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Novel insights into delayed bitterness control in traditional *Huangjiu:* Regulating bacterial community by inoculated with *Saccharomyces cerevisiae* SC-6

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ABSTRACT

Under semi-open brewing conditions, traditional *Huangjiu* often suffers from unstable flavor quality, including occasional delayed bitterness. To address this issue, a yeast strain, *Saccharomyces cerevisiae* SC-6, was screened for its ability to reduce delayed bitterness. The effects of SC-6 on the flavor and microbial composition of *Huangjiu* exhibiting high levels of delayed bitterness were also investigated. GC–MS analysis indicated that *S. cerevisiae* SC-6 significantly lowered the levels of key delayed bitter compounds, including octanoic acid, ethyl caprylate, benzaldehyde, and phenol, by 54.80 %, 12.21 %, 31.38 %, and 43.23 %, respectively. PCoA analysis demonstrated that SC-6 altered the microbial community of traditional *Huangjiu*. Correlation analysis indicated that reduced bitter compounds correlated with changed abundances of *Rhodopseudomonas,* unclassified_f_Micromonosporaceae, *Lachnoclostridium, Lactobacillus, Streptomyces,* Clostridium_sensu_stricto_1, *Staphylococcus*, *Pseudomonas*, unclassified_o_Enterobacterales, and *Candida*. These results provide effective guidance for controlling off-flavors in traditional *Huangjiu* and clarify the role of microorganisms in the production of bitter substances.

1. Introduction

Traditional *Huangjiu* is one of the three most ancient fermented wines in the world, with a history of more than 5000 years (Jiao et al., 2017). It is made from glutinous rice and wheat koji using a unique bilateral fermentation process with saccharification and fermentation (Chen et al., 2012) in a semi-open environment for 70–90 d (Yu et al., 2021) in winter. The unique raw materials and fermentation process endow traditional *Huangjiu* with a pleasant and fragrant aroma, as well as a mellow, sweet-bitter, and refreshing taste (Wang, 2023). Appropriately balanced bitterness makes traditional *Huangjiu* taste refreshing and strong (Wang et al., 2013), whereas excessive bitterness or prolonged aftertaste, referred to as delayed bitterness, can destroy the coordination of *Huangjiu* (Yu, Li, et al., 2022). Therefore, reducing delayed bitterness is crucial to preserving the high-quality flavor of traditional *Huangjiu*.

Bitter sunstances in *Huangjiu* include amino acids, fusel oil, tyrosol, and others (Yu, Xie, Xie, Ai, & Tian, 2019; Yu, Xie, Xie, & Tian, 2019). Bitter amino acids and peptides are commonly regarded as a pivotal source of the delayed bitterness of *Huangjiu* (Lu et al., 2021). Yu et al. identified that polypeptides with the amino acid sequence Leu-Pro-Thr-Leu exhibited a delayed bitter in traditional *Huangjiu* (Yu, Wang, et al., 2022). The taste characteristics of traditional *Huangjiu* are attributed not only to the contribution of non-volatile compounds but also to the cross-modal effects of volatile substances. Based on gas chromatography-olfactometry (GC-O), a total of 11 bitter-tasting volatile compounds were identified in traditional *Huangjiu*, including benzaldehyde, 2-furaldehyde, isoamyl alcohol, phenethyl alcohol, 2-methyl-1-propanol, ethyl caprylate, ethyl nonanoate, ethyl palmitate, phenol, octanoic acid, acetophenone (Wang, 2023). Appropriate amounts of the aforementioned flavor compounds can enhance the taste complexity of traditional *Huangjiu*, whereas excessively high concentrations may lead to lingering delayed bitterness, thereby adversely affecting the drinking quality of traditional *Huangjiu*.

Microorganisms play a pivotal role in the flavor formation of fermented alcoholic beverages (Böhmer et al., 2020; Wu et al., 2024). Recent studies indicated that bioturbation can drive the reconstruction of the microbial community structure during fermentation, potentially enhancing the quality of fermented products (Zhang et al., 2023). Tong's research (Tong et al., 2024) demonstrated that bioaugmentation of

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Bacillus velezensis DQA21 in the fermentation process of *baijiu*, significantly changed the microbial community composition, as well as notable enhanced the concentrations of key flavor compounds. Similarly, He et al. employed *Bacillus velezensis* and *Bacillus subtilis* to increase the contents of major flavor compounds by altered the diversity and complexity of interspecies interactions in liquor (He et al., 2020). Qiu's study showed that the addition of a strain of *Saccharomyces cerevisiae (S. cerevisiae)* with high organic acid tolerance and ethyl hexanoate production induced changes in the microbial community and elevated levels of ethanol and ethyl caproate of strong-flavor *baijiu* (Qiu et al., 2024). Numerous strains of *S. cerevisiae* have been selected to attain certain objectives, including flavor enrichment, reduction of unwanted byproducts, and controllable fermentation processes (Cadiere et al., 2012). Therefore, employing *S. cerevisiae* that inhibit the formation of delayed bitterness compounds during fermentation process may be an effective strategy for improving the controllability of the flavor quality of traditional *Huangjiu*.

The objectives of this study are: (1) to screen *S. cerevisiae* strains with the ability to reduce delayed bitterness in traditional *Huangjiu* through a combined approach of GC–MS and sensory evaluation; (2) to investigate the impact of obtained *S. cerevisiae* on the content of delayed bitterness compounds in *Huangjiu*; (3) to analyze effects of this strain on the flavor and microbial composition during the fermentation of *Huangjiu*; (4) to examine the correlation between microbial communities and key delayed bitterness compounds based on metabolomic and bioinformatic analysis.

2. Materials and methods

2.1. Chemicals

Ethanol (≥99.7 %), dichloromethane (≥99.5 %), 2-octanol (≥99.0 %), and n-alkanes (C_6-C_{30}) were chromatically pure (Sigma-Aldrich Company). TIANamp Stool DNAKit Stool Genome DNA extraction kit, nucleic acid primer (Shanghai Sangon Biological Co., Ltd.). DL10,000 DNAMarker (Takara Bio (Beijing)). Malt powder, liquefaction enzyme (2000 μ/g), saccharifying enzyme (90 %, A2000) (Shanghai Adamas Reagent Co., Ltd.). PDA liquid medium (Beijing Land Bridge Technology Co., Ltd). 2 x goldstar best master mix polymerase (Cowin Biotech Co., Ltd). Angel Yeast (Angel Yeast Co., LTD).

2.2. Huangjiu simulated fermentation

2.2.1. Simulated Huangjiu medium

Steamed rice and water were mixed in a mass ratio of 1:4. To this mixture, 10 % malt flour, raw wheat bran, and enzymes including 1.1 ‰ (per thousand) of liquefying enzyme and saccharifying enzyme were added for saccharification. The saccharification process was conducted at 60 °C for 4 h. Subsequently, the mixture was filtered through eight layers of gauze and then sterilized at 115 °C for 20 min to produce the simulated *Huangjiu* medium.

2.2.2. Yeast strains

Twenty-eight strains of *S. cerevisiae*, screened from the traditional *Huangjiu* mash at Zhejiang Tapai Shaoxing Wine Co., Ltd., were used in this study. The detailed information of strains were shown in Table 1. All strains were inoculated into Yeast Extract Peptone Dextrose (YPD) liquid medium with an inoculum size of 2 % (volume ratio), respectively. Each strain was cultivated for 24 h at 28 °C and 200 rpm of shaking, with the process being repeated for three generations. Then the bacterial suspension obtained from the third generation was centrifuged at a low temperature of 4 °C (4500 rpm for 10 min). The precipitated cells were washed 2 to 3 times with sterile water, and an equal volume of sterile water was added to obtain a working solution that contains 10^7 cfu/mL. Then these working solution of the bacterial strain were inoculated into a simulated rice wine medium at a volume ratio of 10 % and fermented at 28 °C with shaking at 200 rpm for 48 h, yielding a simulated fermentation broth of the bacterial strain. The simulated fermentation broth was employed in the strain screening process to evaluate corresponding indicators.

Table 1
Strain information.

Serial number	Strain category	Strain number
1	*Saccharomyces cerevisiae*	SC-1
2		SC-3
3		SC-39
4		SC-6
5		SC-J4
6		SC-KL4
7		SC-L1018
8		SC-L30
9		SC-M3
10		SC-NL29
11		SC-NL16
12		SC-NL17
13		SC-NL21
14		SC-NL4
15		SC-NL64
16		SC-S5R1-1
17		SC-T14
18		SC-SE11
19		SC-SE23
20		SC-YLL20
21		SC-TC9
22		SC-WFC-SC-14
23		SC-WFC-SC-72
24		SC-AH-6
25		SC-ML3
26		SC-S2-6
27		SC-91
28		SC-YI19

2.2.3. Evaluation of delayed bitterness control in selected strains during fermentation

In laboratory-scale *Huangjiu* brewing experiments, we investigated whether strains capable of reducing the production of key delayed bitter compounds (LD strains) also possess the ability to inhibit the synthesis of these compounds when co-cultured with high-producers of key delayed bitter compounds (HD strains). Activated working solutions of the LD and HD strains were mixed in a 1:10 ratio and then inoculation into the simulated *Huangjiu* medium at a volume ratio of 5 %, to prepare a simplified starter culture for *Huangjiu* fermentation aimed at controlling the formation of delayed bitterness. Similarly, the working solution of the HD strain was inoculated into the simulated *Huangjiu* medium at a volume ratio of 5 % to produce another simplified starter culture for *Huangjiu* fermentation with excessive delayed bitterness. Both cultures were incubated separately at 28 °C with shaking at 200 rpm for 24–48 h to obtain a simplified yeast starter. *Huangjiu* brewing was conducted according to the material ratios listed in Table 2. The primary fermentation temperature was maintained at 28 °C for 120 h, with stirring and ventilation twice within the first 24 h, followed by stirring and ventilation once every 20–24 h thereafter. The secondary fermentation was carried out at 15 °C for 15 days. Following fermentation, the fermented mash was centrifuged at 4500 rpm and 4 °C for 10 min. A control group,

Table 2
Huangjiu fermentation ingredients list.

Raw materials	Control group (Ratio)	Experimental group (Ratio)
Raw glutinous rice	standard	standard
Rice	150 %	150 %
Deionized water	125 %	125 %
Simplified yeast starter	–	11.4 %
Raw wheat koji	13.6 %	13.6 %
Angel koji	0.5 %	0.5 %

consisting of fermented *Huangjiu* without the introduction of any external strains, was established to evaluate the effectiveness of the LD strain in reducing delayed bitterness.

2.3. Quantitative descriptive sensory evaluation

The training of evaluators and sensory evaluation experiments were conducted in a sensory evaluation laboratory with a controlled temperature of 20 °C according to the National Standard of China (GB/T 17946-2008). Each candidate voluntarily consented to participate, and informed consent was obtained before participation.

Twenty-four candidates with previous olfactory testing experience participated in the preliminary training for the sensory evaluation experiments, but they were not informed of the purpose of this experiment. The candidates underwent a one-month training (30 min per day) to describe and identify the odor qualities of aroma compounds. Out of these candidates, 10 evaluators (5 males and 5 females, aged 20–30) with high olfactory discrimination ability were selected to form a sensory evaluation expert panel.

Before conducting the formal sensory experiments, the evaluators underwent retraining to ensure accurate and stable assessments of aroma attributes. The evaluators were required to smell the standard reference samples for each aroma attribute and repeatedly evaluate them until all evaluators could correctly identify the standard references with 100 % accuracy. Eight aroma attribute indicators were selected for this experiment, namely, sour aroma, ester aroma, sauce aroma, sweet aroma, alcoholic aroma, caramel aroma, fruity aroma, and koji aroma (Yu, Xie, Xie, Ai, & Tian, 2019).

2.4. Electronic tongue

Using tartaric acid/potassium chloride solution as the reference liquid, the taste information of the samples was collected at a room temperature of 25 °C. The TS-5000Z electronic tongue system (Japanese INSENT) underwent self-inspection, diagnosis, and calibration procedures before proceeding to the sample information collection phase. The sampling time was set at 120 s, with a frequency of 1 time per second. Before and after each collection, the sensors were placed in distilled water for 90 s for washing, and the collection of each sample was repeated five times.

2.5. Solid-phase microextraction - gas chromatography−mass spectroscopy (SPME-GC–MS)

Delayed bitterness volatile components were extracted from the samples by SPME. 5 g *Huangjiu* samples and 20 μL internal standard (2-octanol, 220 μg/mL) were placed in a 20 mL headspace bottle. After equilibrium at 50 °C for 5 min, the HS-SPME extraction head (100 μm, DVB/CAR/PDMS coating) was exposed to the headspace environment of the headspace bottle for extraction for 50 min. During the extraction process, the rotor was continuously stirred at a speed of 250 r/min.

The procedure and method of GC–MS (Agilent Corporation) analysis refer to the previous study (Yu, Xie, Xie, Ai, & Tian, 2019).

Aroma substances were searched through the NIST17 spectrum database, and their retention index (RI) was compared with literature value qualitatively, and an internal standard method was used to quantify aroma substances.

2.6. High-performance liquid chromatography (HPLC) analysis

The content of delayed bitterness amino acids in traditional *Huangjiu* was determined by HPLC. The method originated from Yu, Li, et al. (2022).

2.7. High-throughput sequencing

The fermented traditional *Huangjiu* was subjected to centrifugation for sedimentation, and subsequently, high-throughput sequencing was performed on the obtained precipitates, and the experimental procedures were performed following previous studies (Böhmer et al., 2020). DNA from sediment bacteria was extracted using TIANamp Stool DNA Kit for stool genome DNA extraction. After gel extraction of PCR products, microbial diversity sequencing was performed using the Illumina MiSeq platform.

2.8. Data analysis

All data were analyzed using Duncan's multiple range test for variance analysis in SPSS (Inc., Chicago, Illinois), with a significant difference level set at 0.05. Radar charts and bar graphs were plotted using Origin 9.0 (OriginLab Corporation, Northampton, MA, USA). The Pearson correlation coefficient between microflora and bitter substances in traditional *Huangjiu* samples was calculated by using R. Clustering correlation heatmap with signs was performed using the OmicStudio tools at https://www.omicstudio.cn.

3. Results and discussion

3.1. The ability of S. cerevisiae strains on producing delayed bitter substances

S. cerevisiae was the predominant yeast present in the fermentation mash and contributed to the production of flavor substances in *Huangjiu*. To evaluate the performance of strains in producing delayed bitter substances, 28 strains of *S. cerevisiae* that isolated from traditional *Huangjiu* fermentation mash were used as the starter for *Huangjiu* production. The total concentrations of delayed bitterness substances, including isoamyl alcohol, benzaldehyde, ethyl caprylate, phenethyl alcohol, octanoic acid, 2-methyl-1-propanol, phenol, 2-furaldehyde, ethyl nonanoate, ethyl palmitate, and acetophenone in the fermentation broth of each strain in the simulated *Huangjiu* medium were analyzed using GC–MS (Fig. 1A). Due to metabolic variations during *Huangjiu* fermentation, different strains significantly influenced the concentration of delayed bitter compounds. Notably, SC-6 exhibited the lowest concentration among all samples, measuring only 7010.89 μg/kg, followed by SC-NL4, SC-NL17, SC-WFC-SC-14, SC-NL29, and SC-SE23. Conversely, SC-YI19 demonstrated the highest capacity to produce delayed bitterness compounds among the screened strains, with a concentration of up to 16,618.42 μg/kg. The concentrations of three delayed bitter compounds—phenethyl alcohol, ethyl octanoate, and octanoic acid showed significant strain-specific variations. The production of phenethyl alcohol by SC-YI19 was notably higher compared to other groups (Fig. 1B). In contrast, SC-6, SC-NL4, SC-NL17, SC-WFC-SC-14, SC-NL29, and SC-SE23 exhibited limited capacity to produce these three delayed bitter compounds.

A more detailed analysis of delayed bitterness and bitterness intensity within the fermentation broths of selected strains was undertaken through quantitative descriptive sensory evaluation. As shown in Fig. 1C, the bitterness and delayed bitterness scores of strains SC-NL29, SC-NL4, SC-NL17, SC-WFC-SC-14, and SC-6 decreased sequentially, with SC-6 having the lowest score. Conversely, SC-YI19, SC-AH-6, SC-YLL20, and SC-SE23 exhibited a significantly higher delayed bitterness score. Notably, the trend in bitterness scores closely mirrored the trend in delayed bitterness scores. Additionally, the sensory evaluation results for delayed bitterness in the fermentation broths of these strains were generally consistent with the content of key delayed bitterness compounds obtained through GC–MS analysis. The bitterness scores were significantly correlated with the kinds and contents of bitter substances, as reported by Yu, Li, et al. (2022). Based on the integration of GC–MS analysis and sensory evaluation results, SC-6, which exhibited a lower

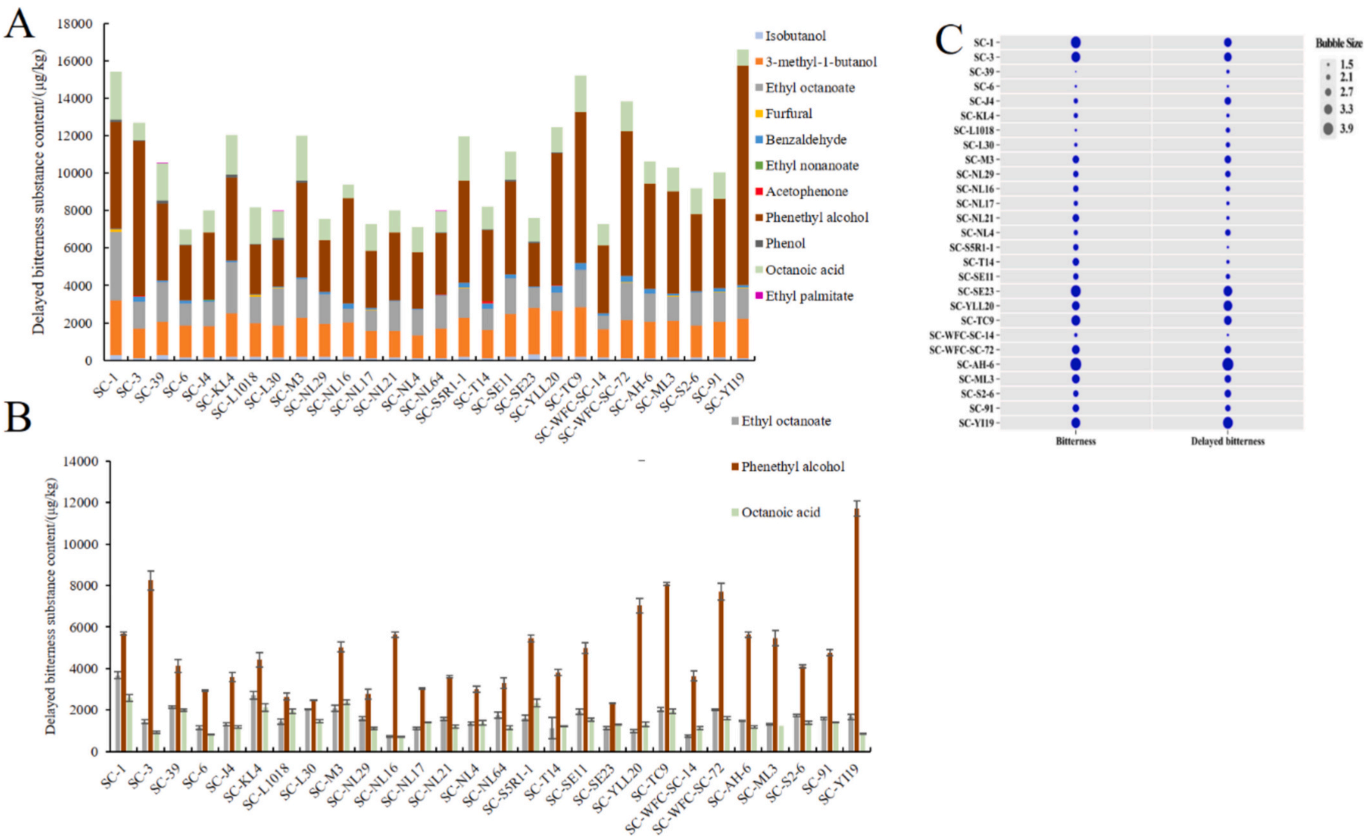

Fig. 1. Comparison of bitter substances (A and B), sensory evaluation of bitteness and delayed bitterness (C) in fermentation broth of different strains.

ability to produce key delayed bitterness compounds, was selected as the target strain. Furthermore, SC-YI19, which exhibited high delayed bitterness production, was chosen to produce traditional *Huangjiu* with excessive delayed bitterness (EDB).

3.2. Validation of the ability of S. cerevisiae SC-6 in Huangjiu with excessive delayed bitterness

3.2.1. S. cerevisiae SC-6 reduced the delayed bitterness of Huangjiu

Synergistic fermentation is an effective strategy for utilizing functional microorganisms during food fermentation to enhance the flavor and quality of fermented foods (Fan et al., 2023). To evaluate the ameliorative effect of SC-6 on the delayed bitterness of *Huangjiu*, SC-6 was co-inoculated with SC-YI19. The experiment included a control group without additional strains (CTRL), a group undergoing fermentation with SC-YI19 (EDB), and a mixed fermentation group consisting of SC-YI19 and SC-6 (EDB_SC_6). Utilizing sensory evaluation methodologies in conjunction with electronic tongue technology, a comprehensive assessment of the bitterness and delayed bitterness of *Huangjiu* was conducted (Table 3). The results of both sensory evalustion and electronic tongue analyses indicated that the score of bitterness and delayed bitterness were significantly increased by the introduction of SC-YI19 alone, suggesting that *Huangjiu* fermented by SC-YI19 exhibited the typical characteristics of excessive delayed bitterness. The interactions between different strains affect the production of flavor compounds (Fleet, 2003). A comparison between EDB_SC_6 and EDB revealed that the incorporation of SC-6 significantly diminished both the bitterness and the delayed bitterness in *Huangjiu*. This finding underscored the unique efficacy of SC-6 in the traditional brewing process of *Huangjiu*, particularly in significantly mitigating the delayed bitterness.

Table 3
Bitterness and delayed bitterness of fermented *Huangjiu*.

Sample	Sensory evaluation		Electronic tongue	
	Bitterness	Delayed bitterness	Bitterness	Delayed bitterness
CTRL	6.08 ± 0.13^b	4.93 ± 0.29^b	3.98 ± 0.61^b	1.48 ± 0.12^b
EDB	6.53 ± 0.76^a	5.48 ± 0.79^a	4.64 ± 0.15^a	1.72 ± 0.10^a
EDB_SC_6	4.33 ± 0.35^c	4.13 ± 0.57^c	3.07 ± 0.08^c	0.75 ± 0.06^c

Differential letters denoted the differences between the various groups.

3.2.2. S. cerevisiae SC-6 inhibited delayed bitterness substances formation

To investigate the factors contributing to the change in bitterness of EDB-*Huangjiu* following SC-6 inoculation, we analyzed the variations in delayed bitter compounds present in *Huangjiu*. The concentrations of these bitter compounds were rigorously analyzed using GC–MS (Fig. 2). The total concentration of delayed bitterness compounds in the EDB group was higher than that in the CTRL group. However, in the EDB_SC_6 group, the total concentration of these delayed bitterness compounds was reduced, with particularly significant decreases in the levels of octanoic acid, ethyl caprylate, benzaldehyde, and phenol, by 54.80 %, 12.21 %, 31.38 %, and 43.23 %, respectively.

In addition to volatile substances, amino acids are also important components affecting the flavor of traditional *Huangjiu* (Zhao et al., 2021). Besides freshness, sweetness, and astringency, amino acids can also bind to bitter receptors on the human tongue through their hydrophobic side chains, which results in the perception of bitterness (Luo et al., 2020). In traditional *Huangjiu*, amino acids associated with bitterness include histidine (His), arginine (Arg), valine (Val), phenylalanine (Phe), leucine (Leu), lysine (Lys), tyrosine (Tyr), and isoleucine (Ile) (Dung et al., 2007). The threshold values (Yu, Li, et al., 2022)and concentrations of bitter-tasting amino acids in three types of traditional *Huangjiu* were presented in Table 4. Compared to EDB and CTRL,

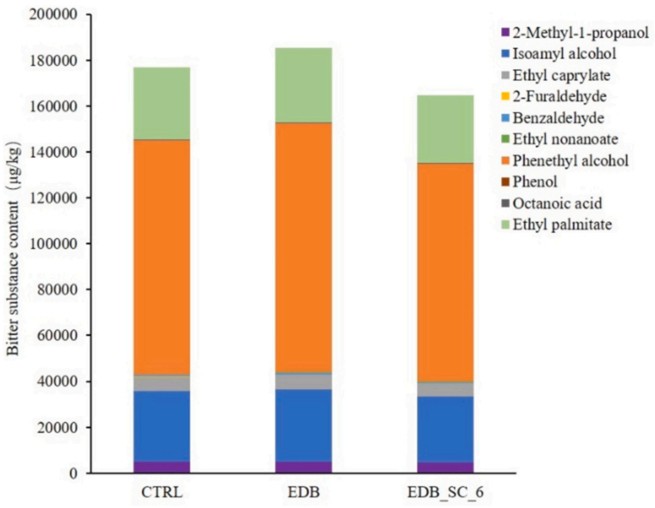

Fig. 2. Contents of delayed bitterness substances in three types of traditional *Huangjiu*.

Table 4
Amino acid threshold and concentration of fermented *Huangjiu*.

Amino acid	Threshold (mg/kg)	Concentration (mg/kg)		
		CTRL	EDB	EDB_SC_6
Val	400	192.75 ± 0.13^b	172.34 ± 3.25^c	267.44 ± 0.23^a
His	175	196.30 ± 0.26^a	114.20 ± 4.36^b	96.91 ± 2.74^c
Arg	320	550.41 ± 0.17^b	562.16 ± 10.65^a	411.36 ± 1.30^c
Ile	900	63.81 ± 0.52^c	118.24 ± 1.64^b	181.66 ± 0.16^a
Leu	3550	267.37 ± 0.94^b	291.02 ± 5.53^a	226.89 ± 4.18^c
Phe	800	250.64 ± 1.07^c	255.77 ± 2.30^b	289.37 ± 0.91^a
Lys	600	302.47 ± 3.92^a	285.10 ± 3.39^b	242.48 ± 0.61^c
Tyr	700	235.88 ± 3.82^b	256.40 ± 0.81^a	216.22 ± 8.80^c

Differential letters denoted the differences between the various groups.

EDB_SC_6 exhibited significant reductions in His, Arg, Leu, Lys, and Try. Arg accounted for approximately 25.20 % of all bitter amino acids, ranking it first among them, and it has a relatively low threshold of 320 mg/kg. After the addition of SC-6, the content of Arg decreased significantly by approximately 6 %. Given that Arg has a lower bitterness threshold, it likely plays a leading role in the perception of bitterness in *Huangjiu* (Yu, Li, et al., 2022). Similarly, the reduction in His content, which has a lower bitter threshold (175 mg/kg), also significantly impacts bitter perception.With the addition of SC-6, although the contents of Val, Ile, and Phe increased, these changes did not reach critical threshold levels and thus had minimal effect on the delayed bitterness of traditional *Huangjiu*. These results indicated that SC-6 introduction could significantly reduce the bitterness of traditional *Huangjiu* by reducing the production of both volatile delayed bitterness compounds and non-volatile bitter amino acids.

3.3. *Influence of fermentation of S. cerevisiae SC-6 on traditional Huangjiu flavor*

The unique raw materials and brewing technology provide traditional *Huangjiu* with alcoholic, caramel, fruity, herbal, incense smoke, Qu and honey flavor. Compared to the EDB sample, the sweetness and fruity aroma intensity of the EDB_SC-6 sample were significantly enhanced (Fig. 3A), reflecting the typical characteristics of southern *Huangjiu* (Yu et al., 2021; Yu, Li, et al., 2022), which may be attributed to the higher content of key esters (Li et al., 2023). Otherwise, estery, mellow and sauce notes with strong intensities in northern traditional *Huangjiu* were weaker in EDB_SC-6 sample (Ye et al., 2022).

The sensory evaluation of *Huangjiu* includes four descriptors: sweet, sour, astringent and umami (Lu et al., 2022). The electronic tongue analysis revealed distinct differences in the taste characteristics of the EDB sample before and after SC-6 inoculation. As shown in Fig. 3B, the EDB samples exhibited sour, astringent, and umami taste attributes, while sweetness was the weakest. However, following the introduction of SC-6, sweetness became more pronounced and sourness was reduced. It was evident that the introduction of SC-6 altered the flavor profile of EDB-*Huangjiu*, bringing it closer to that of the control group.

3.4. *Microbial diversity and composition of traditional Huangjiu fermented by SC-6*

3.4.1. *Analysis of microbial diversity*

Alpha diversity is primarily employed to reflect the richness and diversity of microbial community. The alpha diversity analysis of microbiota in traditional *Huangjiu* was conducted based on ace, chao, simpson and shannon indexes. As shown in Fig. 4 (A represented the bacterial group and B represented the fungal group), the ace index (Fig. 4A$_1$) and chao index (Fig. 4A$_2$) of EDB and EDB_SC_6 samples were significantly higher than those in the CTRL group. These results implied that introduction of additional strains increased richness of bacterial communities in traditional *Huangjiu* (Elizabeth et al., 2009). Similarly, the Shannon index (Fig. 4A$_3$) also increased after the introduction of additional strains, indicating a higher richness component and rarer cover types of bacterial communities. Meanwhile, the simpson index, which emphasizes evenness and dominant cover types, was higher in the CTRL samples than EDB and EDB_SC_6. Furthermore, no significant difference was observed between EDB and EDB_SC_6 in the alpha diversity of the bacterial community. For the fungi, the ace and chao indices showed no significant difference between groups (Fig. 4B$_1$ and 4B$_2$). Similar to bacterial communities, the simpson index values were higher, and the shannon index values were lower in EDB and EDB_SC_6 compared to CTRL, but no significant difference was observed between the CTRL and EDB_SC_6 (Fig. 4B$_3$ and 4B$_4$). Based on these observations, the richness and diversity of the microbial community in *Huangjiu* were influenced by the inoculation of exogenous strains, which was independent of the specificities of the strains themselves. Notably, bacterial communities were more significantly affected by exogenous strains than fungal communities.

Principal Coordinates Analysis (PCoA) is an unconstrained method for dimensionality reduction that can be used to study the similarities or differences in the composition of sample communities. The distribution area and distance of each sample in the PCoA score chart were used to determine the similarity between samples (Wang et al., 2024). As shown in Fig. 5A, the three groups were distributed across different quadrants, with the greatest distance observed between the CTRL and EDB, and a relatively smaller difference between the CTRL and EDB_SC_6. This suggested that the bacterial community in EDB-*Huangjiu* was normalized by the introduction of SC-6. According to Fig. 5B, there was minimal variation in the fungal community structure between EDB and EDB_SC_6. However, the obvious difference could been observed in the fungal community structure between CTRL and EDB, EDB_SC_6 group. The results also indicated that the influence of SC-6 on the bacterial community of EDB-*Huangjiu* was greater than its effect on the fungal community.

3.4.2. *Differences of bacterial flora structure in three kinds of traditional Huangjiu*

The bacterial community composition of three kinds of *Huangjiu* was

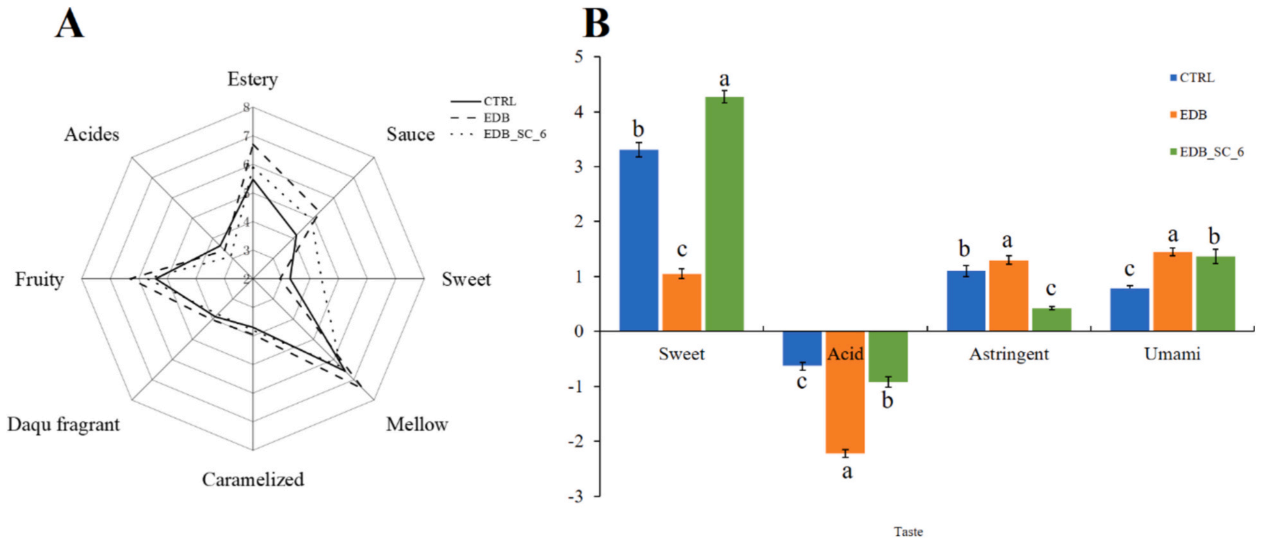

Fig. 3. Sensory evaluation of aroma (A) and electronic tongue results (B) in three types of traditional *Huangjiu*. Differential letters denoted the differences between the various groups.

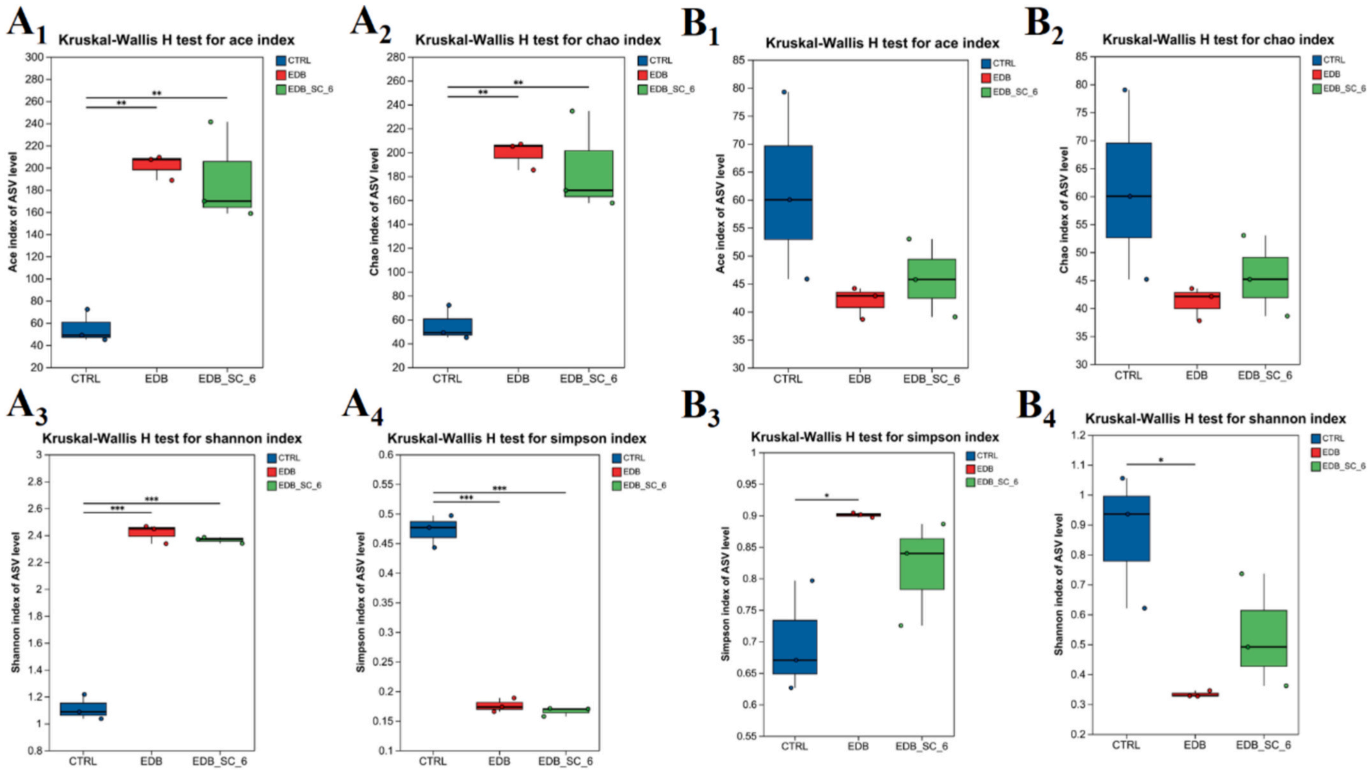

Fig. 4. Analysis of bacterial (A) and fungal (B) Alpha diversity in three types of traditional *Huangjiu*.

identified, as shown in Fig. 6A. Four predominant bacterial phyla with relative abundances exceeding 0.01 % at the phylum level were identified: Firmicutes, Actinobacteria, Proteobacteria, and Cyanobacteria. In the fermentation process of *Huangjiu*, Firmicutes, and Proteobacteria are the main bacteria in the fermentation of *Huangjiu* (Xie et al., 2013). After the addition of SC-6, the abundance of Firmicutes in EDB_SC_6 significantly decreased compared to that in EDB. Firmicutes were negatively correlated with 2-methyl-1-propanol, so an increase in Firmicutes will lead to a decrease of 2-methyl-1-propanol in *Huangjiu* samples (Liu et al., 2021). Fig. 6B showed the specific proportions of the microbial communities. The proportion of Actinobacteriota in EDB_SC_6 decreased by 28 % compared to that in EDB. Similarly, after the introduction of SC-6, the relative proportions of both Cyanobacteria and Proteobacteria in the EDB_SC_6 increased by 17 % compared to the EDB. Proteobacteria plays a pivotal role in a diverse range of fermented wines, exerting a significant influence on their quality and flavor profile (Zhao et al., 2021).

The primary bacterial genera, with relative abundances exceeding 0.01 at the genus level, numbered a total of ten, as depicted in Fig. 7A. Upon the addition of SC-6, the concentrations of *Weissella* and *Pediococcus* decreased significantly. *Weissella* contributed to the production of ethyl esters and higher alcohols (Guo et al., 2024). While, the reduction in *Pediococcus* leads to a decrease in the production of off-

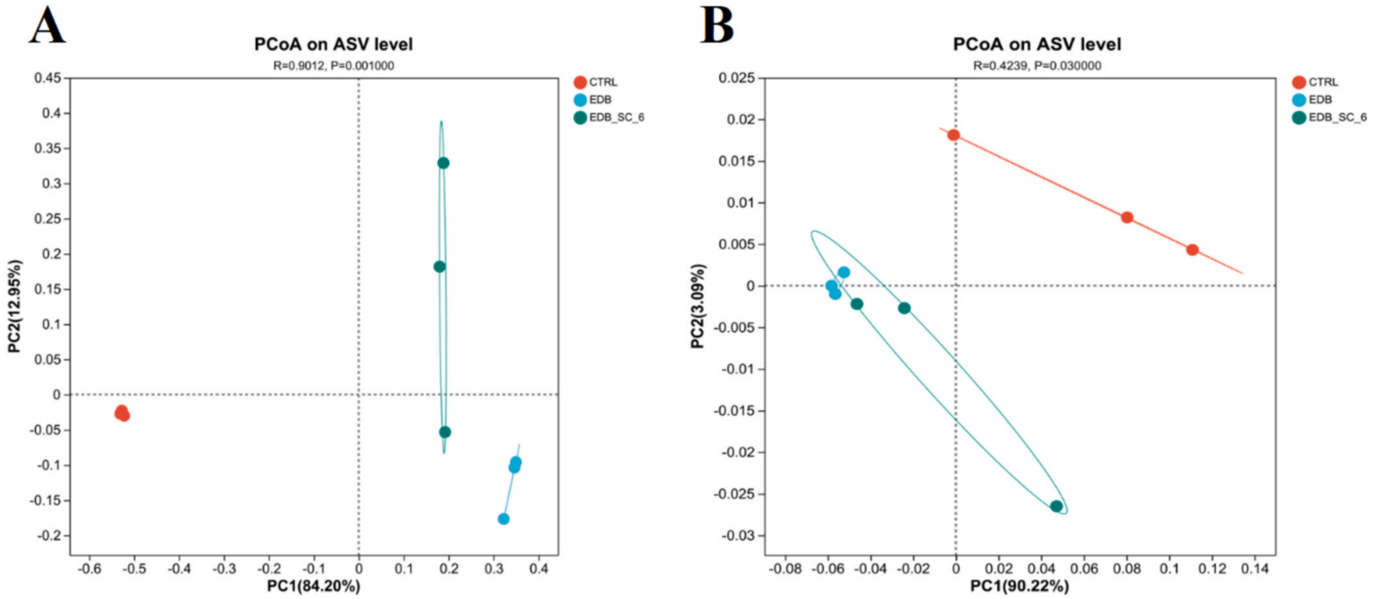

Fig. 5. PCoA diagram of bacteria (A) and fungi (B) in three types of traditional *Huangjiu*.

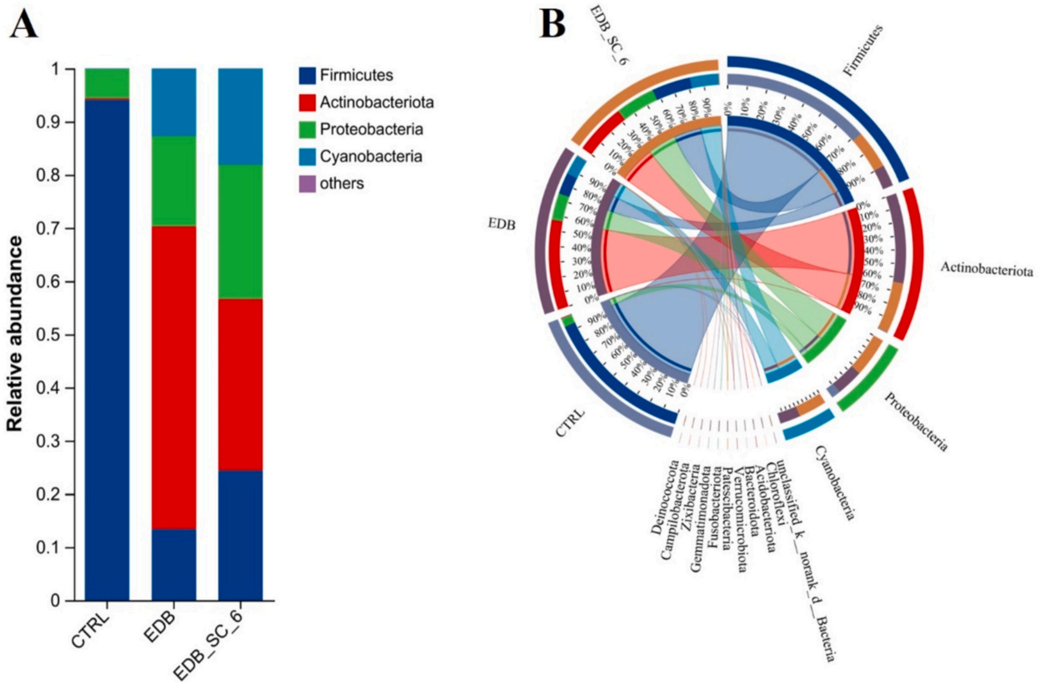

Fig. 6. Comparisons of bacterial phylum among three types of traditional *Huangjiu*.

flavors in the wine (Wade et al., 2018). Compared to EDB, the addition of SC-6 resulted in a significant increase in the abundance of norank-f-norank-o-Chloroplast and norank-f-Mitochondria.

The Linear Discriminant Analysis (LDA) value was employed to quantify the impact of species on the differential effect, implying that such species may hold a pivotal role in the process of environmental change. As shown in Fig. 7B, *Weissella*, *Klebsiella* and *Enterococcus* were considered as biomarkers of control traditional *Huangjiu*. The LDA plot revealed that after the introduction of SC-6, the abundances of *Enterobacterales*, *Staphylococcus*, and *Pseudomonas* were significantly higher than those in the other two groups. *Enterobacterales* contributes to the development of a more diverse and rich flavor profile in food (Ritschard et al., 2022). *Staphylococcus* species can enhance the ester aroma in fermented foods (Zhang et al., 2023). *Pseudomonas* is a common bacterium during the fermentation process and has a significant impact on the formation of wine flavors. Furthermore, it exhibits a strong positive correlation with compounds such as isopentanol, isobutanol, ethyl octanoate, and ethyl hexanoate (Wang et al., 2022). Conversely, the abundance of eight strains within the EDB was significantly higher compared to the other two groups. These strains include *Saccharopolyspora*, unclassified_f_Micromonosporaceae, *Rhodopseudomonas*, *Lactobacillus*, *Lachnoclostridium*, *Streptomyces*, *Bacillus*, and *Clostridium_sensu_stricto_1*. *Streptomyces* reduced the flavor compounds (alcohols and esters) contributed by yeasts (Du et al., 2015). The differential abundance of these taxa may be utilized to develop biomarkers for delayed bitterness in quality control of traditional *Huangjiu*.

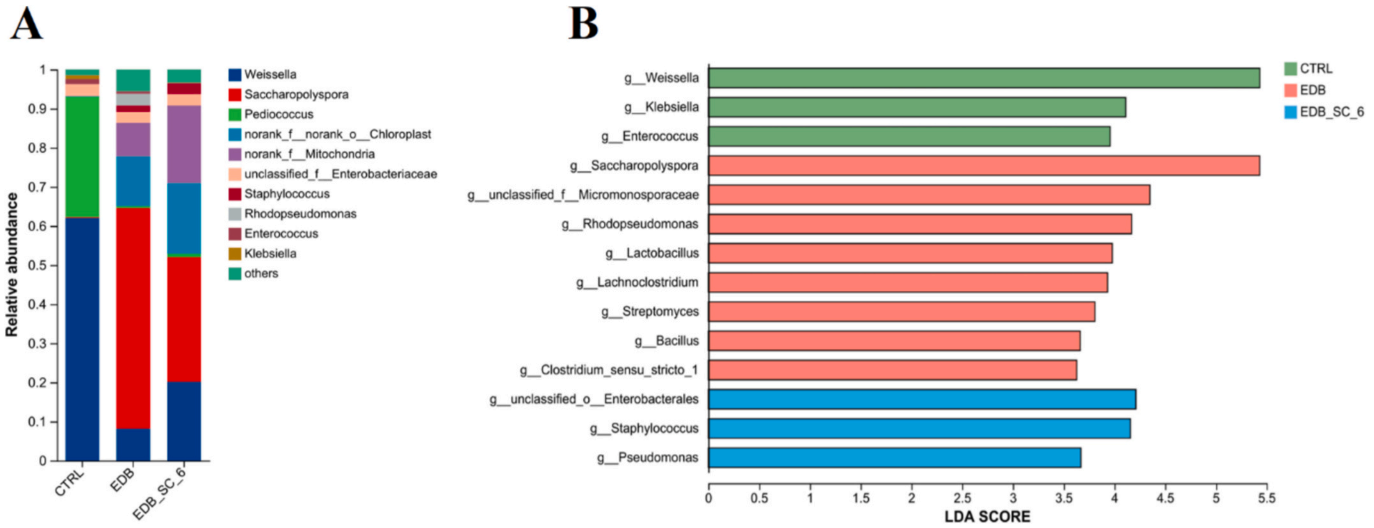

Fig. 7. Comparisons of bacterial genus level (A) and LDA (B) among three types of traditional *Huangjiu.*

3.4.3. Flora structure of fungi in three kinds of traditional Huangjiu

At the phylum level, a total of five fungal phyla with relative abundances exceeding 0.01 were detected (Fig. 8A). Among these, Ascomycota, the most dominant phylum, represents a key group in fungi, playing a crucial role during the fermentation process (Liu et al., 2021). Fig. 8B shown the specific proportions of the microbial communities. Compared to EDB, the EDB-*huangjiu* introduced with SC-6 showed a significant 14 % increase in Basidiomycota, while no significant changes were observed in other fungal phyla at the phylum level.

At the genus level of fungi (Fig. 9A), three primary fungi were detected: *Saccharomyces*, *Aspergillus*, and unclassified_f_Aspergillaceae. *Saccharomyces* and *Aspergillus* play crucial roles in the production of non-volatile differential metabolites (Zhao et al., 2021). *Saccharomyces* was identified as the most dominant fungal species in the three groups. After the inoculation of SC-6, *Saccharomyces* exhibited no significant change compared to EDB, while *Aspergillus* showed a slight increase. *Aspergillus* may play a role in the degradation of cellulose and is widely used as the primary saccharifying agent in naturally fermented traditional foods (Li et al., 2015; Liu et al., 2023). As shown in Fig. 9B, *Saccharomyces* were considered as biomarkers of EDB-*Huangjiu*, while *Candida* served as biomarkers in EDB_SC_6. *Candida* may contribute to the distinctive taste of the EDB_SC_6 *Huangjiu*. *Candida* species facilitate aroma release

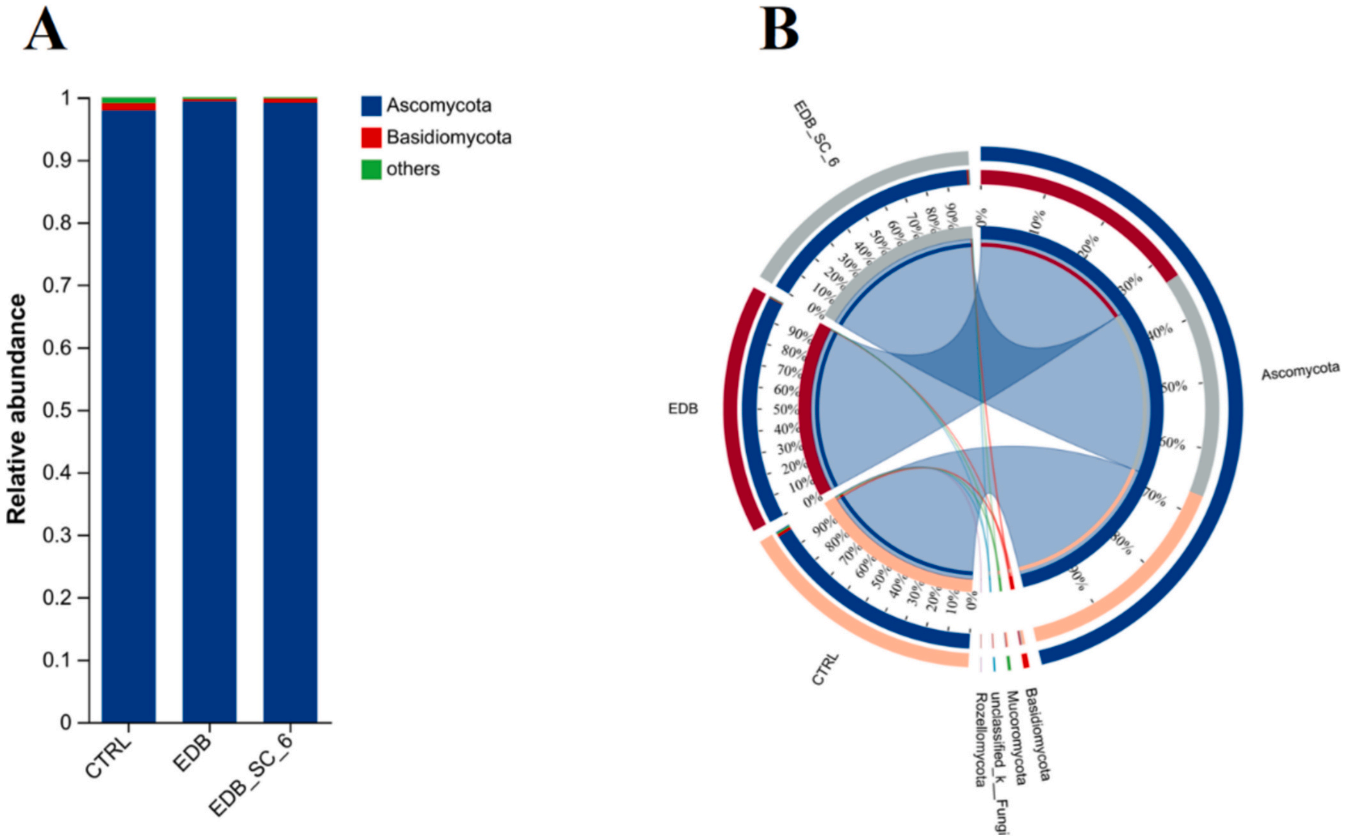

Fig. 8. Comparisons of fungal phylum among three types of traditional *Huangjiu.*

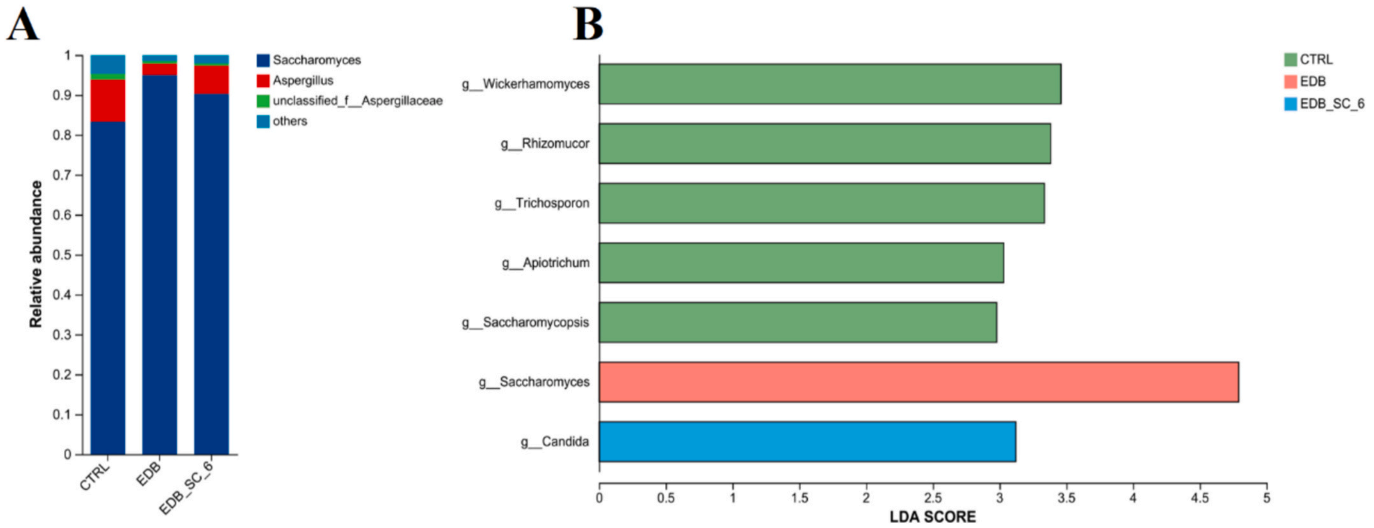

Fig. 9. Comparisons of fungal genus level (A) and LDA (B) among three types of traditional *Huangjiu*.

through the secretion of proteins, primarily enzymes, and the synthesis of new secondary metabolites. Additionally, they contribute to the stability of colored wine without depleting the available sugars in the must (Rodríguez et al., 2020).

3.4.4. *Relationship between flora and delayed bitterness substances*

To delve into the core functional microorganisms influencing the formation of delayed bitterness in traditional *Huangjiu*, this study conducted a correlation analysis between the microbial biomarkers identified through LDA and the18 bitter compounds present in traditional *Huangjiu*. Subsequently, a correlation heatmap was generated based on these data, wherein microbial names were aligned along the right side of the map, and bitter compounds in traditional *Huangjiu* were sequentially arranged at the bottom. Within this heatmap, red regions signify

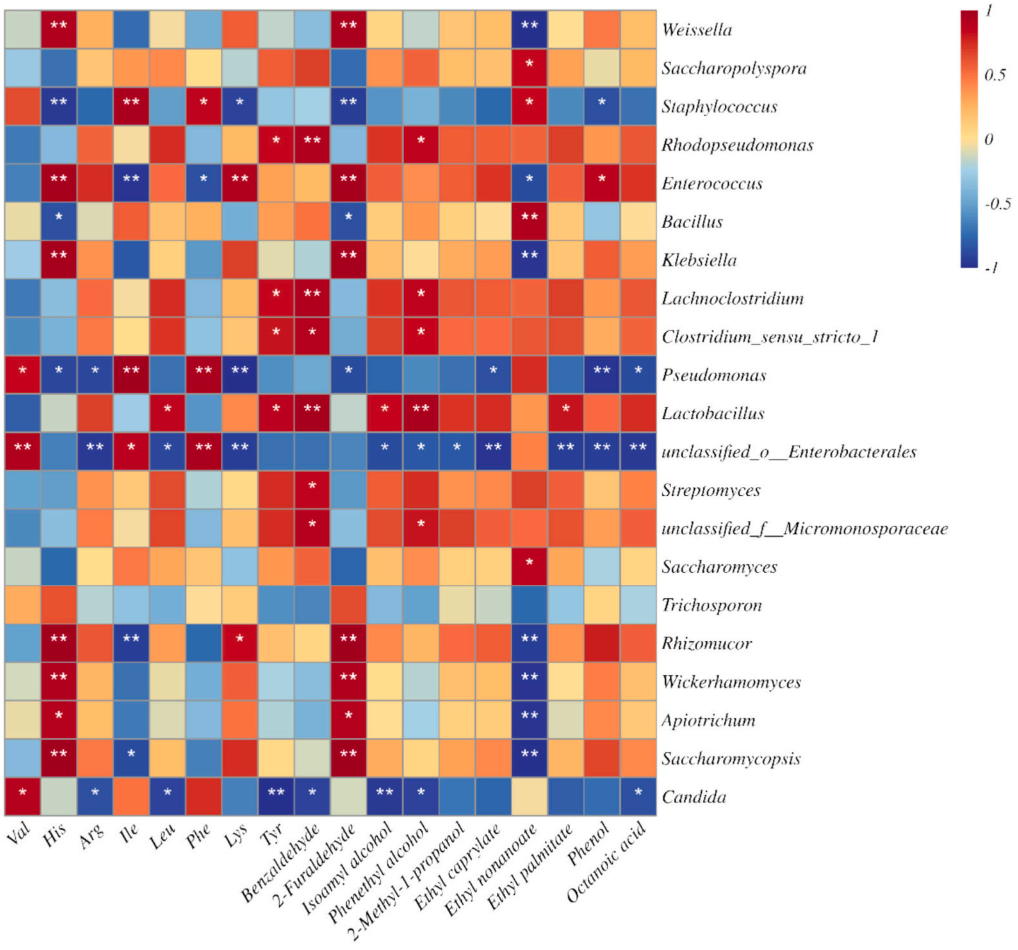

Fig. 10. Heatmap of Microbial-delayed bitterness compound correlations in traditional *Huangjiu*.

significant positive correlations, whereas blue regions indicate significant negative correlations.

As depicted in Fig. 10, numerous positive and negative correlations were observed. The aforementioned research indicated that His, Arg, ethyl caprylate, phenol, octanoic acid, and benzaldehyde had significant influences on the delayed bitterness of traditional *Huangjiu*. *Rhodopseudomonas,* unclassified_f_Micromonosporaceae, *Lachnoclostridium, Lactobacillus, Streptomyces,* and Clostridium_sensu_stricto_1 showed positive correlations with benzaldehyde. Additionally, *Rhizomucor, Wickerhamomyces, and Saccharomycopsis* demonstrated significant positive correlations with His and 2-furaldehyde, while *Enterococcus* correlated positively with His, 2-furaldehyde, and phenol. Upon adding SC-6, the relative abundances of *Rhodopseudomonas,* unclassified_f_Micromonosporaceae*, Lachnoclostridium, Lactobacillus, Streptomyces,* Clostridium_sensu_stricto_1 were significantly reduced compared to EDB. This reduction effectively decreased the production of flavor compounds associated with delayed bitterness, thereby mitigating the delayed bitterness characteristic in traditional *Huangjiu*.

Conversely, several significant negative correlations were also observed. Notably, *Staphylococcus* exhibited significant negative correlations with His and phenol. *Pseudomonas* negatively correlated with His, Arg, ethyl caprylate, phenol, and octanoic acid. Unclassified_o_Enterobacterales negatively correlated with Arg and ethyl caprylate, and *Candida* negatively correlated with Arg, benzaldehyde, and octanoic acid. In comparison to EDB, the addition of SC-6 resulted in increased relative abundances of *Staphylococcus*, *Pseudomonas*, unclassified_o_Enterobacterales, and *Candida*. The addition of SC-6 led to an increase in the abundance of these strains, which subsequently reduced the production of flavor compounds associated with delayed bitterness, thereby mitigating the a delayed bitterness in traditional *Huangjiu*.

Based on the correlation analysis, it can be preliminarily inferred that the content of delayed bitterness compounds were strongly correlated with *Rhodopseudomonas,* unclassified_f_Micromonosporaceae*, Lachnoclostridium, Lactobacillus, Streptomyces,* Clostridium_sensu_stricto_1, *Staphylococcus*, *Pseudomonas*, unclassified_o_Enterobacterales, and *Candida,* suggesting that these microbial genera may significantly contribute to the delayed bitterness in traditional *Huangjiu*. These microorganisms can impact the delayed bitterness of traditional *Huangjiu* by influencing the production of flavor compounds associated with delayed bitterness. Therefore, it is essential to control the excessive growth of these microorganisms associated with bitterness production during the fermentation process.

4. Conclusion

This study focused on the fermentation of traditional *Huangjiu* using inoculated *S. cerevisiae* strains, yielding significant findings relevant to managing bitterness in this beverage. Notably, fermentation with SC-6 significantly reduced delayed bitterness, correlating with decreased levels of both volatile delayed bitterness compounds and non-volatile bitter amino acids.

High-throughput sequencing and correlation analyses revealed that specific microbial populations, including *Rhodopseudomonas,* unclassified_f_Micromonosporaceae, *Lachnoclostridium, Lactobacillus, Streptomyces,* Clostridium_sensu_stricto_1, *Staphylococcus*, *Pseudomonas*, unclassified_o_Enterobacterales, and *Candida* were associated with bitterness and delayed bitterness levels. This finding may explain the superior performance of *S. cerevisiae* SC-6 in reducing delayed bitterness. Bioaugmentation with functional microorganisms was an effective strategy for reducing the delayed bitterness of traditional *Huangjiu* and improving its overall flavor by modulating the microbial community in traditional *Huangjiu*. However, this improvement may be accompanied by subtle alterations in its overall flavor profile. This research offered valuable methods and theoretical insights for regulating delayed bitterness in traditional *Huangjiu*. However, there remained ample room for further research in reducing delayed bitterness and enhancing the overall flavor of traditional *Huangjiu*. In light of this, future research and practice may consider incorporating strains with different characteristics for co-fermentation, aiming to reduce the disharmonious flavor, while further refining and enhancing the overall flavor dimensions of the *Huangjiu*.

Ethical statement

The content of the sensory evaluation experiment has been informed consent of the candidates and has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

CRediT authorship contribution statement

Haiyan Yu: Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Formal analysis, Conceptualization. **Shiqi Wu:** Writing – original draft, Investigation, Formal analysis. **Qiaowei Li:** Methodology, Investigation. **Chen Chen:** Supervision, Resources. **Qian Chen:** Writing – review & editing, Writing – original draft, Project administration. **Huaixiang Tian:** Supervision, Resources, Project administration.

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

Data will be made available on request.

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