

# Microbial interactions in mixed-species biofilms on the surfaces of *Baijiu* brewing environments



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## ABSTRACT

Environmental microorganisms commonly inhabit dense multispecies biofilms, fostering mutualistic relationships and co-evolution. However, the mechanisms underlying biofilm formation and microbial interactions within the *Baijiu* fermentation microecosystem remain poorly understood. Hence, the objective of this study was to investigate the composition, structure, and interactions of microorganisms residing in biofilms on environmental surfaces in *Baijiu* production. The results revealed a shift in the bacteria-fungi interaction network following fermentation, transitioning from a cooperative/symbiotic relationship to a competitive/antagonistic dynamic. Core microbiota within the biofilms comprised lactic acid bacteria (LAB), yeast, and filamentous fungi. From the environmental surface samples, we isolated two strains of LAB (*Lactiplantibacillus pentosus* EB27 and *Pediococcus pentosaceus* EB35) and one strain of yeast (*Pichia kudriavzevii* EF8), all displaying remarkable biofilm formation and fermentation potential. Co-culturing LAB and yeast demonstrated a superior capacity for dual-species biofilm formation compared to mono-species biofilms. The dual-species biofilm displayed a two-layer structure, with LAB in the lower layer and serving as the foundation for the yeast community in the upper layer. The upper layer exhibited a dense distribution of yeast, enhancing aerobic respiration. Metabolic activities in the dual-species biofilm, such as ABC transporter, oxidative phosphorylation, citric acid cycle, sulfur metabolism, glycine, serine, threonine metabolism, lysine degradation, and cysteine and methionine metabolism, showed significant alterations compared to LAB mono-species biofilms. Moreover, bacterial chemotaxis, starch, and sucrose metabolism in the dual-species biofilm exhibited distinct patterns from those observed in the yeast mono-species biofilm. This study demonstrated that a core microbiota with fermentation potential may exist in the form of a biofilm on the surface of a *Baijiu* brewing environment. These findings provide a novel strategy for employing synthetic stable microbiotas in the intelligent brewing of *Baijiu*.

## 1. Introduction

Environmental microorganisms commonly reside in biofilms, which are dense multispecies communities (Nadell et al., 2016). Biofilms are a widespread microbial survival strategy, with an estimated 40–80 % of microbial cells worldwide living in biofilms (Flemming and Wuertz, 2019). Compared to planktonic cells, biofilm structures provide enhanced stability and tolerance for microbiota in ecosystems (Rabin et al., 2015; Yao et al., 2022). Biofilms exhibit a distinct spatial

organization, and the arrangement of different strains and species within them significantly impacts the relative fitness benefits of cooperative and competitive phenotypes (Nadell et al., 2016). Food fermentation primarily takes place among microorganisms coexisting in multispecies biofilms (Yao et al., 2022). Recently, there has been a growing interest in mixed-species biofilms within the food industry. Yeast and bacterial strains have the ability to form metabolically active biofilms, facilitating efficient initiation of alcoholic and malolactic fermentation in wine (Palud et al., 2024). Moreover, the mixed-species

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biofilm of *Saccharomyces cerevisiae* and *Lactiplantibacillus plantarum* has displayed promising characteristics as an immobilized cell system for ethanol fermentation, exhibiting high ethanol productivity and robustness (Abe et al., 2013).

The *Baijiu* brewing process, known for its open solid-state fermentation, provides an ideal environment for the development and growth of diverse mixed-species biofilms. This is attributed by the presence of varied environmental conditions and a wide range of microbial populations involved in *Baijiu* production (Tan et al., 2022). Spontaneous fermentation relies heavily on the indigenous microbiota present in the environment or used raw materials (Du et al., 2019; Pang et al., 2021; Pang et al., 2018; Pang et al., 2020). Environmental microorganisms play an important role as a microbial source in *Baijiu* brewing. Previous research indicates that a significant proportion, ranging from 62.61 % to 90.90 %, of bacteria and 20.00 % to 38.94 % of fungi involved in the fermentation of light-flavor *Baijiu* mainly originate from the ground and surfaces of tools within the production environment (Wang et al., 2018). In long-term continuous, multi-round open fermentation systems, environmental microorganisms undergo adaptations and evolution as a response to changes in biotic and abiotic factors. For instance, commercial wine yeast strains have been found to form biofilms and adhere to soft plastics of commonly used winery hoses, thereby impacting the indigenous microbiota profile (Tek et al., 2018). Similarly, *Oenococcus oeni*, when existing in a biofilm lifestyle on various winemaking surfaces such as stainless steel and oak chips, contributes to the enhancement of malolactic fermentation outcomes and imparts aromatic properties to wine (Tofalo et al., 2021). The presence of biofilms plays a vital role in establishing a stable brewing microecology, which can resist adverse environmental conditions and improve fermentation quality. Therefore, it is important to investigate the composition and structure of the multispecies biofilm formed by environmental microorganisms.

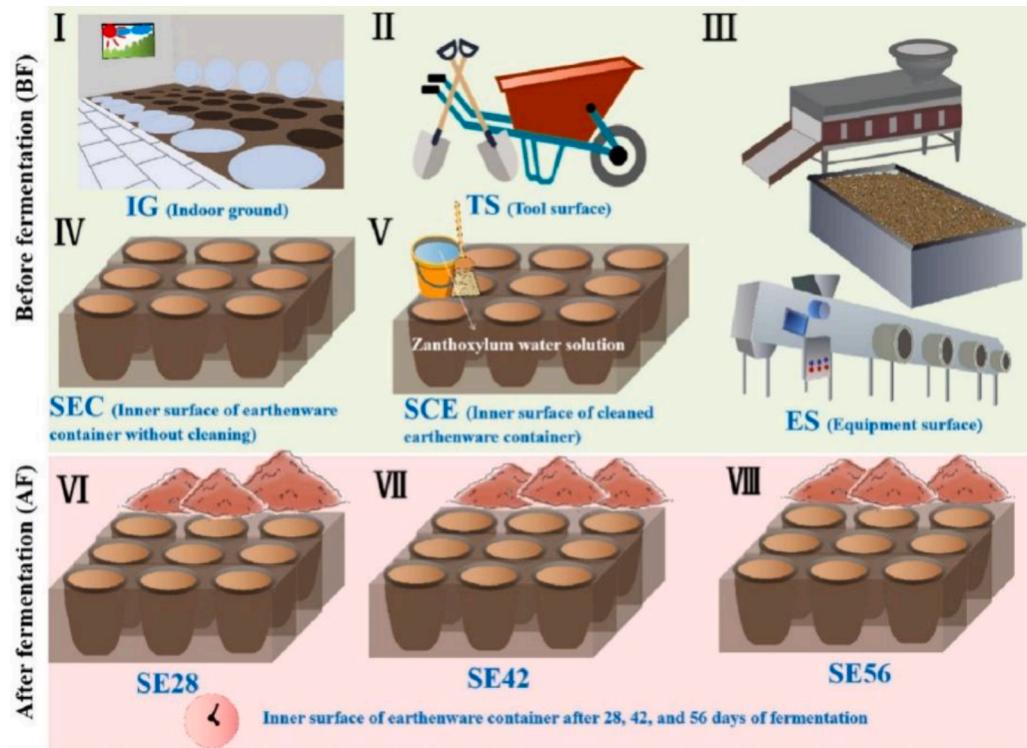
Previous studies have investigated the biofilms present in *Daqu* (fermentation starter) and fermented grains, which are essential

elements of the *Baijiu* brewing system. Fan et al. (2020) identified biofilms formed by non-cultivable lactic acid bacteria (LAB) and *S. cerevisiae* in *Daqu*, allowing them to thrive despite limited nutrients. Zeng et al. (2023) examined the biofilm-producing microbes present in the fermented grains of strong-flavor *Baijiu*. They found that biofilms can enhance the diversity of esters while simultaneously inhibiting their content. Nevertheless, limited research has explored on the structure and interactions of multispecies biofilms on different environmental surfaces during *Baijiu* production, despite these surfaces serving as natural adhesion sites. Therefore, this study aims to analyze the microbial community and core microorganisms on environmental contact surfaces at various stages of light-flavor *Baijiu* production using high-throughput sequencing technology. Additionally, environmental microorganisms will be isolated and screened for their biofilm-forming capacity and fermentation potential. Finally, we will characterize the capacity of environmental bacteria and fungi to form mixed-species biofilms and analyze their metabolic interactions within the biofilm.

## 2. Materials and methods

### 2.1. Experimental design and sample collection

Environmental surface samples were collected from a well-known light-flavor *Baijiu* manufacturer in Shanxi Province, China. The manufacturer performed a traditional brewing process of light-flavor *Baijiu*, with sorghum as raw material and low-temperature *Daqu* as starter for two rounds of fermentation process in large earthenware containers (Kang et al., 2022a). The study included a total of 8 sampling sites, as illustrated in Fig. 1. These sites included indoor ground, tool surfaces, equipment surfaces, and the inner surface of earthware containers before and after cleaning, as well as the inner surface of earthware containers after fermentation at different fermentation time points (28, 42, and 56 days). The samples were labeled as IG, TS, ES, SEC, SCE,



**Fig. 1.** Process diagram illustrating the sampling sites within the *Baijiu* production environment. The abbreviations used for the sampling sites are as follows: IG (Indoor ground), TS (Tool surface), ES (Equipment surface), SEC (Inner surface of earthenware container without cleaning), SCE (Inner surface of cleaned earthenware container), SE28 (Inner surface of earthenware container after 28 days of fermentation), SE42 (Inner surface of earthenware container after 42 days of fermentation), and SE56 (Inner surface of earthenware container after 56 days of fermentation).

SE28, SE42, and SE56, respectively.

To collect samples from direct contact surfaces such as indoor grounds and inner surface of earthware containers, nine sampling points were selected within each workshop. From these points, three earthenware containers were selected for swabbing and scraping. Also, nine samples included equipment and tool surfaces involved in the pre-treatment of sorghum and *Daqu*, as well as the transportation process of fermented grains. These samples were obtained by swabbing the contact surfaces according to the actual production. Environmental surface samples were collected using a 3 M Swab-sampler (3 MTM, United States). The swab was used to scrape a 10 cm × 10 cm area horizontally and vertically, ensuring 10 repetitions of each. Afterward, the swab was placed in a neutralizing buffer and agitated. The collected samples from the same workshop within each group were mixed and labeled. All 24 samples were then sent to the laboratory for further analysis. Each set of samples was divided into two portions: one was preserved at -80 °C for high-throughput sequencing, and the other was used to isolate and identify strains within 24 h of sampling.

## 2.2. Microbial community structure analysis

The total DNA of microbial communities on environmental surfaces was extracted using an improved CTAB method, following the protocol described by Huang et al. (2020). The quality of the extracted DNA was assessed through 1 % agarose gel electrophoresis. For bacteria, the V3-V4 domains of the 16S rRNA genes were amplified using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). For fungi, the ITS region of each sample was amplified using the forward primer ITS5F (5'-GGAAGTAAAGTCGTAACAAGG-3') and the reverse primer ITS1R (5'-GCTCGTTCTTCATCGATGC-3'). The PCR products were purified and then subjected to paired-end sequencing on the NovaSeq 6000 PE250 platform (Illumina, San Diego, CA, USA). The raw sequencing data were submitted to the NCBI SRA database with the BioProject accession number PRJNA933955.

The sequencing reads were clustered into Amplicon Sequence Variants (ASVs) using the "uniose3" plugin in USEARCH11. The representative sequences of bacterial ASVs were compared against the SILVA 138.1 and EZBioCloud databases available at <https://www.ezbiocloud.net>. Similarly, the representative sequences of fungal ASVs were compared against the UNITE 8.2 database and the Central Bureau of Fungal Cultures database (CBSKNAW) accessible at <https://www.wi.knaw.nl>. The "core diversity" plugin in QIIME2 was used to calculate alpha and beta diversity indices.

## 2.3. Isolation and screening of environmental bacteria and yeasts with excellent biofilm formation ability and fermentation potential

According to the method previously described by Kang et al. (2021), bacteria and yeasts present in the environmental samples were isolated and identified. A total of 40 strains of bacteria (15 genera and 28 species) and 10 strains of yeasts (5 genera and 7 species) were obtained. These strains were identified using primers for the 16S rRNA and ITS or 26S rRNA genes. The resulting phylogenetic tree are shown in Fig. S1 (bacteria) and Fig. S2 (yeast).

To evaluate the biofilm formation capacity of the bacterial and yeast isolates, a semi-quantitative determination of crystal violet in 96-well plates was used, as described by Kang et al. (2020). The broth-only wells were used as negative control. The cutoff value (ODc) for biofilm formation capacity was determined by calculating the mean of the negative controls (OD<sub>negative</sub>) plus three standard deviations (SDs). Weak biofilm formation capacity was represented by ODc ≤ OD ≤ 2 × ODc, moderate biofilm formation capacity by 2 × ODc ≤ OD ≤ 4 × ODc, and strong biofilm formation capacity by OD ≥ 4 × ODc, following the criteria established by Diaz et al. (2016). Fig. S3 illustrates the results of this assessment, showing that a total of 14 strains (10 bacteria and 4

yeasts) exhibited a strong biofilm formation capacity.

Subsequently, we evaluated the fermentation characteristics of these 14 strains, including their acid and alcohol tolerance (Huang et al., 2021), esterase activity (Huang et al., 2021), acid protease activity, and beta-glucosidase activity (Hu et al., 2016). Finally, the results obtained from the technological characterization of 14 strains with strong biofilm formation were subjected to Principal Component Analysis (PCA) (Fig. S4). The analysis revealed that three strains, namely *Pediococcus pentosaceus* EB35, *Lactiplantibacillus pentosus* EB27, and *Pichia kudriavzevii* EF8, showed higher fermentation characteristics and were selected for further investigation.

## 2.4. Analysis of the interaction between LAB and yeast in mixed-species biofilms

Three strains that exhibited strong biofilm formation capacity and fermentation characteristics were cultured in a sorghum extract medium to assess their capacity to form mono-species and dual-species biofilms. The sorghum extract medium was prepared following the method described by Deng et al. (2020). The isolated LAB and yeast were individually activated using MRS and YPD broth, respectively, and their suspensions were adjusted to a concentration of approximately 1 × 10<sup>6</sup> CFU/mL.

For dual-species biofilms, the LAB and yeast suspensions were mixed in equal proportions. These suspensions, along with the sorghum extract medium (at a ratio of 1:100, v/v), were added to 96-well plates and incubated at 30°C for 3 d. The determination of the formation capacity of mono-species and dual-species biofilms was conducted according to the method of Kang et al. (2020).

To determine the biomass of LAB and yeast in mono- and dual-species biofilms on stainless steel surfaces, the colony counting method was used. A stainless steel sheet measuring 1 cm × 1 cm × 0.1 cm was ultrasonically cleaned in acetone for 30 min, followed by rinsing with 75 % ethanol and sterile water. The sheet was then sterilized for further use. Next, the sterile stainless steel sheet was placed in a 6-well plate, and 2 mL of sorghum extract medium with 10 µL of the microbial suspensions was added. The plate was incubated at 30°C for 3 d. After that, the sheet was carefully removed and rinsed with normal saline three times to remove planktonic cells. Subsequently, the sheet and normal saline was added to 50 mL tubes and ultrasound treatment for 5 min was performed to harvest the biofilm-state microbial suspensions. The LAB and yeast were cultured in MRS medium containing 500 µg/mL natamycin and YPD medium containing 200 mg/mL chloramphenicol, respectively. The counting results were expressed as CFU/cm<sup>2</sup>.

To analyze the morphology of mono-species and mixed-species biofilms, a Field Emission Scanning Electron Microscope (FESEM, S-4800, Hitachi, Tokyo, Japan) was used, following the method outlined by Kang et al. (2022b).

## 2.5. Untargeted metabolomic analysis

Untargeted metabolomic analysis of biofilm samples was carried out following the method described by Kang et al. (2022b), with slight modifications. Each mono- or dual-species biofilm-state cell sample was combined with 400 µL methanol and sonicated for 60 s. Then, 60 µL of 2-chlorophenylalanine (0.2 mg/mL) was added and further sonicated for 60 s. The mixture was centrifuged at 12000 × g at 4°C for 10 min, and the entire supernatant was transferred to a new centrifugal tube and freeze-dried using a vacuum concentrator. In addition, 60 µL of methoxylamine hydrochloride solution was added to the sample and heated at 80°C for 30 min. Subsequently, 60 µL of BSTFA reagent (containing 1 % trimethylchlorosilane) was added for derivatization at 70°C for 90 min. After cooling the sample to room temperature, 5 µL of FAMEs were added. The sample was then centrifuged at 12000 × g for 15 min, and 100 µL of supernatant was collected in a vial for further analysis.

The injection and ion source temperatures were set at 280 °C and 230 °C, respectively. Full-scan mode was used for acquiring the mass spectrometry data over the *m/z* range of 35 to 750. The temperature program started at 60 °C for 2 min, followed by a 10 °C/min increase until reaching 300 °C, and then maintained at 300 °C for 8 min. Once the raw data were obtained, data analyses were performed using the MS-DIAL 4.3.8 software, which utilized the Fiehn database (Tsugawa et al., 2015).

## 2.6. Bioinformatics and statistical analysis

The significance analysis of intergroup differences was conducted using the Duncan test in a one-way ANOVA within SPSS 23.0. A significance level of  $P < 0.05$  was used to determine the presence of intergroup differences. Graph plotting was performed using Origin 2021 software. For the difference analysis of microbial communities, the Bray-Curtis distance algorithm was utilized, which included principal coordinate analysis (PCoA), ANOSIM similarity analysis, MRPP inter-group difference analysis, and Adonis multivariate analysis of variance. These analyses were conducted using the vegan software package in R platform. In the microbial co-occurrence network analysis, the selection threshold was set as  $|Spearman's\ rho| > 0.85$  and  $P < 0.01$ . The resulting network was visualized using Gephi software (version 0.9.2). To identify key nodes in the network, within-module connectivity ( $Z_i$ ) and among-module connectivity ( $P_i$ ) were calculated. Nodes with  $Z_i > 2.5$  and  $P_i < 0.62$  were defined as module hubs, while nodes with  $Z_i < 2.5$  and  $P_i > 0.62$  were considered connector hubs. Network hubs were defined as nodes with  $Z_i > 2.5$  and  $P_i > 0.62$  (Deng et al., 2012). MetaboAnalyst 5.0, available at <https://www.metaboanalyst.ca/>, was used for the

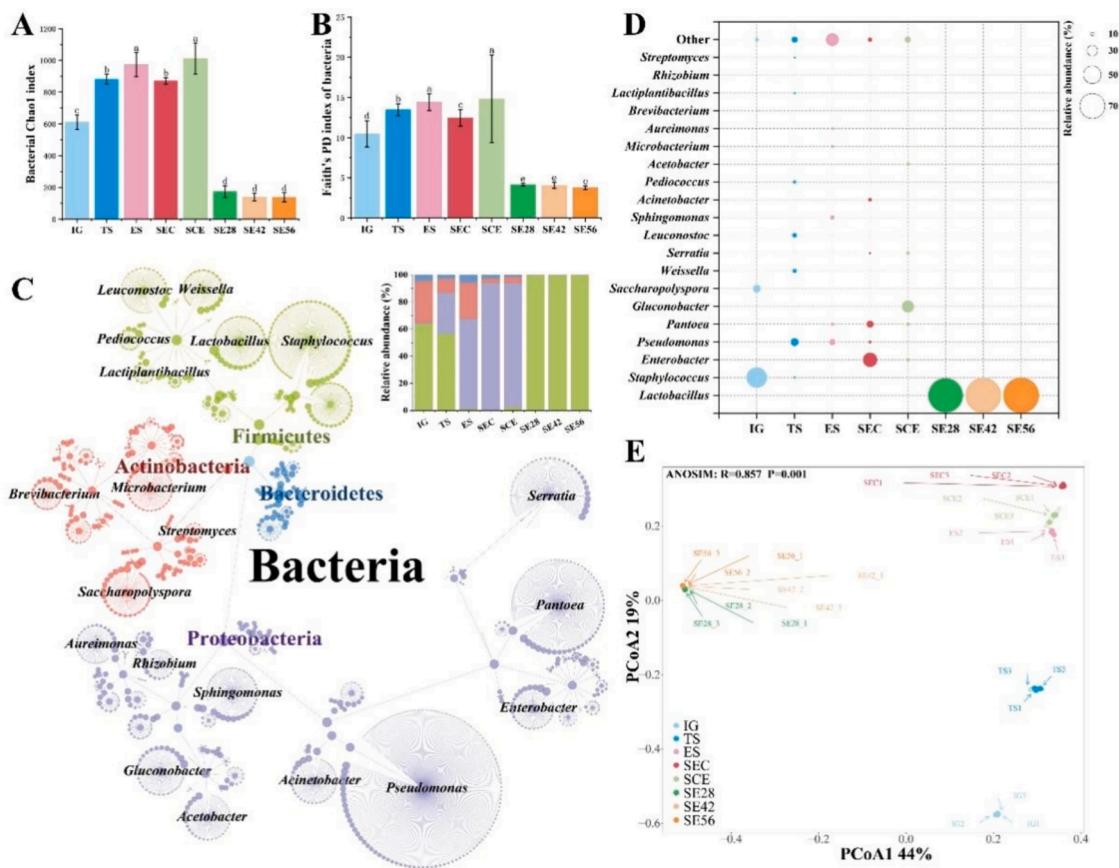
multivariate statistical analysis and pathway enrichment analysis of untargeted metabolomic.

## 3. Results

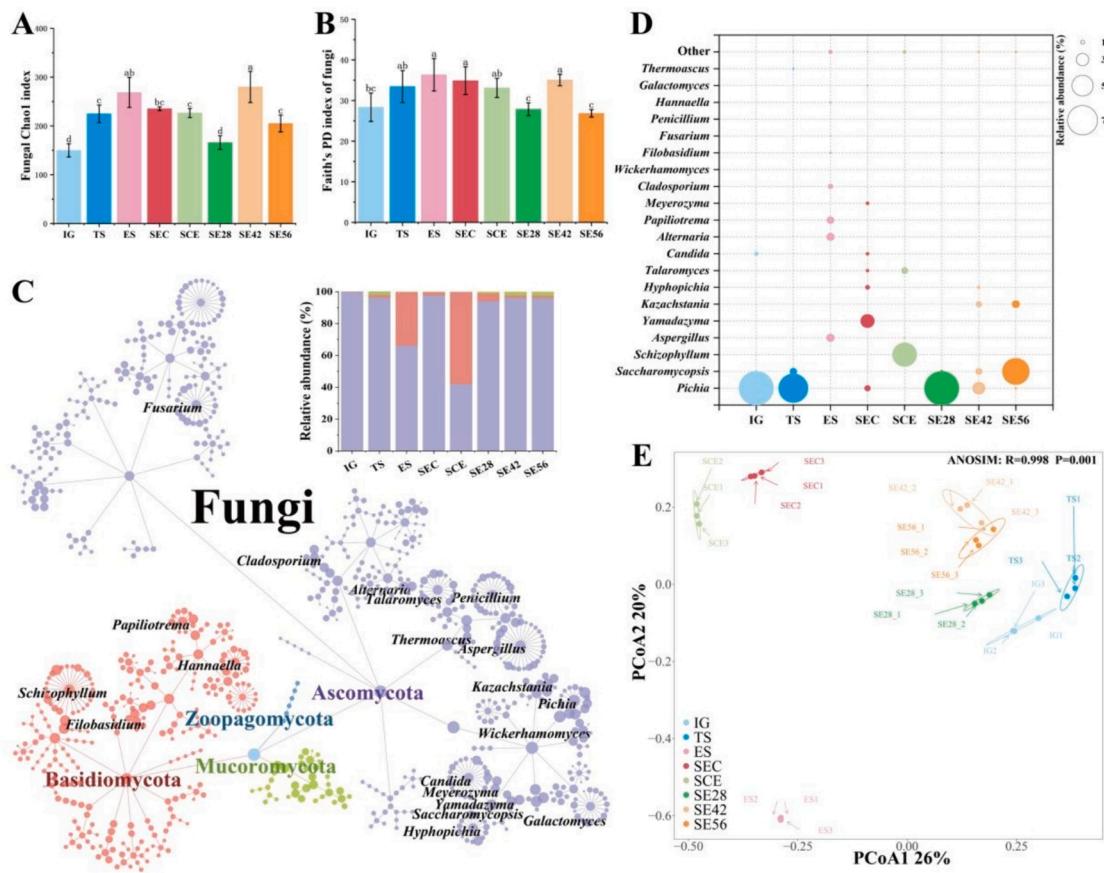
### 3.1. Microbial community composition on the surfaces of Baijiu brewing environments

High-throughput sequencing was used to investigate the microbial communities within the *Baijiu* brewing environment, resulting in the identification of 153 bacterial genera and 161 fungal genera across all samples obtained from various environmental surfaces. Notably, we observed variations in both the diversity and richness of microorganisms among different surfaces in the *Baijiu* brewing environment. After fermentation, the richness and phylogenetic diversity of bacterial communities on the inner surfaces of the earthenware container were significantly lower compared to the environmental surface samples collected prior to fermentation (Fig. 2A and B). Moreover, SE42 and ES exhibited higher fungal community richness compared to other environmental surface samples, while IG and SE28 had significantly lower fungal community richness compared to the others (Fig. 3A). Furthermore, with the exception of SE28 and SE56, the phylogenetic diversity indices of fungal communities on other surface samples were similar (Fig. 3B).

For the bacterial community, Firmicutes was the dominant bacterial phylum in IG, TS, SE28, SE42, and SE56, whereas Proteobacteria was dominant in ES, SEC, and SCE (Fig. 2C). As shown in Fig. 2D, the dominant bacterial genera in IG were *Staphylococcus* and *Saccharopolyspora*, accounting for 56.83 % and 21.42 % of the total abundance,



**Fig. 2.** Comparison of the bacterial community characteristics on surfaces in varying environments. A: Comparison of bacterial richness indices; B: Comparison of bacterial diversity indices; C: Classification tree diagram of bacterial ASV (different colors represent different phylum, each point represents the corresponding classification level, and the stacking histogram shows the distribution of bacterial phylum level in different environmental surfaces); D: Bubble diagram of relative abundance of bacterial genera (Top 20); E: PCoA plot of bacterial community structure.



**Fig. 3.** Comparison of the fungal community characteristics on surfaces in varying environments. A: Comparison of fungal richness indices; B: Comparison of fungal diversity indices; C: Classification tree diagram of fungal ASV (different colors represent different phylum, each point represents the corresponding classification level, and the stacking histogram shows the distribution of fungal phylum level in different environmental surfaces); D: Bubble diagram of relative abundance of fungal genera (Top 20); E: PCoA plot of fungal community structure.

respectively. TS exhibited dominance of *Pseudomonas*, *Leuconostoc*, and *Weissella*, each with relative abundances exceeding 10 %. *Pseudomonas*, *Sphingomonas*, and *Pantoea* were the dominant genera in ES, comprising 40.38 % of the total abundance. *Enterobacter* and *Pantoea* represented over 50 % of the bacterial genera in SEC, while *Glucoronobacter* dominated in SCE. After fermentation, *Lactobacillus* became the dominant genus, with relative abundances exceeding 96 % at all three sampling sites on the inner surfaces of the earthenware container (SE28, SE42, and SE56).

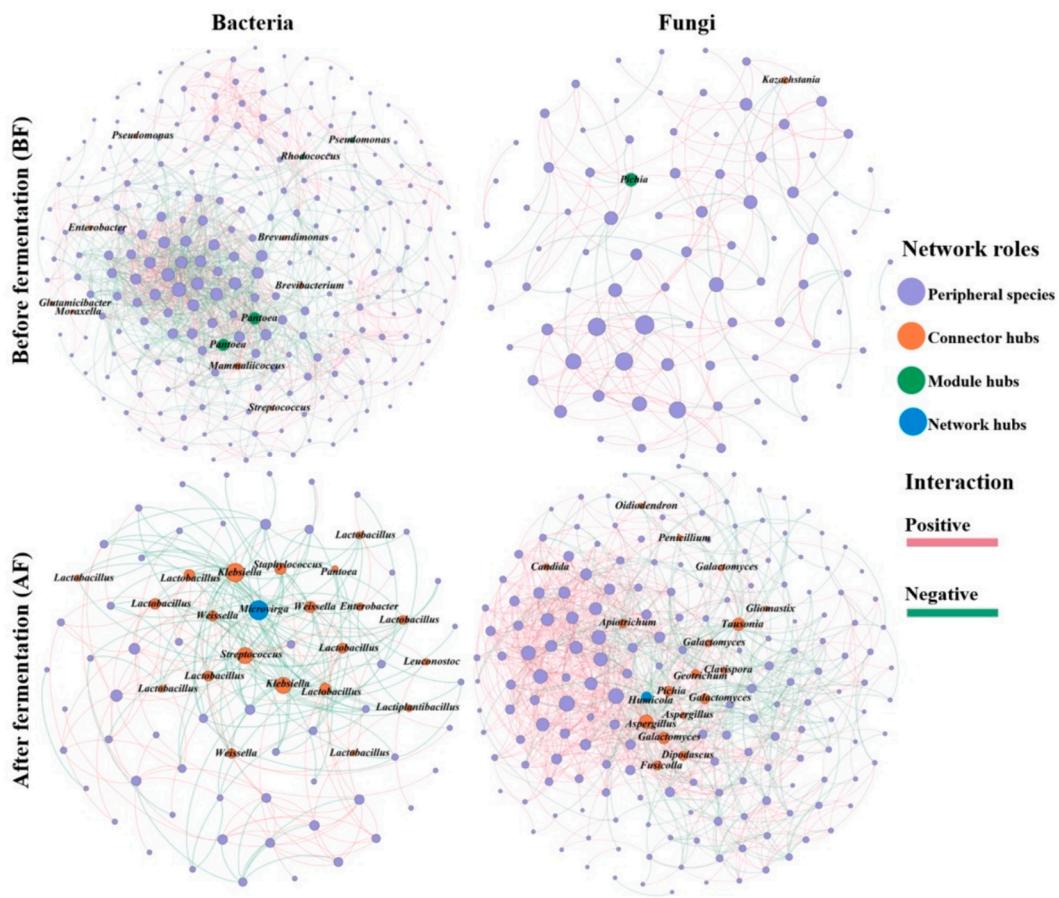
To assess the differences in microbial community structure between samples, PCoA was performed, with the first and second principal component axes explaining 44 % and 19 % of the total variation, respectively. Notably, SE28, SE42, and SE56 clustered together after fermentation (Fig. 2E). Moreover, the results of ANOSIM, MRPP, and Adonis non-parametric dissimilarity tests confirmed significant differences in bacterial community structure between the environmental surface before and after fermentation (Table S1).

In terms of fungal communities, Ascomycota dominated the environmental surface in most samples, except for SCE where Basidiomycota and Ascomycota were dominant (Fig. 3C). As shown in Fig. 3D, *Pichia* showed relatively high abundance in IG, TS, and SE28, with proportions of 81.13 %, 69.31 %, and 81.49 %, respectively. Conversely, ES exhibited the lowest relative abundance of *Pichia* at only 0.23 %. *Aspergillus*, *Alternaria*, *Papiliotrema*, and *Cladosporium* were the dominant fungal genera in ES, each with relative abundances exceeding 10 %. In SEC, the dominant genera were *Yamadazyma*, *Pichia*, and *Hyphopichia*, all with relative abundances greater than 10 %. *Pichia*, *Saccharomyopsis*, *Kazachstania*, and *Hyphopichia* were the dominant fungal genera in SE42 and SE56 (Fig. 3D).

The PCoA analysis of fungal communities revealed that the first and second principal component axes accounted for 26 % and 20 % of the variation, respectively. However, in contrast to the bacterial communities, there was only a slight difference in fungal community structure before and after fermentation (Fig. 3E). These findings were further supported by the results of ANOSIM, MRPP, and Adonis non-parametric dissimilarity tests, which indicated no significant differences in fungal communities between the groups before and after fermentation (Table S1).

### 3.2. Co-occurrence network of microbial communities on environmental surfaces

To analyze the interactions of bacteria and fungi on the environmental surface before and after fermentation, co-occurrence networks were constructed for both. The results revealed interesting patterns. Following fermentation, the bacterial network experienced a reduction in size and complexity, decreasing from 248 nodes and 1365 edges to 79 nodes and 242 edges. In contrast, the fungal network displayed the opposite trend, increasing from 88 nodes and 192 edges to 194 nodes and 1185 edges (Fig. 4 and Table S2). Moreover, the density values of the microbial network increased after fermentation, with the bacterial network changing from 0.045 to 0.079, and the fungal network changing from 0.050 to 0.063. The correlations of bacterial and fungal networks, both before and after fermentation, were predominantly positive (Table S2), indicating a cooperative or symbiotic relationship between the bacterial or fungal communities on the environmental surfaces. Notably, the proportion of positive link increased for bacteria after



**Fig. 4.** Co-occurrence network of bacterial and fungal communities on environmental surfaces before and after fermentation. Each node represents an ASV, nodes are colored according to their network role, and the size of the node represents the degree of each node. The red and green lines in the network represent positive and negative correlations, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fermentation, while it decreased for fungi. Additionally, when compared to random networks, the empirical networks exhibited significantly higher topological parameters (Table S2), suggesting that all four networks displayed typical small-world network characteristics.

The  $Z_i$  and  $P_i$  values were employed to identify changes in key microbes before and after fermentation (Fig. 4). Following fermentation, there was an increase in the number of keystone species in both the bacterial and fungal networks. Prior to fermentation, 12 keystone species were identified in the bacterial network, including 4 module hubs and 8 connector hubs. After fermentation, a total of 22 keystone species were identified in the bacterial network, consisting of 1 network hub and 21 connector hubs, with most annotated as LAB. Similarly, prior to fermentation, two fungal keystone species were identified (1 connector hub and 1 module hub), annotated as *Kazachstania* and *Pichia*, respectively. After fermentation, 18 keystone species were identified in the fungal network, including 1 network hub and 17 connector hubs, mainly annotated as *Humicola*, *Galactomyces*, *Aspergillus*, *Candida*, and *Pichia*.

### 3.3. Interdomain interaction network of bacteria and fungi on environmental surfaces

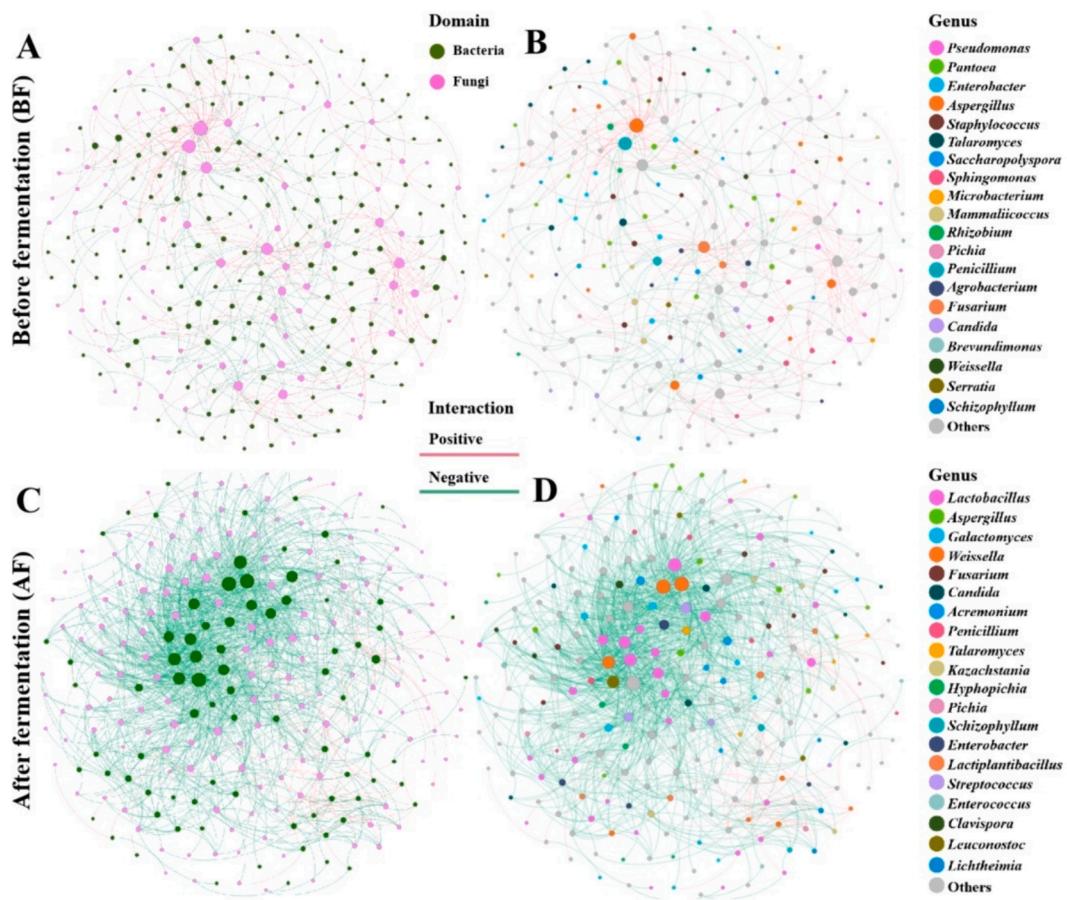
The interaction between bacteria and fungi on the *Baijiu* brewing environment surface, both before and after fermentation, was analyzed using the interdomain network (Fig. 5 and Table S3). Following fermentation, the number of nodes between bacteria and fungi decreased (bacteria: 171 vs 82, fungi: 191 vs 169), while the association number increased (506 vs 1270), indicating a stronger interaction after fermentation. Furthermore, the network's robustness improved (bacteria: 0.747 vs 0.803, fungi: 0.690 vs 0.871). The negative correlation

between bacteria and fungi was enhanced after fermentation (38.14 % vs 92.68 %). The results suggested that negative correlation (competition) dominated the bacterial-fungal interaction on the environmental surfaces after fermentation, and most of the nodes involved belong to LAB and filamentous fungi (Fig. 5 and Table S3).

### 3.4. Interaction between LAB and yeast in mixed-species biofilm

Most of the bacteria and yeasts with strong biofilm capacity, isolated from the surface of the *Baijiu*-brewing environment, showed excellent fermentation potential (Fig. S4A). These findings suggested that fermentation functional microorganisms in the *Baijiu*-brewing environment may exist on environmental surfaces as biofilm. Thus, we evaluated the interaction between bacteria and fungi in a mixed-species biofilm in using LAB (*La. pentosus* EB27 and *Pe. pentosaceus* EB35) and yeast (*Pi. kudriavzevii* EF8) cultured in a sorghum extract medium. Three strains were selected for their excellent biofilm formation capacity and fermentation potential.

As shown in Fig. 6A, the biofilm formation capacity of *La. pentosus* EB27 and *Pe. pentosaceus* EB35 was significantly higher than that of *Pi. kudriavzevii* EF8. Interestingly, when *La. pentosus* EB27 was co-cultured with *Pi. kudriavzevii* EF8 and when *Pe. pentosaceus* EB35 was co-cultured with *Pi. kudriavzevii* EF8, the mixed-species biofilm formation capacity was higher compared to the mono-species biofilms. Furthermore, we measured the biomass of the three strains for the formation of mono- and dual-species biofilms on stainless steel surfaces (Fig. 6B). It was shown that the biomass of LAB (*La. pentosus* EB27 and *Pe. pentosaceus* EB35) decreased, while the biomass of *Pi. kudriavzevii* EF8 increased after the formation of dual-species biofilm, in comparison to the respective mono-



**Fig. 5.** Bacterium-fungus interdomain network of environmental surfaces before and after fermentation: A and B represent the bacterium-fungus network before fermentation, while C and D depict the bacterium-fungus network after fermentation. In panels A and C, the node color indicates the domain-level classification, whereas in panels B and D, the color of nodes represents the classification at the microbial genus level.

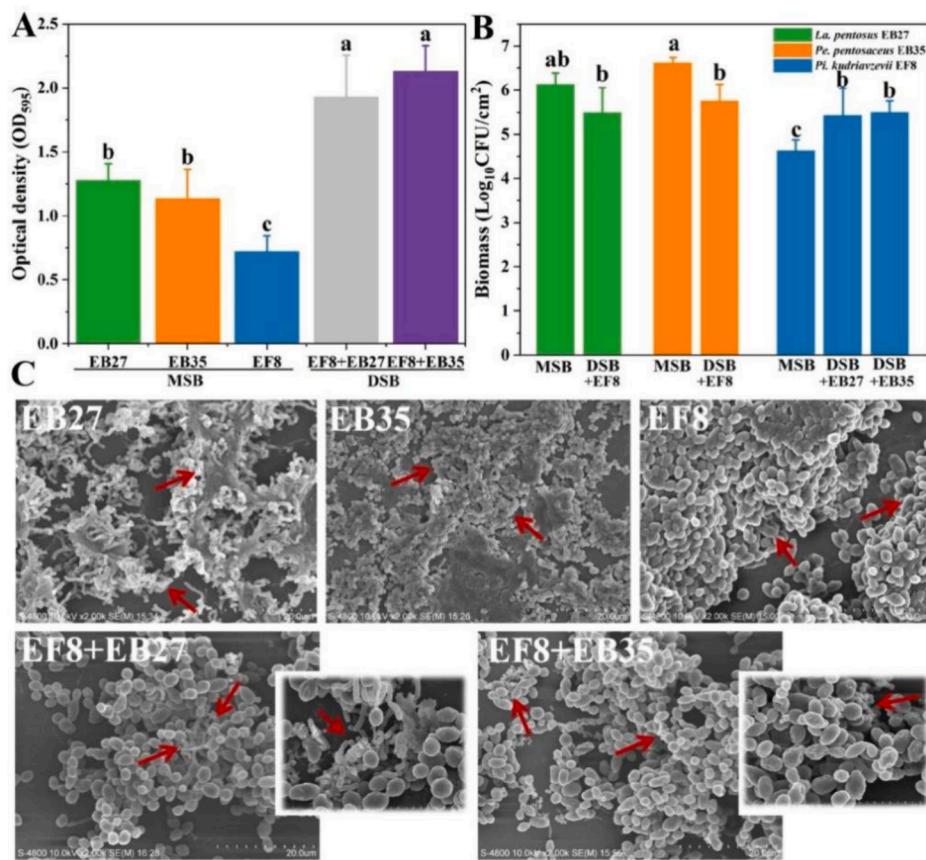
species biofilms. Additionally, FESEM analysis was used to examine the morphology of mono- and dual-species biofilms (Fig. 6C). The mixed-species biofilm formed by *La. pentosus* EB27 or *Pe. pentosaceus* EB35 and *Pi. kudriavzevii* EF8 exhibited a double-layer structure, with *Pi. kudriavzevii* EF8 occupying the upper layer and LAB covering the bottom layer.

### 3.5. Metabolic characteristics of LAB and yeast in mixed-species biofilm

To further evaluate the interaction between LAB and yeast in the biofilm state, the metabolic differences between mono- and dual-species biofilms were analyzed via using untargeted metabolomic technology and an OPLS-DA model. The stability and reliability of the model were confirmed by the high values of  $R^2X(\text{cum})$ ,  $R^2Y(\text{cum})$ , and  $Q^2(\text{cum})$  parameters, as shown in Table S4. Differential expression metabolites (DEMs) were selected based on the criteria of  $\text{VIP} > 1$  and  $P < 0.05$ , and a volcano plot was generated by calculating the fold change. The metabolite profiles of mono- and dual-species biofilms of LAB and yeast showed significant differences, as indicated in Fig. 7A, 7D, 8A, and 8D. In *La. pentosus* EF27 mono-species biofilms, we identified 29 up-regulated and 23 down-regulated DEMs compared to dual-species biofilms of *Pi. kudriavzevii* EF8 and *La. pentosus* EB27 (Fig. 7B). Similarly, in the dual-species biofilms, we found 10 up-regulated and 9 down-regulated DEMs compared to *Pi. kudriavzevii* EF8 mono-species biofilms (Fig. 7E). Overall, there was a greater metabolic difference in the mono-species biofilm formed by *La. pentosus* EB27 than in *Pi. kudriavzevii* EF8, when compared to the dual-species biofilm formed by *Pi. kudriavzevii* EF8 and *La. pentosus* EB27 together.

Metabolic function enrichment analysis of KEGG highlighted the most significant pathways in the dual-species biofilms of *Pi. kudriavzevii* EF8 and *La. pentosus* EB27 compared to *La. pentosus* EB27, including ABC transporters, phosphotransferase system, citrate cycle, as well as glyoxylate and dicarboxylate metabolism (Fig. 7C). In comparison to the mono-species biofilm formed by *Pi. kudriavzevii* EF8, the dual-species biofilms exhibited significant enrichment in taurine and hypotaurine metabolism and valine, leucine, and isoleucine biosynthesis pathways. Additionally, bacterial chemotaxis, bacterial biofilm, and ABC transporters pathways were also significantly enriched (Fig. 7F).

For the dual-species biofilm formed by *Pe. pentosaceus* EB35 and *Pi. kudriavzevii* EF8, 19 DEMs were up-regulated and 9 DEMs were down-regulated compared to *Pe. pentosaceus* EB35 mono-species biofilms (Fig. 8B). Similarly, in comparison to *Pi. kudriavzevii* EF8 mono-species biofilms, the dual-species biofilm exhibited 2 up-regulated DEMs and 9 down-regulated DEMs (Fig. 8E). The metabolic difference was greater in the mono-species biofilm formed by *Pe. pentosaceus* EB35 than in *Pi. kudriavzevii* EF8, when compared with the dual-species biofilm formed by both strains. Additionally, in the dual-species biofilm compared to *Pe. pentosaceus* EB35 mono-species biofilms, significantly enriched metabolic pathways included glyoxylate and dicarboxylate metabolism, taurine and hypotaurine metabolism, alanine, aspartate, and glutamate metabolism, as well as sulfur metabolism (Fig. 8C). Moreover, compared to *Pi. kudriavzevii* EF8 mono-species biofilms, enriched metabolic pathways included the cAMP signaling pathway, pyruvate metabolism, propanoate metabolism, phenylalanine metabolism, and bacterial chemotaxis (Fig. 8F).



**Fig. 6.** Biofilm formation capacity and morphological characterization of *La. pentosus* EB27 or *Pe. pentosaceus* EB35 and *Pi. kudriavzevii* EF8 in mono- and dual-species biofilms. A: crystal violet quantification of biofilm; B: biofilm formation on stainless steel surfaces, MSB: mono-species biofilms, DSB: dual-species biofilms; C: FESEM observation of biofilms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

#### 4. Discussion

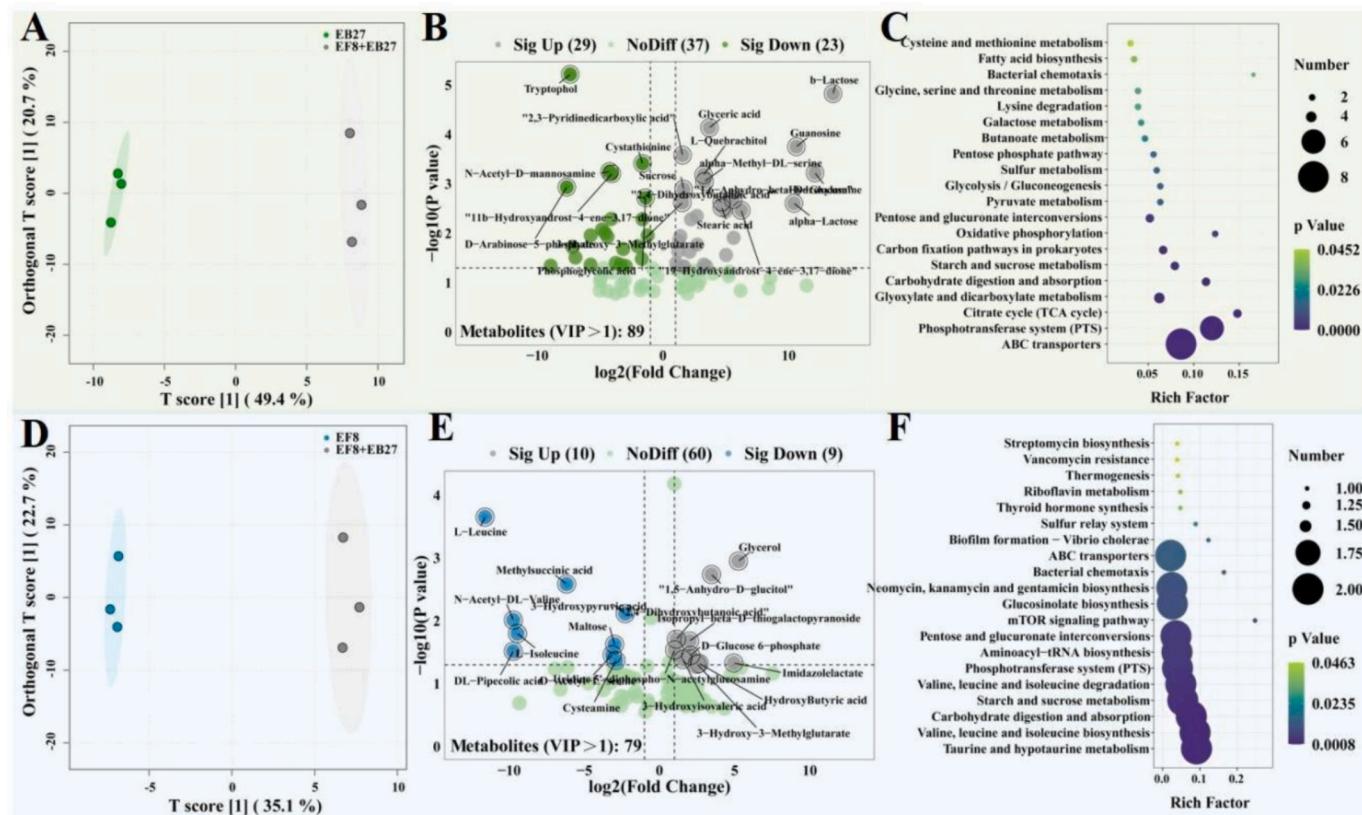
Environmental microbiotas play a vital role in both microbial succession and flavor formation during *Baijiu* fermentation (Pang et al., 2018; Wang et al., 2018). This study investigated the composition and metabolic characteristics of mixed-species biofilms formed by environmental microorganisms on the surface of the *Baijiu* brewing environment. The results confirmed the presence of core microbiotas, such as LAB and yeast, adhering to the contact surface as a double-layered mixed-species biofilm. Previous studies have shown that *Baijiu* fermentation leads to increased acidity and moisture in the fermented grains (Huang et al., 2020; Pang et al., 2018), while oxygen content decreases (Qian et al., 2021). Oxygen depletion leads to accelerated LAB growth and the preferential production of lactic acid (Bartle et al., 2019). As a result, LAB became the dominant microorganism on the inner surface of earthware containers after the fermentation of grains, which facilitated the succession of fermentative microorganisms on the surface of the *Baijiu* brewing environment.

During light-flavor *Baijiu* fermentation, LAB was identified as the predominant bacterial community (Huang et al., 2020; Pang et al., 2018). LAB not only produces a wide array of flavor compounds but also synthesizes organic acids, which serve as essential stress factors in the fermentation process (Huang et al., 2020; Kang et al., 2022a; Li et al., 2023; Pang et al., 2021). In this study, *Pichia*, an important yeast for alcohol production in *Baijiu* manufacturing, was detected in all environmental surface samples. Zhang et al. (2021) found that *Pichia*, primarily originating from the environment, significantly contributes to the formation of the microbial community in *Baijiu* fermentation, highlighting the crucial role of environmental microbiota in regulation and facilitation. Notably, the fungal community structure showed more

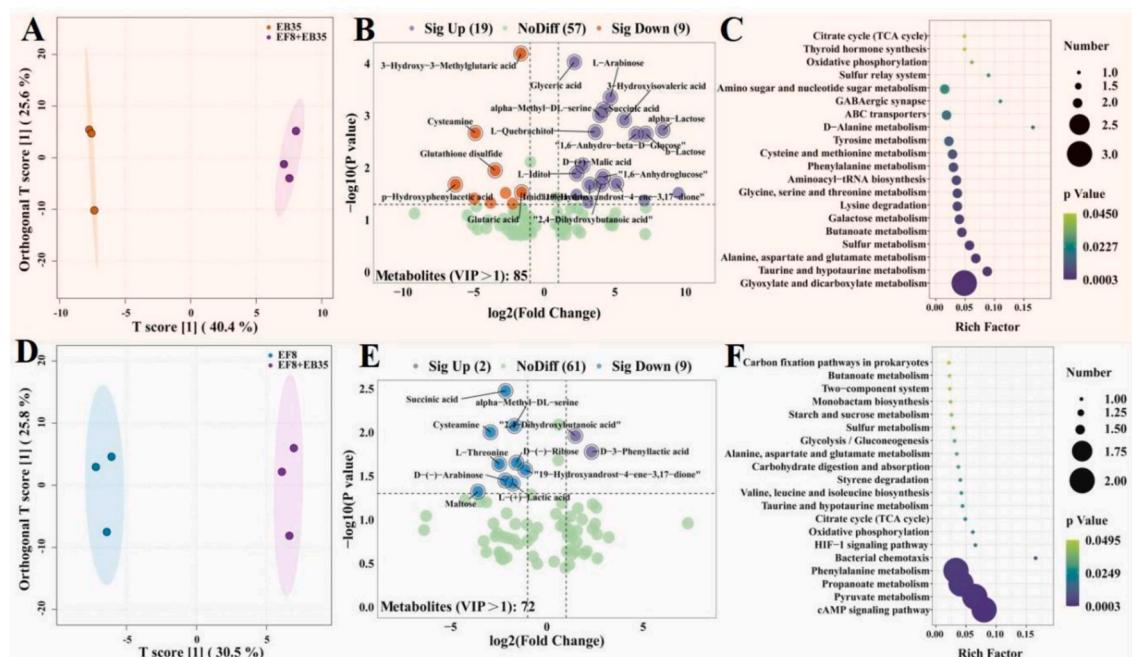
similarity pre- and post-fermentation compared to the bacterial community. Similarly, Kang et al. (2022b) found that fungal communities were less affected by dynamic abiotic factors in the second-round fermentation of light-flavor *Baijiu*, when compared to bacteria.

Fermentation acts as a stress factor, leading to the development of a more stable network of environmental surface microorganisms centered around functional microorganisms. Our findings indicated that, post-fermentation, the size and complexity of bacterial co-occurrence networks decreased, while the opposite was observed for fungi. This can be attributed to the susceptibility of most surface bacteria to the stress caused by dynamic abiotic factors during fermentation (Li et al., 2023; Qian et al., 2021). Increased stress likely reduces bacterial interaction on environmental surfaces but enhances fungal interaction. de Vries et al. (2018) demonstrated that soil bacterial networks were less stable than fungal networks under drought conditions, with drought having a more significant impact on soil bacterial communities. Moreover, *Pi. kudriavzevii* effectively reduces lactic acid concentration in the presence of high levels, promoting the growth of *S. cerevisiae*. Conversely, under lower concentrations, both *S. cerevisiae* and *Pi. kudriavzevii* can cooperatively utilize lactic acid (Deng et al., 2020).

We observed that the interaction network between bacteria and fungi became tighter and more stable post-fermentation. Their relationship shifted from primarily cooperative symbiosis before fermentation to predominantly competitive or antagonistic after fermentation. LAB and fungi constituted the majority of nodes in the microbial network after fermentation, providing further evidence for the competitive or antagonistic relationship between these two types of microorganisms in the *Baijiu* brewing environment. Wang et al. (2022) also reported a negative correlation between LAB and filamentous fungi in brewing fermentation. As *Baijiu* fermentation progressed, the rapid



**Fig. 7.** Comparison of metabolic characteristics between mono- and dual-species biofilms formed by *Pi. kudriavzevii* EF8 and *La. pentosus* EB27. A and D: OPLS-DA score plot; B and E: Volcano plots of metabolites with VIP > 1; C and F: Bubble diagram of KEGG function enrichment of DEMs; A-C: Dual-species biofilms of *La. pentosus* EB27 and *Pi. kudriavzevii* EF8 vs *La. pentosus* EB27 mono-species biofilms; D-F: Dual-species biofilms of *La. pentosus* EB27 and *Pi. kudriavzevii* EF8 vs *Pi. kudriavzevii* EF8 mono-species biofilms.



**Fig. 8.** Comparison of metabolic characteristics between mono- and dual-species biofilms biofilms formed by *Pe. pentosaceus* EB35 and *Pi. kudriavzevii* EF8. A and B: OPLS-DA score plot; C and D: Volcano plots of metabolites with VIP > 1; E and F: Bubble diagram of KEGG function enrichment of DEMs; A-C: Dual-species biofilms of *Pe. pentosaceus* EB35 and *Pi. kudriavzevii* EF8 vs *Pe. pentosaceus* EB35 mono-species biofilms; D-F: Dual-species biofilms of *Pe. pentosaceus* EB35 and *Pi. kudriavzevii* EF8 vs *Pi. kudriavzevii* EF8 mono-species biofilms.

proliferation of LAB led to a decrease in pH value and oxygen content, as well as an increase in acidity in the environment, conditions unfavorable for the survival of filamentous fungi (Wang et al., 2022). However, the connectivity of the interdomain network of bacteria and fungi was only 0.033 and 0.092 (Table S2), indicating that many other bacteria-fungi relationships were not represented in the network. Wang et al. (2019) found a positive correlation between LAB and yeasts during *Baijiu* fermentation, but a negative correlation between them and other dominant microorganisms. Du et al. (2015) revealed that *Streptomyces* produced macrolide antibiotics that inhibited the growth of yeast and mold, consequently affecting the *Baijiu* brewing process. In summary, a more complex and stable interaction network formed between microorganisms after fermentation.

In food fermentation systems, biofilm formation by mixed microbial communities plays an important role in promoting communication, cooperation, and resistance to environmental stressors among microorganisms (Bartle et al., 2019; Yao et al., 2022). In our study, we isolated two strains of LAB and one strain of yeast from the surfaces of *Baijiu* brewing environments, and these strains exhibited strong biofilm formation ability and fermentation potential. When co-cultured, the LAB and yeast strains formed a double-layered biofilm, with LAB composing the lower layer and yeast the upper layer. Interestingly, the biomass of LAB decreased while that of yeast increased after the biofilm formed on the stainless steel surface. The ability of microorganisms to form biofilms varies between abiotic and biotic surfaces (Dominguez-Manzano et al., 2012). The formation of multispecies biofilms during food fermentation may be influenced by interspecies interactions and the microenvironment (Yao et al., 2022). In addition, due to the possibility of microorganisms existing in a viable but non-culturable state post-fermentation stress, relying solely on culture-dependent techniques may result in inaccurate enumeration and identification of microorganisms in the biofilm state (De Roos et al., 2019; Fan et al., 2020).

The layered spatial distribution of mixed-species biofilms can accommodate the diverse requirements of microorganisms for microenvironments with varying levels of dissolved oxygen (Yao et al., 2022). This finding offers a possible explanation for the spatial structure observed in the biofilm formed by the two strains in our study. The positioning of yeast in the upper layer of the biofilm may create a more suitable environment for aerobic respiration, while LAB are better suited to adhere to the contact surface of the environment and form the biofilm. Fan et al. (2020) conducted a study on the biofilm structure of LAB and *S. cerevisiae* in low-temperature *Daqu* and also observed a double-layer structure in the mixed-species biofilm formed by *Lactobacillus* sp. and *S. cerevisiae*. *Lactobacillus* primarily occupied the lower layer, which represented a low-oxygen environment, while *S. cerevisiae* was predominantly found in the upper layer, exposing it to higher levels of oxygen. The mixed-species biofilm of LAB and yeast exhibited a double-layered structure, with the lower layer consisting of LAB cells and the upper layer composed of co-aggregates of LAB and yeast cells. The ability of LAB to adhere to both solid surfaces and yeast cells facilitated the formation and maintenance of the biofilm as an ecosystem for LAB and yeast (Furukawa et al., 2010; Hirayama et al., 2019).

The metabolic characteristics of microbes in dual-species biofilms, comprised of LAB and yeast, differed from those observed in mono-species biofilms. These differences encompassed energy metabolism, carbohydrate metabolism, amino acid metabolism, and cell motility. Nutrient cross-feeding serves as a significant mode of cooperation among microorganisms in the biofilm lifestyle (Yao et al., 2022). Specifically, yeast consumes nitrogen in the form of amino acids, which can impede the growth of LAB. However, yeast lees act as a nutrient source for LAB, enabling them to scavenge amino acids, sugars, and peptides (Bartle et al., 2019). Intriguingly, the metabolic characteristics of LAB-yeast within the dual-species biofilm demonstrated greater similarity to those of yeast in mono-species biofilms rather than LAB in mono-species biofilms. These findings indicated that the interaction between LAB and yeast within the biofilm state influenced their respective

metabolic functions, potentially providing yeast with an advantageous role. The study conducted by Fan et al. (2020) exhibited similar findings. Furthermore, interspecific interactions were frequently observed among biofilm microorganisms comprising mixed species, being essential for their survival and fermentation processes (Yao et al., 2022).

In winemaking, the formation of mixed-species biofilms benefits yeast by providing access to nitrogen and carbon resources that are typically scarce. Moreover, it confers enhanced resistance to low pH and high ethanol content for LAB (Bartle et al., 2019). Ponomarova et al. (2017) demonstrated that in nitrogen-rich environments, *S. cerevisiae* regulates its metabolism by secreting amino acids, thereby supporting the survival of LAB. Additionally, the introduction of a mixed-species biofilm consisting of *O. oeni* and *S. cerevisiae* accelerated alcohol fermentation. Biofilm-state microbes displayed resilience to the stresses associated with wine fermentation due to the regulation of redox and amino acid metabolism (Palud et al., 2024). Notably, the biofilm structure not only enabled environmental microorganisms to withstand stress but also served as a hub for the migration and transformation of functional microorganisms during *Baijiu* fermentation. The biofilm, adhering to the surface of wooden tools, proved to be an efficient delivery system for LAB, facilitating their release into cheese fermentation (Lortal et al., 2014). Furthermore, it was discovered that the inner wall of the barrel served as an additional microbial inoculation for Lambic beer production. The presence of biofilm-associated genes in *Dekkera bruxellensis* suggested its potential to adhere to the inner wall of the barrel, providing protection against chemical detergents and disinfectants (De Roos et al., 2019).

## 5. Conclusion

In conclusion, this study highlighted the significance of fermentation production as a stress-inducing factor that drove the microbial interactions within mixed-species biofilms on environmental surfaces in *Baijiu* brewing. The LAB-yeast biofilm exhibited a distinctive double-layer structure, with LAB primarily located in the lower layer tightly adhered to the surface, while yeast is densely distributed in the upper layer. The biofilm structure serves as a vital survival strategy for the environment microbiota and acts as a reservoir of functional microorganisms within the *Baijiu* brewing microecosystem. Future research using multi-omics technology should focus on understanding the impact of environmental microorganisms in the biofilm state on *Baijiu* brewing.

## CRediT authorship contribution statement

**Jiamu Kang:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Conceptualization. **Rengshu Li:** Writing – original draft, Methodology. **Yunan Hu:** Methodology, Investigation, Conceptualization. **Xiaoning Huang:** Writing – review & editing. **Xiao-Xue Chen:** Investigation, Funding acquisition. **Bei-Zhong Han:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

## 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.foodres.2024.114698>.

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