple points. Floor, wall and tool surface samples were taken at multiple locations with skim cotton wetted with PBS (0.1M). Air samples were collected using glass fiber membrane (25 mm, Pall Life Sciences, USA) with a flow rate of 1.05 m^3 /min for 6 h. Three parallels were taken in each workshop.

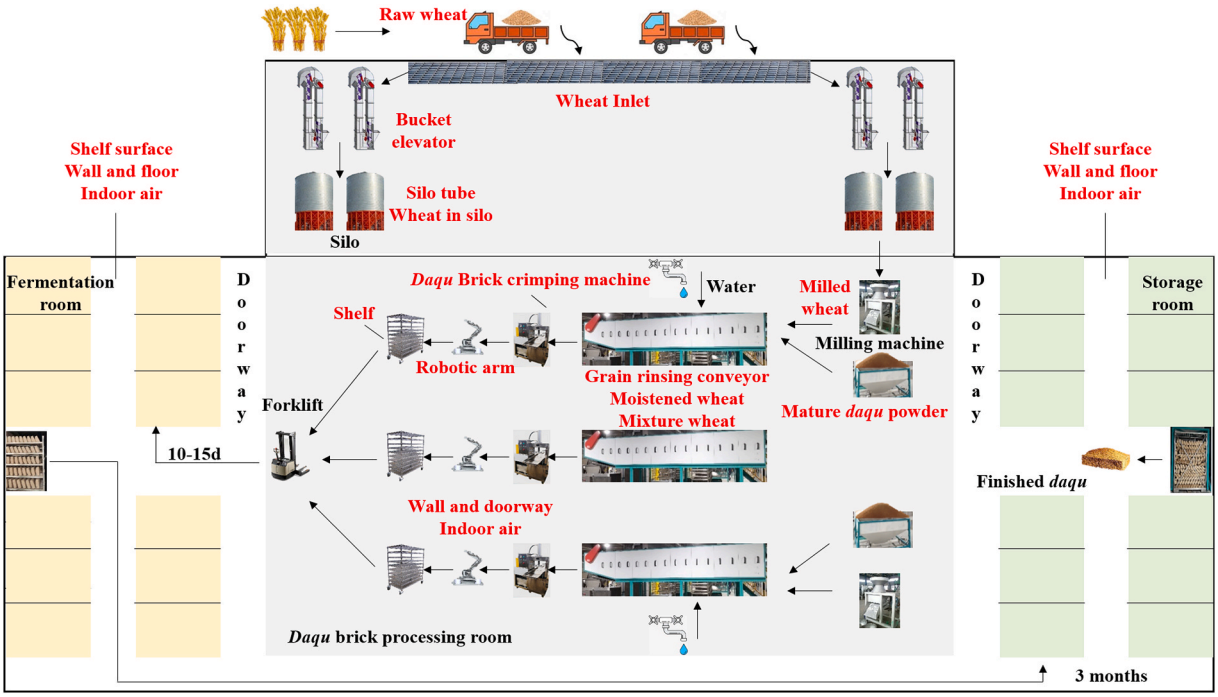

Fig. 1. Schematic diagram of production process and sampling points of mechanically strong-flavor *daqu*

2.2. DNA extraction, PCR amplification and high-throughput amplicon sequencing

DNA was extracted using the CTAB method. The 16S rRNA gene and ITS region were amplified using universal primer 27F_1492R (5′-AGGRGTTTGATYNTGGCTCAG-3'/5′-TASGGHTACCTTGTTASGACTT-3′) and ITS1_ITS4 (5′- CTTGGTTCATTTAGAGGAAGTAA-3'/5′-TCCTCCGCTTATTGATATGC-3′) with barcode. PCR products were measured and concentrated by Thermo Scientific NanoDrop 8000 UV–Vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE). SMRT Bell libraries were created as required, then purified using AM Pure PB columns and the library fragments sizes were assayed using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Sequencing was performed with PacBio Sequel II instrument.

2.3. Determination and analysis of physicochemical indicators

Determination of moisture content was performed with gravimetric method by drying *daqu* under 105 °C for 4 h (Liu et al., 2020). Acidity and amino nitrogen were determined according to acid-base titration method (Gong et al., 2020). Amylase and glucoamylase activities were measured referring to DNS and iodometric titration method respectively (Yu et al., 2021).

2.4. Statistics

The raw image data obtained from sequencing was converted to sequence data by Base calling and saved in BAM (Binary Alignment/Map format) file. For quality control of sequencing data, the Arrow algorithm was used to obtain high precision raw CCS sequences. Raw CCS sequences were obtained for each sample by identifying the different Barcode sequences using Lima software. CCS sequences with over 99% accuracy were selected, and Blast software was adapted to match the intercepted target sequences (primer regions were retained by default) and correct the sequence orientation. For 16S rRNA sequences, sequences over 1500 bp in length were selected into the next step analysis. ITS sequences smaller than 600 bp were removed before downstream analysis. Chimeric sequences were removed using the UCHIME algorithm. All Clean CCS were further processed by QIIME 2 (Caporaso et al., 2010). Sequences were clustered into different amplicon sequence variants (ASVs) based on 100% similarity using DADA2. Each representative sequence from clustered ASV was compared with the Silva database (v 13.8) and the UNITE database (v 12.11) to obtain taxonomic information (Christian, 2013; Nilsson et al., 2019). Single ASVs were discarded before further analysis to eliminate errors (Li et al., 2022).

Chao1 and Shannon index were calculated using QIIME 2. Duncan method was applied to determine significance of difference according to p value. Clustering analysis of microorganisms based on Bray-Curtis distance using Past software (v 4.10) was applied to determine *daqu* fermentation stages. Restricted principal co-ordinate analysis (CPCoA) was performed using the Vegan package. ADONIS/ANOSIM analyses were used to determine the difference significance among samples. In order to investigate the way of microbial community construction during fermentation, NST and Hmisc package were used for neutral model NCM analysis and β-NTI value calculation. Spearman correlation-based Mantel test and CCA analysis were used to investigate the relationship between physicochemical parameters and microbial communities. Spearman correlation was used to analyze the interaction among microorganisms and visualized with Cytoscape (v 3.9.0). SourceTracker (v 1.0.0) was applied to explore the origin of microorganisms during *daqu* processing and fermentation.

3. Results

3.1. Dynamics of physicochemical indexes during daqu fermentation process

The fermentation process was divided into 3 stages (stage I, 0–4 d; stage II, 4–11 d; stage III, 11d-end) according to the temperature changes, similar to traditional *daqu* (Zhu, Cheng, et al., 2022) (Fig. 2A). Moisture content decreased rapidly to 15.97% ± 1.77% during stacking and heating process in Stage I and II. *Daqu* was transferred to the storage room for stacking and fermentation again after 10 d, thus the temperature decreased slowly at the initialization of stage III but rapidly decreased to room temperature later. Acidity and amino nitrogen content showed an overall slow increasing trend. Glucoamylase activity reached a maximum value of 1713.41 ± 479.17 U/g at 1 d, then rapidly decreasing to the end (Fig. 2B). Amylase activity showed a general trend of decreasing and then increasing, and suddenly decreasing to 1.29 ± 0.12 U/g at 62 d.

3.2. Microbial diversity profiles during daqu process

In terms of 16S rRNA and ITS gene sequencing data, 2,602,934 and 3,095,151 high-quality reads were obtained in total (average length 1465 and 741 bp). After removing chimeras and individuals, 2,017,263 and 2,128,660 reads were clustered into 2458 and 2212 ASVs respectively based on 100% similarity.

The abundance and diversity of microorganisms were expressed by Chao1 and Shannon index, respectively. The microbial growth and metabolism were more active at the beginning of fermentation, with bacterial diversity, fungal diversity and richness increasing rapidly, then gradually decreasing and leveling off (Fig. S2). Bacterial richness fluctuated greatly throughout fermentation process.

Based on the characteristics of microbial community succession, the fermentation process could be divided into three stages (0–4 d, 4–11 d and 11 d-end) (Fig. S3). CPCoA and ADONIS/ANOSIM analyses showed significant differences ($p < 0.01$) in *daqu* microbial structure at different stages (Table 1, Fig. S3). The β-NTI index and neutral model analysis (NCM) were calculated to investigate in microbial formation process in *daqu* (Sloan et al., 2006). The construction of the *daqu* microbiota was influenced by both deterministic and stochastic processes, and the contribution of deterministic processes to bacterial and fungal composition was 56.63% and 44.09%, respectively (Fig. S4).

3.3. Structural succession of microbial communities in daqu

3.3.1. Structure of prokaryotic communities

At the genus level, the dominant bacteria were *Acetobacter, Leuconostoc,* lactic acid bacteria (LABs)*, Staphylococcus, Thermoactinomyces, Weissella* and *Ralstonia* (Fig. S5A). Initial bacteria in *daqu* mainly consisted of *Acetobacter, Levilactobacillus* and *Companilactobacillus*. Dominant genera became *Leuconostoc* and *Weissella* in 2–4 d. *Staphylococcus* and *Thermoactinomyces* showed dominance in later stages. Notably, sample in 8 d differed from others, with *Herbaspirillum* and *Ralstonia* as main genera.

At the species level, the initial bacteria mainly contained *Acetobacter* sp. *HBB7* and *Companilactobacillus paralimentarius* (Fig. 3A). *Weissella confusa* and *Leuconostoc citreum* increased significantly in abundance, becoming dominant species in 1–4 d. As the temperature increased, *Thermoactinomyces daqus/vulgaris* turned into dominant species after stage II.

3.3.2. Structure of eukaryotic community

The main fungal genera during fermentation were *Pichia, Saccharomyces, Saccharomycopsis, Lichtheimia, Rhizomucor* and *Rhizopus* (Fig. S5B). Dominant genera gradually changed from *Saccharomyces* and *Pichia* to *Rhizomucor* and *Saccharomycopsis* from 0 to 6 d. After 8 d,

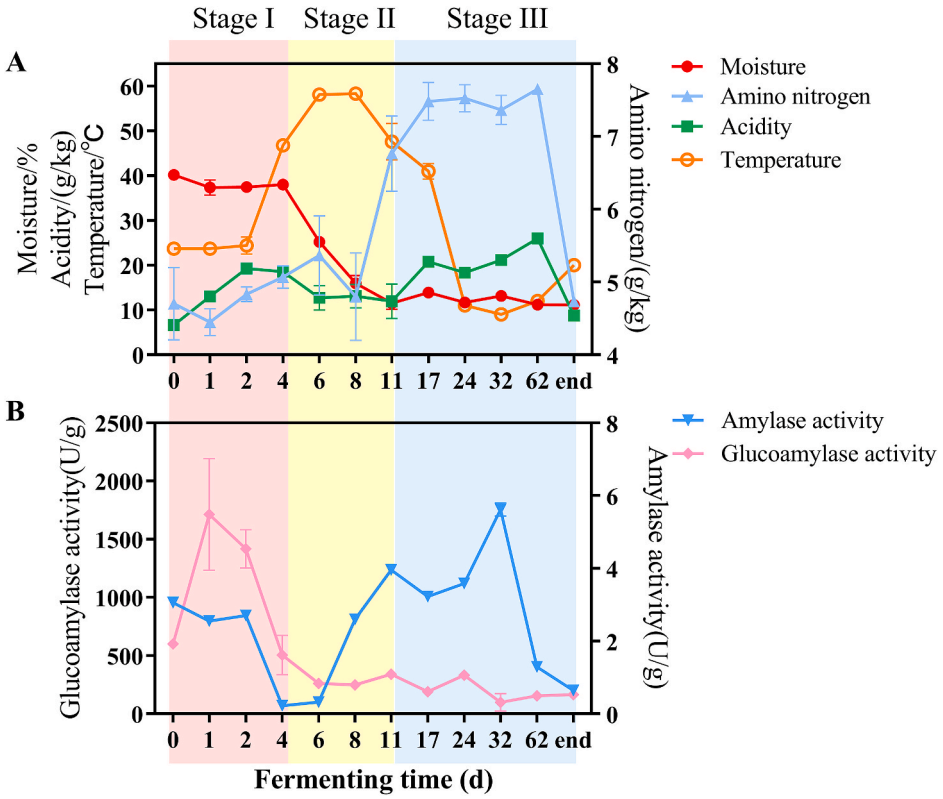

Fig. 2. Dynamics of physicochemical indexes (A), amylase and glucoamylase activities (B) in mechanically strong-flavor *daqu* throughout fermentation.

Table 1
ADONIS/ANOSIM analyses to test for differences in microbial communities of three phases in mechanically strong-flavor *daqu*.

	ANOSIM		ADONIS	
	R	P	R^2	P
Bacteria	0.4746	0.001	0.1547	0.001
Fungi	0.5685	0.001	0.3090	0.001

proportion of *Rhizopus* increased significantly but increased significantly after 32 d, turned dominant fungi together with *Saccharomycopsis* and *Thermomyces*.

Dominant species changed from *Pichia kudriavzevii* to *Saccharomycopsis fibuligera*, *Wickerhamomyces anomalus* and *Rhizomucor Pusillus* from stage I–II (0–6 d) (Fig. 3B). In addition, *Rhizopus oryzae/arrhizus* were also the dominant species in *daqu* in stage III.

3.4. Microbial diversity profiles in daqu producing environment

A slight increase could be observed in microbial diversity and abundance in silo wheat compared to raw wheat (Fig. S6). For *daqu* brick making process, all Alpha indices showed a decrease after the wheat milling step, except for fungal richness. Generally, bacterial richness, fungal diversity and richness indices all obviously decreased after addition of mature *daqu* powder and water, with latter two changes being significant ($p < 0.01$). All indices in the finished *daqu* have increased compared to the initial fermentation. Alpha indices of fungi varied in different processing and fermentation rooms ($p < 0.05$), while bacterial diversity showed no significant difference. Air diversity and abundance were higher in fermentation/storage rooms than in brick process room and the warehouse, however, all indices, except bacterial diversity, decreased or only slightly increased on the floor and walls in these locations (Table S2). For the equipment surface samples, the highest microbial diversity and abundance were detected in the silo and bucket elevator tubes. It is worth mentioning that the bacterial indices in storage room shelves obviously reduced compared to the fermentation and brick process rooms, while the fungal indices tended to increase.

3.5. Microbial structure in daqu producing environment

3.5.1. Structure of prokaryotic communities

Bacterial composition of the air in *daqu* producing places has undergone significant changes as fermentation proceeded, with increasing proportion of dominant species in *daqu* (0–4 d) such as *Ralstonia* sp., *Acetobacter* sp. *HBB7* and *Lactococcus garvieae*. External bacterial composition of the air was significantly different from that in warehouse ($p < 0.05$) (Fig. S7). Compared with the raw wheat, the percentage of *Staphylococcus saprophyticus* decreased in the wheat in silo, while *Pseudomonas fluorescens*, *Ralstonia* sp. and *Sphingomonas* sp. increased. It's estimated that the environment inside the silo with high temperature and low moisture could significantly improve the bacterial composition of the raw material, thus affecting the fermentation process of the *daqu*. *Acetobacter* sp. *HBB7* was enriched in moistened and mixture wheat, probably originating from the air in *daqu* brick processing room. *Latilactobacillus curvatus* was distributed in moistened wheat, grain rinsing conveyor surface and shelf. Dominant species in the shelves in fermentation/storage room, such as *S. saprophyticus* and *Ralstonia* sp. were also abundant in *daqu* fermentation process (8–32 d).

3.5.2. Structure of eukaryotic community

Fungal composition in air inside the workshop was partially coincident with the dominant species during *daqu* fermentation, including *Rhizopus microsporus/arrhizus/oryzae*, *Lichteimia ramosa*, *Mucor racemosus*, *P. kudriavzevii* and *S. fibuligera* (Fig. S8). Relative abundance of *Aspergillus penicillioides* and *Epicoccum nigrum* in the wheat in the silo increased significantly compared to the raw wheat, probably enriching from the silo tubes. *P. kudriavzevii* and *W. anomalus* as mainly initial fungi in *daqu* was also enriched in the air and tool surfaces in brick

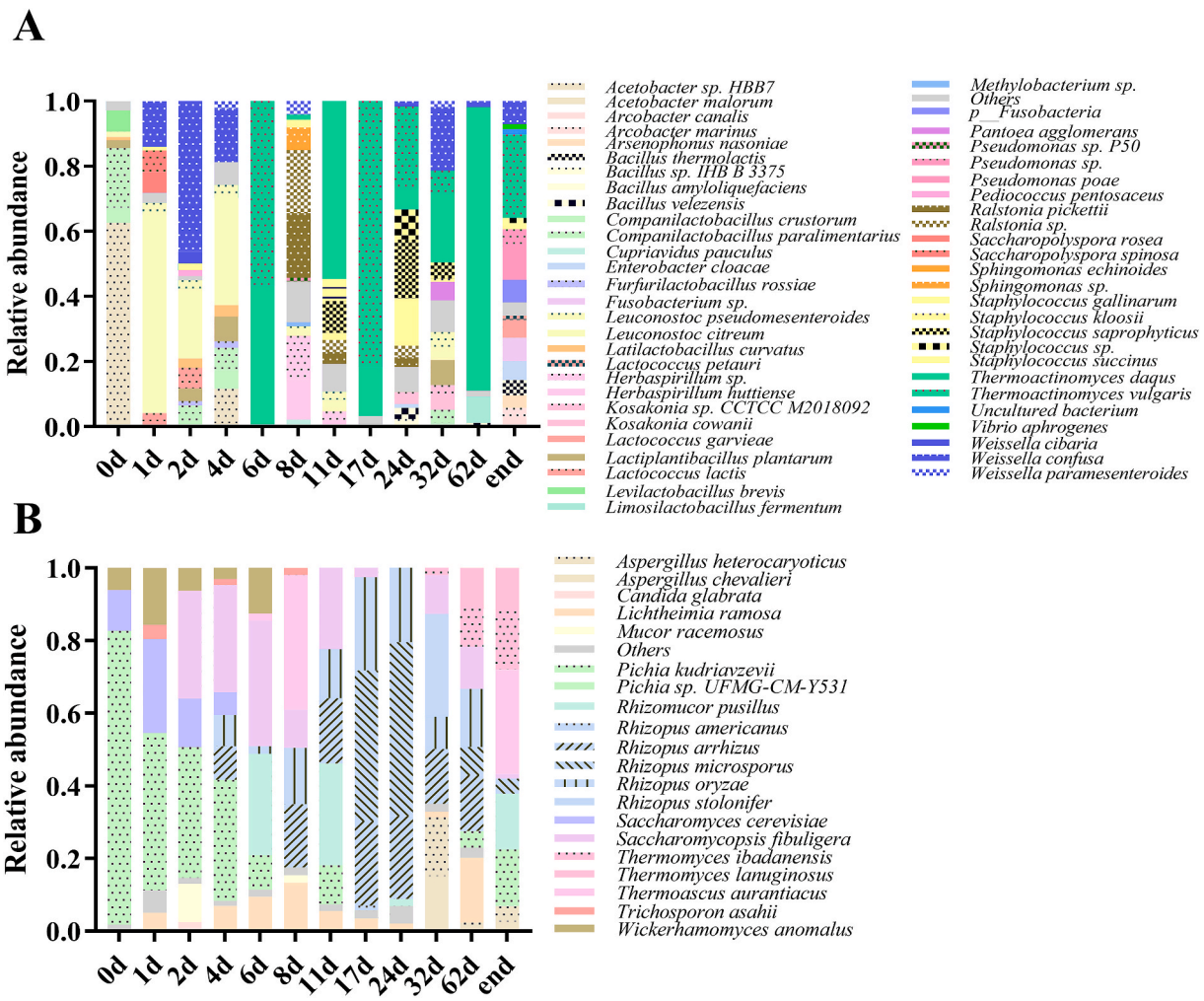

Fig. 3. Bacterial (A) and fungal (B) community structure in *daqu* at species level. Species with relative abundance less than 1% were classified as others.

processing room. It is worth mentioning that the abundance of *P. kudriavzevii* rose significantly in moistened and mixture wheat compared to milled wheat, which was estimated to originate from the equipment surface and mature *daqu* powder. Shelves in both the fermentation and storage room were dominated by *S. fibuligera*, and *R. oryzae/arrhizus*, which were also the main species during fermentation. The fungal communities in the air and on the floor of different rooms were significantly different.

3.6. Contribution of raw material and environment to the microbial structure of daqu by source tracking analysis

Source tracking was applied to analyze the influence of environment and raw materials on the *daqu* brick processing and fermentation. Microorganisms in milled wheat were mainly from raw wheat and bucket elevator tubes (Fig. 4A and B). Mature *daqu* powder and processing environment had a great impact on microbial community during *daqu* bricks production and early stage of fermentation. Bacteria in moistened/mixture wheat and 0 d *daqu* mainly originated from the air in brick processing room and mature *daqu* powder, while fungi in mixture wheat and 0 d *daqu* mainly came from the surface of grain rinsing conveyor. Interestingly, the floor in brick processing room contributed 16.93% of the bacteria in moistened wheat, probably resulting from mature *daqu* powder scattered on the floor. For the finished product, mature *daqu* powder contributed 93.25% of the fungi and 58.87% of the bacteria. Meanwhile, the floor in the fermentation room was also the main source of bacteria even without contact, probably due to the scattering of powder on the floor.

Interactions between main microorganism during *daqu* production and fermentation were analyzed (Fig. 4E). A total of 28 nodes and 88 edges were obtained. Overall, microbial interactions were complex and more edges were found in fungi. Among the co-occurrence networks, *Aspergillus heterocaryoticus*, *C. paralimentarius* and *L. citreum* were relatively more closely related to other microorganisms. Species assigned to *Rhizopus* were negatively correlated with most LABs. Significant positive correlations were found between thermophilic microorganisms such as *Thermomyces lanuginosus/ibadanensis* and *Thermoascus aurantiacus*. *Alternaria infectoria* was negatively correlated with other species.

We further analyzed the effect of environment and raw materials on the main microorganisms at the beginning of the fermentation and in the finished product. The mature *daqu* powder and the air in brick processing room supplied the main microorganisms in 0 d sample, including *Levilactobacillus brevis*, *C. paralimentarius* and *P. kudriavzevii* (Fig. 4C). Among all, the air outside the workshop was also the main source of *Acetobacter* sp. *HBB7*, which may be brought into production through the raw material. *T. vulgaris*, *Pseudomonas poae*, *W. confuse*, *Enterobacter cloacae* and *L. garvieae* in the finished *daqu* were mostly contributed by mature *daqu* powder and processing environment, including raw material transport tubes, the brick production equipment and storage room environment (Fig. 4D). For the fungi, surface of grain rinsing conveyor and mature *daqu* powder provided most of *T. aurantiacus*, *T. ibadanensis/lanuginosus*, *P. kudriavzevii* and *R. pusillus* in the finished *daqu*.

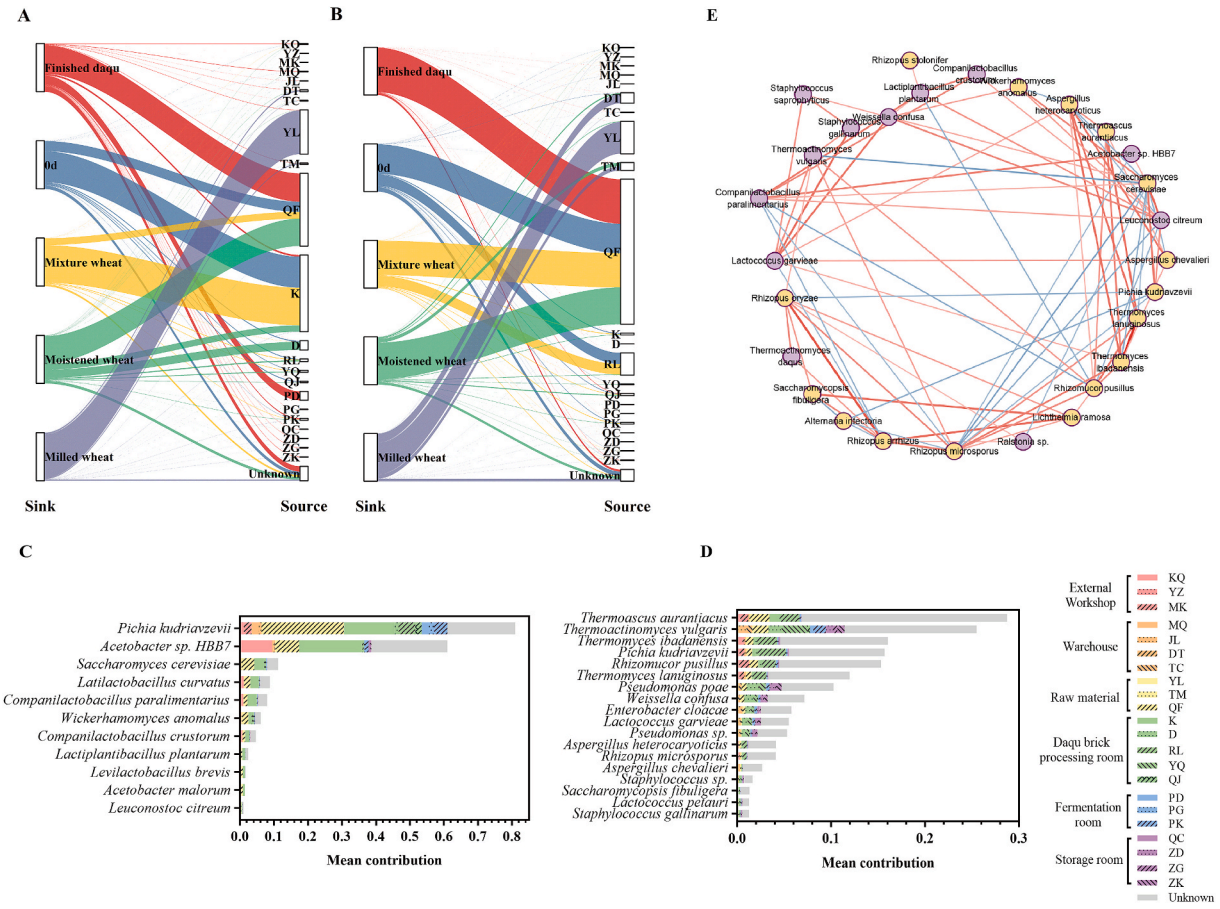

Fig. 4. Contribution of environment to bacterial (A) and fungal (B) structure in samples during *daqu* brick processing and mature *daqu* by source tracking analysis. Source tracking analysis showed the environmental source of main microorganisms (relative abundance > 1%) in (C) 0 d *daqu* brick and (D) finished *daqu*. (E) Co-occurrence network of main microorganisms (relative abundance ≥ 0.5%) during *daqu* brick processing and fermentation. The purple nodes indicate bacteria while the orange ones represent fungi. Red and blue edges indicate positive and negative interactions between nodes. The edge widths indicate the absolute value of Spearman's r correlation. Only |r| ≥ 0.3 and p ≤ 0.05 correlations were shown in the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Correlation between daqu microbial communities and physicochemical parameters

Mantel test was used to describe the relationship between *daqu* microorganisms and physicochemical parameters. The results showed that bacterial communities were positively correlated with glucoamylase activity, moisture content, amino nitrogen and temperature ($p < 0.05$), while fungal communities were positively correlated with temperature, moisture content and amino nitrogen ($p < 0.05$) (Table 2). CCA analysis was conducted to further investigate the contribution rate of physicochemical factor on *daqu* community formation. Bacterial community was highly influenced by moisture content and temperature, with explaining rate of 25.9% and 17.0%, mainly driving bacterial community in 0–4 d and 11d-end samples (Fig. 5). Temperature and moisture content explained 19.8% and 34.6% of fungal community changes respectively in 0-11d samples.

Table 2
Mantel test analysis on the fermentation process of mechanically strong-flavor *daqu* with physicochemical factors based on Spearman correlation. $p < 0.05$ indicates significant correlation.

	Bacteria		Fungi	
	Mantel's r	p-value	Mantel's r	p-value
Glucoamylase activity	0.3693	0.012	0.2754	0.053
Amylase activity	-0.3524	0.99	-0.2172	0.91
Moisture	0.5270	0.0030	0.4931	0.0040
Acidity	0.05274	0.34	0.05188	0.34
Amino nitrogen	0.1688	0.12	0.4404	0.013
Temperature	0.3693	0.0090	0.4763	0.015

4. Discussion

Daqu is used as saccharifying and fermenting agent in the production of Baijiu and Chinese vinegar (Zheng et al., 2012), and can be classified into sauce-flavor, strong-flavor and light-flavor *daqu* according to flavor type of liquor. The functional microorganisms in *daqu* provide enzymes and flavor precursors for the fermentation of liquor (Sakandar et al., 2020). *Daqu* is produced in an open environment, enriching a variety of microorganisms from raw materials and processing environment, which can significantly influence the microbial community in *daqu* and ultimately shape the characteristics of the product (Guan et al., 2021; Hu, Huang, et al., 2021). Currently, mechanical technology has been widely applied in food industry. However, process improvements may affect the quality of traditional brewed food products (Gao et al., 2019, 2020b; Hu, Yang, et al., 2021). There have been no reports on the production process and the environmental microbial formation mechanism of mechanically strong-flavor *daqu*. In this study, we analyzed the mechanisms and the source of the microbial construction in *daqu* by SMRT sequencing and source tracking analysis.

Ninety-seven bacteria and fourty-six fungi species were identified in our study. Reprnsetative genera in mechanically strong-flavor *daqu* included *Staphylococcus*, LABs, *Bacillu*, *Weissella*, *Leuconostoc*,

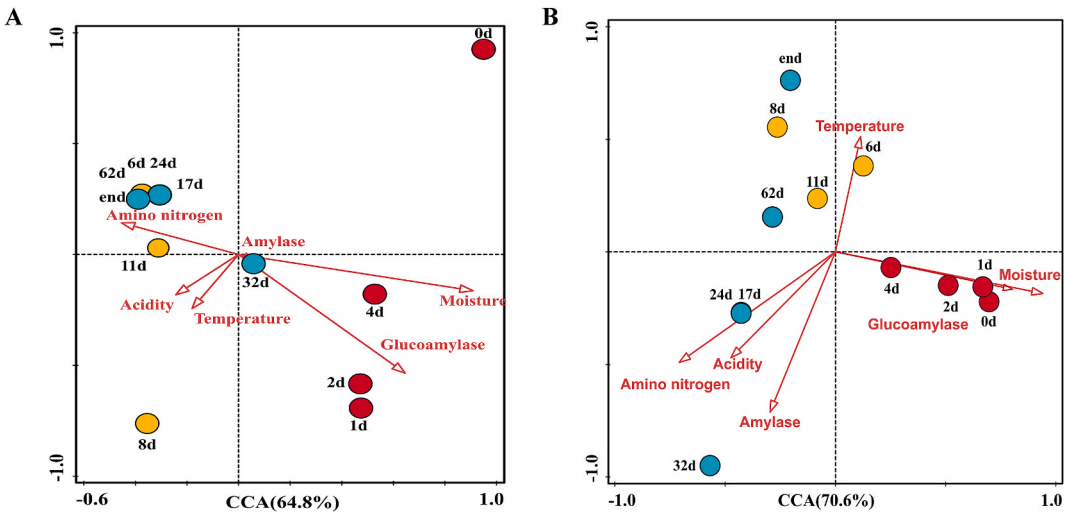

Fig. 5. CCA Analysis showed the correlation between bacterial (A) and fungal (B) structure with environmental factors.

Thermoactinomyces, *Aspergillus*, *Thermoascus*, *Thermomyces*, *Saccharomycopsis*, *Rhizomucor*, *Rhizopus* and *Pichia*, also detected in traditional strong-flavor *daqu* previously (Gou et al., 2015). Twelve bacterial and nine fungal genera were firstly detected in strong-flavor *daqu*, mainly including LABs (*Companilactobacillus*, *Lactiplantibacillus*, *Levilactobacillus* and *Furfurilactobacillus*), *Trichosporonaceae* (*Trichosporon*, *Cutaneotrichosporon*), *Herbaspirillum*, *Ralstonia* and *Hansenia*. These newly discovered LABs were usually found in fermented vegetables, sourdough or spoiled beer, dominating at the beginning of fermentation (0–4 d), producing large amounts of organic acids through EMP and other pathways using glucose as raw material together with *Acetobacter*. These organic acids can inhibit the growth of spoilage microorganisms to some extent, while causing rise in the acidity in stage I, also leading to sour smell in *daqu* (Gullo et al., 2009; Rojan et al., 2005; Yang et al., 2021; Zhao et al., 2022; Zheng et al., 2020). *Ralstonia pickettii* were often detected in soil, water and plants, propably brought into *daqu* through wheat (Stelzmueller et al., 2006). *Hanseniaspora uvarum* is commonly observed in grape and wine fermentation, used as fermentation starter along with *Saccharomyces cerevisiae* (Albertin et al., 2016; Pietrafesa et al., 2020). Emergence of differential species may result from changes in the production environment. The SMRT sequencing used in this study allowed the identification of more microorganisms and their annotation to the species level.

Changes in acidity and enzyme activity during the fermentation of mechanically strong-flavor *daqu* were somewhat different from traditional one, indicating that production methods had a great impact on the internal physicochemical environment in *daqu* (Zhu, Zheng, et al., 2022). The moisture content was high (>37%) at stage I (0–4 d), being suitable for the growth of most microorganisms with low temperature, with a rapid increase in diversity index (Fig. 1, Fig. S2). The stacking process, confined environment and bioheat generated by microorganisms in the beginning led to a rapid increase in the temperature to above 55 °C. Thereafter, growth of *Pichia*, *Saccharomyces* and *Leucanostoc*, brought in by the wheat and mature *daqu* powder, was significantly inhibited, decreasing rapidly in abundance, while thermophilic bacteria such as *Rhizopus*, *Thermoactinomyces* and *Ralstonia* started to prosper (Fig. S5). In stage II, genus such as *Rhizomucor* began to use organic acids and produce ester through esterification reactions, thus showing a decreasing trend in acidity. Relative abundance of genera such as *Rhizopus* and *Rhizomucor* increased obviously, synthesizing a large amount of glucoamylase. However, ultimate temperature also led to a decrease in glucoamylase activity (López-Belmonte et al., 1997; Suntornsuk & Hang, 1994; Wang et al., 2020). CCA and Mantel test analysis also proved that temperature and moisture content were the main drivers of microbial succession (Fig. 5). It is worth mentioning that the amino nitrogen content increased significantly after 8 d, probably due to the rise of *Rhizopus*, which can synthesize proteases, hydrolyze proteins and release free amino acids (Chen et al., 2009). After 11 d, *daqu* was transferred to storage room and cooled down by turning process. At this time, *daqu* microorganisms secreted α-amylase, glucoamylase, endoglucanase, β-glucosidase and pyruvate dehydrogenase to convert starch, cellulose and protein into unique flavor substances and glucose, etc. Thus, the amino nitrogen content and amylase activity showed a certain degree of recovery in later stage (Chen et al., 2020; Gao et al., 2021; He, Dong, et al., 2019).

In order to further investigate the internal relationships between the microbiota in *daqu* fermentation and production environments, Upset diagrams were used to illustrate the ASVs specific or common to the different sample groups (Fig. 6). The highest microbial diversity was found in the processing environment (505/147 ASVs), followed by raw materials (89/44 ASVs) and the fermentation process (87/39 ASVs). The most abundant unique ASVs were found in processing environment (432/103 ASVs), indicating most species occupied unique ecological positions in the workshop. Nine bacterial and five fungal genera were shared by all groups. Dominant yeasts (*P. kudriavzevii*, *W. anomalus*), *L. citreum* and LABs (*C. crustorum/paralimentarius*, *Furfurilactobacillus rossiae*, *Lactiplantibacillus plantarum*, *L. brevis* and *L. curvatus*) were detected in all samples. Particularly, *A. infectoria* and *Fusarium graminearum* usually appeared in crops such as wheat and corn, which were associated with cellulose degradation in cereals and wheat blast disease (Castañares et al., 2021; Xie et al., 2022). These results suggest that fermenting microorganisms are brought in by raw materials and mature *daqu* powder, and simultaneously modifying the distribution of environmental microorganisms during the production. It is worth mentioning that 13 bacteria and 3 fungal species occurred only in the processing environment and fermentation process, including *E. cloacae*, *Kosakonia cowanii*, *Pantoea agglomerans*, *Sphingomonas* sp., *M. racemosus* and *R. oryzae*, which were the dominant microorganisms during fermentation, mainly distributed on the equipment surface and tubes. In addition, pathogenic bacteria such as *Cupriavidus pauculus*, *Enterococcus faecalis* were also detected in the finished product and processing environment, especially in fermentation and storage rooms (Balish & Warner, 2002; Inkster et al., 2021). The above results emphasized the essential influence of the processing environment on *daqu* fermentation and the possible sources of pollution.

SourceTracker has been widely used for microbial source tracing in food processing, including brewing (Bokulich et al., 2015; Wang et al., 2021), fermented fruits (Penland et al., 2021), dairy products (Bokulich

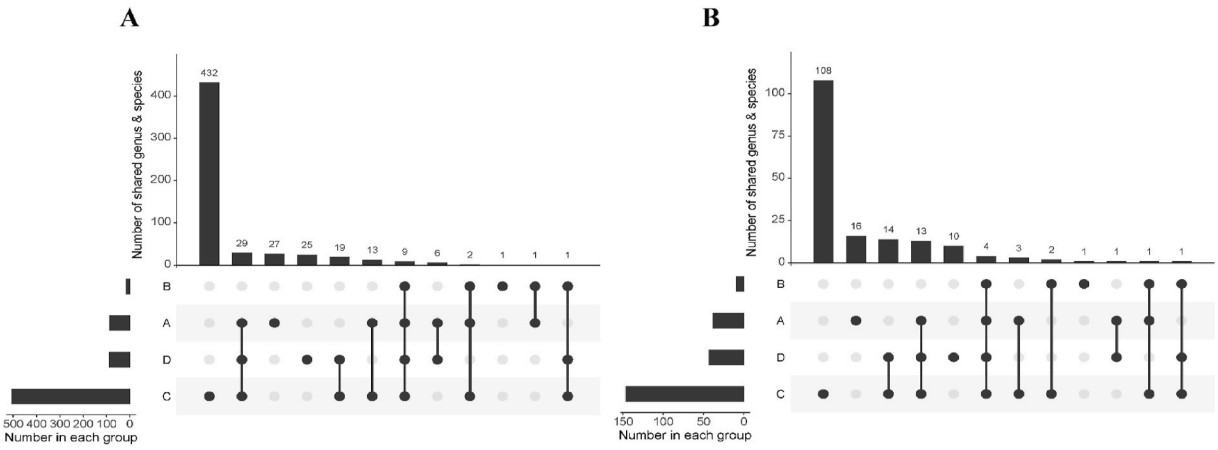

Fig. 6. The Upset plot showed common (A) bacterial and (B) fungal genera and species shared by samples in different groups. Group A: *Daqu* fermentation process. Group B: *Daqu* brick processing. Group C: internal process environment. Group D, raw material.

Nicholas & Mills David, 2013; Doyle Conor et al., 2016) and meat processing (Zwirzitz et al., 2020). Our results showed that microorganisms in milled wheat mainly came from raw wheat and bucket elevator tubes. It's noteworthy that the silo wheat showed significant changes in microbial communities compared to the raw wheat due to high temperature and low moisture in silo, while these differences did not have a significant impact on *daqu* production. However, mature *daqu* powder started becoming the main source of microorganisms in the subsequent samples once being addicted. At the beginning of fermentation, mature *daqu* powder, the air and grain rinsing conveyor in brick processing room contributed the LABs, yeast and *Acetobacter* in *daqu*. LABs can inhibit spoilage bacteria by secreting bacteriocins (Chen et al., 2021), and produce a variety of metabolites together with *Acetobacter*, increasing the sweetness and reducing pungency in Baijiu (Liu et al., 2017). These microorganisms produced bioheat by degrading sugars and proteins, leading to a gradual rise in temperature (Li, Lin, Liu, Wang, & Luo, 2016), thus limiting the growth of LABs and yeast (Fig. 4). Thermophilic bacteria such as *Rhizopus* gradually took dominance, producing heat-resistant and carbohydrate-active enzymes for liquor fermentation (Huang et al., 2017). In addition, almost all microorganisms in the finished product originated from mature *daqu* powder (>55%). The high proportion of unknown sources in the source tracking results of some species may be related to the sample collection and sequencing depth (Zwirzitz et al., 2020). Meanwhile, the results of NCM/β-NTI analyses also indicated *daqu* microbiota was determined by a combination of probabilistic distribution and biotic/abiotic factors (Fig. S4). The above results confirm that raw materials and environment mainly affected the early fermentation in *daqu*. Addition of mature *daqu* powder had a decisive role in microbial enhancement in *daqu*.

5. Conclusion

In summary, mature *daqu* powder and producing environment were the main sources of the initial microorganism in mechanical *daqu*, and mature *daqu* powder were almost the only source of microorganisms in the end. This study showed that adding mature *daqu* powder to *daqu* brick and production environment could promote suitable environment for strong-flavor *daqu* fermentation. This work explained the reason for the stable quality of mechanical *daqu*, providing a reference for the new *daqu* workshop construction.

CRediT author statement

Shuangping Liu: Funding acquisition, Project administration, Writing – review & editing. Yu Zhou: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. Dongna Ma: Data curation, Writing – review & editing. Suyi Zhang: Data curation, Writing – review & editing. Yi Dong: Data curation, Writing – review & editing. Xiu Zhang: Data curation, Writing – review & editing. Jian Mao: Funding acquisition, Supervision, Writing – review & editing.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102467.

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