Saccharomyces cerevisiae strains with low-yield higher alcohols and high-yield acetate esters improve the quality, drinking comfort and safety of *huangjiu*

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A B S T R A C T

Higher alcohols (HAs) and acetate esters (AEs) produced by yeasts are two important volatile flavor substances in fermented alcoholic beverages (FABs). To improve the FABs overall quality, lab-scale *huangjiu* brewing and systematic evaluation were performed using 171 *Saccharomyces cerevisiae* strains. Finally, two *S. cerevisiae* strains that produced lower HAs and higher AEs were obtained and named jiangnan1# and jiangnan3#, respectively. The results of production-scale *huangjiu* fermentation indicated that HAs produced by jiangnan1# sample decreased by 24.99 %, and AEs produced by jiangnan1# increased by 36.35 %. Sensory evaluation showed that the acidic taste, honey aroma attribute intensity were higher in 85# *huagnjiu*, and the fruity aroma attribute intensity was higher in jiangnan1# *huangjiu* ($P < 0.01$). Moreover, urea and ethyl carbamate produced by jiangnan1# strain were degraded by 13.89 % and 45.51 % compared with those of the control strain 85#, indicating the positive effects of jiangnan1# strain on health and safety. Thus, the obtained *S. cerevisiae* strains in this study can better enhance the flavor and improve the drinking safety and comfort of *huangjiu*.

1. Introduction

With the consumer demand for higher quality, healthier, and more diverse fermented alcoholic beverages (FABs), several new types of FABs have appeared and become popular because of their unique flavor characteristics and taste (Suárez-Lepe & Morata, 2012). The flavor profiles of FABs are usually considered to be the most important characteristic; they comprise metabolic intermediates or by-products, which are produced by essential brewing yeasts (Chen, Liu, Tian, Ai, & Yu, 2020; Kitagaki & Kitamoto, 2013). Higher alcohols (HAs) and acetate esters (AEs) are two important volatile flavor substances that ultimately determine the final quality and aroma of common FABs, including *huangjiu* (Chen, Xu, & Qian, 2013), beer (Dack, Black, & Koutsidis, 2017; Tokpohozin, Fischer, & Becker, 2019), wine (Valero, Moyano, Millan, Medina, & Ortega, 2002), and sake (Kitagaki & Kitamoto, 2013). The ratio of the content of esters (especially AEs) to HAs influences the sensory properties; currently, most FABs contain low ester and high HAs content. As the most abundant organoleptic compound, HAs in the

appropriate concentrations and proportions improve quality; however, excessive concentrations of HAs (>400 mg/L) negatively affect quality (an unpleasant flavor), health (headaches and intoxication), drinking safety, and comfort (headaches) (Sun et al., 2020). Compared with HAs and other yeast metabolites, esters are the only trace but the most important aromatic compounds in FABs. AEs such as ethyl acetate (EA, solvent-like aroma; has negative effects in excessively high concentrations of 150–200 mg/L), isoamyl acetate (IA, banana aroma), and 2-phenylethyl acetate (2-PA, roses and honey aroma) are desirable flavor components in esters, and they can positively affect aroma-active yeast metabolites when present below their threshold values in FABs (Steensels, Meersman, Snoek, Saels, & Verstrepen, 2014).

The appropriate content and proportion of HAs and AEs are related to multiple factors, such as yeast response to fermentation parameters (temperature, inoculation density, free amino nitrogen, or the carbon-to-nitrogen (C/N) ratio); however, regulation first relies on an efficient yeast strain (Zheng, Wei, Zhang, Xu, & Li, 2021). Yeasts, especially *Saccharomyces cerevisiae* have been used for at least eight millennia in the production of FABs, and their phylogenetic analyses have suggested a that Chinese origin of *S. cerevisi*ae. Meanwhile, the final aroma-active compounds varies highly among different yeast strains of *S. cerevisiae*, the dominant flavor-producing microorganism (Duan et al., 2018; Kitagaki & Kitamoto, 2013; McGovern et al., 2004). Chinese *huangjiu* (also known as Chinese rice wine or yellow wine) is a traditional FAB with over 9000 years of history, and is one of the world's oldest FABs along with beer and wine (Chen, Xu, & Qian, 2013; Wang et al., 2014). Systematic research on *S. cerevisi*ae in traditional *huangjiu* fermentation can not only improve the quality and safety of modern *huangjiu* (Chen, Xu, & Qian, 2018) but also provide new insights into biodiversity conservation and the evolution of the yeast *S. cerevisi*ae.

Traditional *huangjiu* (Fig. S1A) is typically fermented from steamed rice with wheat Qu (a molded cereal prepared by the natural inoculation of microorganisms) and JIUYAO (a rich source of microorganisms used in brewing), whereas modern *huangjiu* production widely utilizes pure cultured microorganisms (*S. cerevisiae*) to stabilize the product quality and output and achieve continuous production (Chen, Liu, Tian, Ai, & Yu, 2020). The unique brewing process endows *huangjiu* with its local style, high nutritional value, and high ethanol production (15–20 % vol); however, high HA content and relatively low AEs are restrictive factors for *huangjiu*. Yeast assimilable nitrogen effectively reduces the content of HAs in *huangjiu* fermentation, but since it is not controlled from the source, it increases the production costs (Liu et al., 2019; Wang et al., 2014). The raw materials, rice and wheat, used for *huangjiu* production can lead to a high content of amino acids, which can be used by *S. cerevisiae* to produce more HAs (Zhao, Liu, Han, Zhou, & Mao, 2022). There are several dedicated *S. cerevisiae* used in the production of FABs because of their dominant role, while fewer types of this yeast are used in the industrial production of *huangjiu* (Zheng, Wei, Zhang, Xu, & Li, 2021). However, no *S. cerevisiae* strains can be used to regulate the production of HAs and AEs in high-quality modern *huangjiu* owing to the spontaneous fermentation processes with multiple species of microorganisms in *huangjiu* brewing (Zhang et al., 2018). Therefore, it is an effective strategy to use *huangjiu* fermentation to evaluate HAs and AEs production by *S. cerevisiae* for FABs. Many *S. cerevisiae* strains have been selected to achieve certain goals such as enrichment of flavor, reduction of undesired byproducts, and controllable fermentation processes (Cadière, Aguera, Caillé, Ortiz-Julien, & Dequin, 2012). Moreover, new *S. cerevisiae* strains are still being improved and released in the market based on traditional biotechnological methods. Genetically modified (GM) techniques have been developed to improve wine quality by laboratory strains, but few have been successfully applied in the industrial production of FABs using these *S. cerevisiae* strains because of controversy (such as legislation issues and consumer perception) (Husnik et al., 2006). Moreover, the obtained strains adjusted to general fermentation parameters may inhibit the excellent traits of FABs and also have undesired side effects (Pires, Teixeira, Brányik, & Vicente, 2014). It is

difficult to obtain an efficient yeast strain that simultaneously increases the AEs content and decreases HA content and has an acceptable level of undesired side effects. Although many yeast strains are available on the global market, the generation of new strains that display different phenotypes and/or new traits (e.g., low-yielding ethyl carbamate (EC) and an appropriate ratio of HAs to AEs) continue to attract significant industrial interest (Kitagaki & Kitamoto, 2013; Suárez-Lepe & Morata, 2012).

In general, superior industrial yeast strains are the key and prerequisite for obtaining high-quality FABs. Development of a flavor-omics approach has played an important role in the analysis of the flavor compounds in wine. Molecular sensory science, which is used as a systematic research approach, has provided a flavor analysis and characterized key aroma compounds in FABs. Flavor-omics has been widely applied to analyze the volatile and non-volatile organic compounds such as organic acids (OAs), amino acids (AAs) and biogenic amines (BAs) (Guadalupe, Martínez-Pinilla, Garrido, Carrillo, & Ayestarán, 2012; Han et al., 2021; Schmidtke, Blackman, Clark, & Grant-Preece, 2013; Viegas, Esteves, Rocha, Melo, & Ferreira, 2021; Wang et al., 2014). Combining flavor-omics and *huangjiu* simulated fermentation to evaluate *S. cerevisiae* strains may be an effective strategy to obtain excellent strains that can retain the flavor characteristics as well as the desired performance of traditional starters. Therefore, direct tuning of the inherent production of yeast is a much better strategy for obtaining different yeast strains.

In the present study, more than 171 *S. cerevisiae* strains isolated from four typical *huangjiu* brewing wineries in the Shaoxing region were evaluated for their volatile flavor composition. Fermentative performances of the selected strains was also carried out to evaluate the final *huangjiu* quality. Different yeast strains contributed significantly to flavor differences. In particular, the production of HAs and AEs is regulated by the final obtained *S. cerevisiae* jiangnan1#, which can better enhance the flavor and improve the drinking comfort and safety of modern types of industrially produced *huangjiu*. This study establishes a yeast selection strategy without GM techniques to brew safe and high-quality *huangjiu* for the first time, and this can also applicable to other FABs.

2. Materials and methods

2.1. Reagents and chemicals

All chemical standards, internal standards and analytical grade solvents used in this study were purchased from commercial suppliers and used without further purification.

2.2. Collection of samples

A total of 24 fermented mash samples with fermentation times between 2 and 90 days were collected in this study. All samples were manufactured by Jianhu (JH), Shenyonghe (SYH), and Chanyeyuan (CYY) of Guyuelongshan Chinese Rice Wine Co., ltd. (Shaoxing, Zhejiang, China) and Tapai (TP) of the Zhejiang Pagoda Brand Shaoxing Rice Wine Co., Ltd. (Shaoxing, Zhejiang, China) following the standard traditional *huangjiu* making procedures and matured in a pottery jar. All the samples were refrigerated at 4 °C for further analysis. Detailed information on these samples is provided in Table S1. Representatives of these samples were used to determine the flavor of *Shaoxing-jiu* and the selection of flavor-contributing yeasts.

2.3. Isolation of yeast strains

All yeast strains were isolated from the fermented samples. Yeast extract peptone dextrose (YPD) medium was prepared with 20 g glucose, 20 g peptone, 10 g yeast extract, and 1000 mL ddH$_2$O at, a pH of 6.0–6.2. Serial dilutions (10^{-1} to 10^{-5}) of each sample were prepared using sterile water, and 0.1 mL of an appropriate dilution (10^{-3}, 10^{-4}, or

10^{-5}) was spread on YPD agar plates in triplicate. All yeast isolates were stored in an ultra-low temperature refrigerator at -80 °C using a glycerol-based standard storage medium (YPD containing 15 % [v/v] glycerol). In total, 140 yeast isolates were used in this study (Table S2). *S. cerevisiae* strain 85# was included as a control; it is widely used in modern *huangjiu* fermentation in Shaoxing (Zhang et al., 2018).

2.4. Evaluation of fermentation performance of yeast strains in FABs

2.4.1. Preparation of yeast starter culture (YSC)

A rice hydrolysate medium (RHM) was used for the small-scale *huangjiu* fermentation (SSHF) experiments. The raw rice used in the experiment were soaked in water at 60 °C for 30 min and steamed rice was obtained by steam heating. The ratio of the mixture contained raw glutinous rice (benchmark), raw wheat Qu (10 %), and water (400 %), which was then saccharified and liquefied with a saccharifying enzyme (10^6 U/mL) and thermostable α-amylase (2×10^5 U/mL) at 60 °C for 4–6 h. The resulting mixture was filtered through eight layers of sterilized gauze. All media were sterilized at 115 °C for 20 min. Monoclonal colony yeast cells were activated in 15 mL of YPD medium at 30 °C for 24 h by shaking the culture (150 rpm), and then transferred to 135 mL of 13° Brix RHM at 30 °C for 24 h. The harvested cultures were used as yeast starter culture (YSC) for further experiments.

2.4.2. SSHF evaluating the fermentation of S. cerevisiae strains

The YSC (57 mL) mentioned above was used for SSHF in a mixture of steamed glutinous rice (750 g), raw wheat Qu (58.5 g), and cooked wheat Qu (9 g), and water (750 mL) in a 3 L flask. The mash was incubated at 30 °C under stable conditions for 5 days, and post-fermentation was performen at 15 °C for 15 days. All fermentation experiments were performed in triplicate. The ratio of raw materials was increased to evaluate the commercial applicability of the obtained *S. cerevisiae*. The pilot-scale application and performance evaluation were completed in a 150 L fermenter. Analysis of the enological parameters and gas chromatography–mass spectrometry (GC–MS) analysis were used to evaluate different yeast isolates. An additional strain, designated as 85#, was included in all the experiments as a reference strain for the fermentation performance.

2.4.3. Production-scale experiment of fermentation conditions for huangjiu

The industrial application of the obtained strains was carried out in accordance with actual factory production in a 30 kL fermenter. Four batches of *huangjiu* were fermentated (120 kL) with jiangnan1#, 85# (control), jiangnan3#, or 85# (control). According to the factory's modern mechanized production volume and production process, *huangjiu* productions were brewed using the same *S. cerevisiae* strain in four fermentation tanks (usually more than 30 kL) each day. After 3–5 days of pre-fermentation, the content of the four tanks were mixed into the other tank (usually more than 120 kL) for post-fermentation for 15–20 days. Finally, the processes of squeezing, frying, jars, and aging for the obtained *huangjiu* were performed according to the production process in the factory.

2.4.4. Analysis of fermentation parameters

The fermentation parameters (pH, total acid, and amino acid nitrogen) were determined using the method described by Liu et al (). The 3,5-dinitrosalicylic acidmethod was used to detect residual sugars in *huangjiu* samples (Miller, 1959). The urea concentration was determined using diacetyl monoxime reactions (Zhao et al., 2014).

2.4.5. Quantitative analysis of non-volatile substances

Non-volatile substances biogenic amines (BAs), organic acids (OAs), and amino acids (AAs) were determined by HPLC according to the described methods of *huangjiu* (Wang et al., 2014).

2.4.6. Quantitative analysis of volatile flavor compounds

For the rapid determination of volatile flavor compounds in *huangjiu*, the headspace solid phase microextraction (HS-SPME) technique was used to extract the flavor compounds, and dispersive liquid-liquid microextraction was used for determining the main HAs with 4-methyl-2-pentanol (0.4536 g/L in ethanol) as an internal standard. The samples were prepared following a previously described method (Zhou et al., 2020). To determine the key aroma compounds and eliminate variations in extraction efficiency caused by small differences in the sample matrix, 2-octanol (0.1018 g/L in ethanol) was used as a standard to quantify the analytes using GC–MS. The EC in the samples was quantified using GC–MS after extraction by HS-SPME, and *n*-propyl carbamate (nPC) (0.1500 g/L in ethanol) was used for internal standardization (Liu et al., 2018).

2.4.7. Analysis of aroma and taste profiles

The evaluated *huangjiu* samples were obtained at the production-scale mentioned above. The sensory panel test was at National Rice Wine Engineering Technology Research Center (Shaoxing, Zhejiang, China) by a tasting panel composed of twenty-six panellists. Finally, ten trained panellists (six women and four men) between twenty-five and fifty-five years of ages with considerable tasting experience and substantial experience in *huangjiu* sensory analysis. The *huangjiu* description terminology according to a consensus included seven aroma descriptions (alcoholic, smoky, Qu aroma, honey, herb, caramel, and fruity) and five taste attributes (acidic, sweet, bitter, astringent, and umami). The sensory attributes were scored on a five-point scale according to the methods, ranging from 0 (not perceivable) to 5 (strongly perceivable). Information of sensory attributes tested in this study were showed in Table S7 (Chen, Xu, & Qian, 2013). After training and a preliminary test, the panel demonstrated good abilities in consistently, stably, and repeatedly, carrying out sensory analysis of *huangjiu*. Subsequently, two types of *huangjiu* samples were evaluated by the panel.

2.5. Statistical analysis

Principal component analysis (PCA) was performed to analyze the profiles of different *S. cerevisiae* strains and volatile compounds using SIMCA software (ver. 14.1) UMETRICS, Sweden). Statistical analysis was performed using GraphPad Prism 8, and the results were expressed as mean ± standard deviations. One-way analysis of variance (ANOVA) test with Duncan's multiple range test was applied to the data obtained from the chemical quantitative data to identify significant differences ($P < 0.05$) between different *huangjiu* fermented by different yeast isolates.

3. Results and discussion

3.1. Selection of S. cerevisiae strains with superior fermentation performance

To obtain strains with superior fermentation performance, 171 *S. cerevisiae* isolates were used as starters for *huangjiu* production at low temperature and long-term fermentation (approximately 90 days) using traditional production technology (Fig. S1A). The enological parameters of the *huangjiu* samples were determined and the results were shown in Table S2. Further analysis was conducted for the samples using GC-MC; the ethanol content in these samples were equivalent to that of produced by the control strain *S. cerevisiae* 85# (15.7 to 19.0 % vol). Other enological parameters (total acid, residual sugar, and amino acid nitrogen) were within adequate ranges in all the *huangjiu* samples meeting the national standard "*Huangjiu*" (GB/T 13662-2018).

Different yeast strains significantly influenced the concentrations of the volatile compounds, particularly HAs (i.e., *n*-propanol, isobutanol, isoamyl alcohol, and 2-phenylethyl alcohol), because of the metabolic differences in *huangjiu* fermentation. These HAs represent important variables for the differentiation of *S. cerevisiae* strains. However, the

production of other alcohol, aldehyde, acid, and ester compounds was extremely variable and *S. cerevisiae* strains specific. The PCA demonstrated an adequate separation of the different *huangjiu* fermented with different strains in all the four batches; three groups were clearly defined (Fig. 1). When all 28 key volatile flavor compounds and *S. cerevisiae* isolates were included (Fig. 1), the first principal component (PC1) explained 53.6 %, 47.1 %, 56.5 %, and 77.5 % of the total variation and PC2 explained 15.1 %, 19.1 %, 25.8 %, and 10.4 % of the total variation. The first two principal components were statistically significant in explaining the differences between the *S. cerevisiae* strains. Positive loading of PC1 was pivotally related to HAs and AEs, whereas HAs showed the negative loadings and EA, IA, and 2-PA showed positive values (Fig. 1D). For PC2, loadings were characterized by minor values for the three groups, which were not analyzed in this study. The *S. cerevisiae* strains illustrated as (four-pointed stars) and clustered in the same area (purple circle) with the control strain showed high HA and high ester content (HH-HE), while *S. cerevisiae* strains (five-pointed stars) with lower HA and higher ester content (LH-HE) gathered in a black circle. The remaining *S. cerevisiae* strains (triangles) with higher HA and lower ester contents (HH-LE) were distributed on the left and right sides (green circle). Compared with the control, *S. cerevisiae* strains with lower HAs (<600 mg/L, 85# strain 534.34 ± 19.37 mg/L) and higher AEs content (>170 mg/L, 85# strain 182.19 ± 7.69 mg/L) from the two groups (four and five-pointed stars) were selected for further evaluation.

Interestingly, no strains with lower HAs and lower esters content (LH-LE) appeared in the normally fermented *huangjiu* samples. A possible reason could be that the esters in FABs comprise mainly ethyl esters (ethyl acetate and ethyl lactate) that are esterified from fatty acids and ethanol, and AEs (EA, IA, 2-PA) are synthesized from acetyl-coenzyme A (acetyl-CoA) and ethanol or HAs (Fig. S1B) (Verstrepen et al., 2003). The yeast strains used for FABs produce high ethanol, while HAs and esters were the metabolic intermediates or by-products of brewing yeasts (Kitagaki & Kitamoto, 2013; Pires, Teixeira, Brányik, & Vicente, 2014; Tokpohozin, Fischer, & Becker, 2019). The low HA content produced by the strain may be related to the production of esters, which may explain why there was no LH-LE *S. cerevisiae* in this study.

3.2. Comparison of different S. cerevisiae strains with HAs and AEs

Each batch of *S. cerevisiae* isolates exhibited three types of traits, defined as HH-LE, LH-HE, and HH-HE, respectively. However, flavor and fermentation characteristics still differed in *huangjiu* fermented using different *S. cerevisiae*. Some strains showed different performances in primary screening and secondary screening. The long-term post-

fermentation (90 days) of traditional *huangjiu* brewing process could reach the required alcohol content (15–20 % vol), whereas the modern *huangjiu* technology with shortened post-fermentation time (20 days) could not meet production requirements without superior *S. cerevisiae* strains as starters (Fig. S1A). The following fermentation experiments were conducted with modern *huangjiu* technology, because the obtained yeast *S. cerevisiae* strain could be used as a starter in the future. Two rounds of laboratory-scale *huangjiu* fermentation were performed to determine the better LH-HE *S. cerevisiae* by comparing the contents of HAs and AEs produced. In total, 54.43 % (43/79) strains demonstrated good fermentation performance—Jianhu (12/22), Tapai (12/21), Shenyonghe (15/17), and Chanyeyuan (4/19) (Tables S2 and S3). The results of fermentation properties showed that all experimental strains could meet the needs of *huangjiu* fermentation referred to as "Huangjiu", although some indicators differed between different strains (Tables S2 and S3). Seven key flavor substances that contained four HAs and three AEs above were used to evaluate the characteristics of the strains. Moreover, the ability of a strain to produce HAs and AEs was evaluated based on the content produced per unit of ethanol (1 % vol) for industrial applications in other FABs. Considering the volatility of different fermentation batches, an additional round of fermentation was carried out for the excellent strains to ensure accuracy. The results indicated that the target strains performed well and showed high AEs and low HAs characteristics in both batches of fermentation (Figs. S2 and S3). However, the *S. cerevisiae* strains isolated from different factories showed differences under the same process, caused by the traditional brewing of wheat Qu or JIUYAO produced with the varieties and proportions of microorganisms present varied with geographical location, manufacturer, or batch. Yeast *S. cerevisiae* is a starter with varying abilities to metabolize different substrates to produce a diverse array of aroma compounds. They are core microorganisms in JIUYAO and have been shown to affect fermentation and the volatile profile (Cai et al., 2018; Chen, Liu, Tian, Ai, & Yu, 2020). Compared with the control strain 85#, the obtained strains showed good performance in both batches and have the potential to be used in industrial production. Two LH-HE *S. cerevisiae* strains with superior aroma production in each brewery were obtained. A systematic evaluation was performed to obtain the best overall performance of the eight strains obtained in 5 L laboratory-scale *huangjiu* fermentation. Comparison of the eight LH-HE *S. cerevisiae* strains with HAs and AEs in *huangjiu* fermentation, CYY-661 and JH-525 showed better HAs and AEs production, but high and low ethanol production, respectively (Tables S4 and 1). *S. cerevisiae* strains CYY-661 (number CCTCC 2021523) and JH-525 (number CCTCC 2021525) were preserved in China Center for Type Culture Collection and named jiangnan1# and jiangnan3#, respectively, for follow-up research.

Fig. 1. Biplots of the two principal components after principal component analysis (PCA) of 28 key volatile flavor compounds. A: Jianhu. B: Tapai. C: Shenyonghe, and D: Chanyeyuan.

Table 1
Production of higher alcohols and acetate esters in *huangjiu* fermentation by *S. cerevisiae* strains of four factories.

Items	Control	Experimental strains							
	85#	JH-525	JH-506	TP-514	TP-554	SYH-609	SYH-632	CYY-661	CYY-690
n-Propanol	3.94 ± 0.35[bc]	4.44 ± 0.23[cd]	4.83 ± 0.49[d]	5.31 ± 0.60[d]	4.94 ± 0.25[d]	3.80 ± 0.11[b]	4.19 ± 0.07[c]	3.18 ± 0.12[a]	5.04 ± 0.22[d]
Isobutanol	7.49 ± 0.54[c]	6.49 ± 0.18[b]	6.82 ± 0.15[bc]	7.36 ± 0.55[c]	6.81 ± 0.25[bc]	7.48 ± 0.22[c]	7.56 ± 0.25[c]	5.42 ± 0.14[a]	7.76 ± 0.51[c]
Isoamyl alcohols	16.91 ± 0.43[ef]	10.09 ± 0.15[a]	16.32 ± 1.10[ef]	14.62 ± 0.65[de]	16.00 ± 0.56[e]	14.29 ± 0.67d	17.55 ± 0.32[f]	12.34 ± 0.34[c]	11.52 ± 0.20[b]
2-Phenethyl alcohol	7.58 ± 0.29[c]	5.90 ± 0.27[ab]	6.21 ± 0.50[ab]	5.62 ± 0.25[a]	6.88 ± 0.32[b]	6.41 ± 0.33[b]	7.13 ± 0.30[bc]	5.78 ± 0.09[a]	7.78 ± 0.17[c]
Total HAs	35.92 ± 1.52[cd]	26.92 ± 0.25[a]	34.18 ± 1.97[bc]	32.92 ± 1.63[bc]	34.63 ± 0.52[c]	31.99 ± 1.27[bc]	36.44 ± 0.69[d]	26.72 ± 0.66[a]	32.10 ± 1.06[b]
Ethyl acetate	101.45 ± 2.17[a]	139.7 ± 6.88[d]	122.26 ± 2.41[c]	108.84 ± 1.10[b]	101.68 ± 1.23[a]	122.62 ± 1.68[c]	118.22 ± 6.97[c]	127.24 ± 5.83[cd]	117.16 ± 8.02[c]
Isoamyl acetate	18.17 ± 0.24[c]	34.84 ± 1.23[f]	51.45 ± 0.58[g]	28.67 ± 0.44[e]	24.57 ± 0.45[d]	8.89 ± 0.97[a]	12.23 ± 1.01[b]	26.24 ± 1.92[de]	8.37 ± 0.24[a]
2-Phenylethy acetate	15.91 ± 0.33[b]	46.14 ± 1.56[f]	59.69 ± 0.68[g]	16.51 ± 0.36[b]	12.18 ± 0.29[a]	11.81 ± 1.19[a]	14.56 ± 1.99[b]	34.83 ± 1.63[e]	24.43 ± 0.78[c]

The unit of higher alcohols is mg/L/(% vol), ethyl acetate (mg/L), isoamyl acetate (μg/L), and 2-phenylethy acetate (μg/L). Values are means ± standard deviations from at least three independent tests. Values with different letters in the same row are significantly different ($P < 0.05$) from each other using Duncan's multiple comparison tests.

3.3. Evaluation of S. cerevisiae strains jiangnan1# and jiangnan3# with stable production

To verify that the *S. cerevisiae* obtained after natural selection and elimination had stable characteristics, the final two strains preserved in the glycerol tube were successively coated on the plate (six generations) and the yeast were separated after fermentation with the starter. Both the two types of strains were tested to verify fermentation stability by another batch of lab-scale *huangjiu* fermentation. The results of enological parameters, HAs and AEs were not significantly different between the two types of target strains, which indicated that the *S. cerevisiae* strains jiangnan1# and jiangnan3# with unique HAs and AEs production are stable traits of *S. cerevisiae* (Table 2). This suggests that yeast isolates are always genetically stable during *huangjiu* fermentation. Furthermore, *S. cerevisiae* jiangnan1# produced higher amounts of ethanol and acetic acid than 85#, and acetic acid transformed into acetyl-CoA, which related to the formation of esters (Fig. S1B). *S. cerevisiae* jiangnan3# produced higher amounts of acetic acid and lower amounts of ethanol than 85# since ethanol participated in the synthesis of higher EA. As more AEs produced, less acetic acid converted into acetyl-CoA. Previous studies have only considered the production of HAs, and not esters, in FABs (Liu, Ma, et al., 2021; Pires, Teixeira, Brányik, & Vicente, 2014; Shi et al., 2021). Compared with distilled liquor, FABs contain higher HAs, accompanied by a high ethanol content, and lower AEs. The formation of HAs and AEs by yeast involved complex enzymatic and regulatory pathways. It depended on the yeast strains and fermentation conditions in *huangjiu* production, which in turn may be associated with the expression and regulation in key genes of *S. cerevisiae*. Genetic engineering of genes coding for key enzymes is used for their potential of yeast strains in AE and HA biosynthesis. For instance, *ATF1*, *ATF2*, and *BAT2* are important genes of *S. cerevisiae* that are related to AE and HA production, while *ATF1* and *ATF2* can lead to remarkable changes in the AE production (Fig. S1B) (Ma, Huang, Du, Tang, & Xiao, 2017; Zhang et al., 2013). Further studies on the LH-HE strains jiangnan1# and jiangnan3# can explain the differences in formation mechanism and genetic evolution.

3.4. Industrial application of S. cerevisiae strains jiangnan1# and jiangnan3#

Huangjiu fermentation in 150 L fermentation tanks and 5 L laboratory-scale tanks was conducted to verify the performance of the strains before industrial application. The enological parameters of jiangnan1# and jiangnan3# showed that they had the ability to produce higher HAs and lower AEs than 85# (Table S5). To assess the applicability of the obtained *S. cerevisiae* strains for commercial industrial production, a production-scale experiment of the fermentation conditions for *huangjiu* (120 t) was carried out. The *huangjiu* sample fermented with *S. cerevisiae* jiangnan3# was not squeezed separately as the alcohol content (14.6 % vol) could not meet the production requirements. However, this strain had the potential for producing lower alcohol and healthier *huangjiu*, along with HAs and AEs contents, in the future content (Table S6). The pre-fermentation of *S. cerevisiae* jiangnan1# started quickly, which prevented contamination by miscellaneous bacteria and ensured stable production. For the other strain *S. cerevisiae* jiangnan3#, the ethanol production maintained a growth trend for 24 h,

Table 2
Production of of higher alcohols and related acetate esters in *huangjiu* by *S. cerevisiae* strains jiangnan1# and jiangnan3#.

Compound	Concentration					Aroma impression	P-value
	Control-85#	[1]jiangnan1#	[2]jiangnan1#	[1]jiangnan3#	[2]jiangnan3#		
Ethyl acetate	81.91 ± 8.45[a]	100.55 ± 8.12[b]	110.05 ± 5.28[b]	137.66 ± 10.58[c]	123.61 ± 9.85[c]	Fruity, solvent	<0.01
Isoamyl acetate	44.07 ± 6.53[a]	109.67 ± 9.04[b]	112.57 ± 3.54[b]	259.03 ± 15.45[c]	249.53 ± 12.25[c]	Banana	<0.001
Phenylethyl acetate	50.59 ± 7.49[a]	76.05 ± 8.54[b]	76.05 ± 5.17[b]	120.31 ± 10.14[c]	115.65 ± 8.57[c]	Roses, honey	<0.01
n-Propanol	70.08 ± 1.23[b]	64.71 ± 3.76[a]	83.30 ± 3.64[c]	65.45 ± 2.55[a]	81.38 ± 1.25[c]	Alcoholic, sweet	<0.01
Isobutanol	113.19 ± 2.65[b]	107.80 ± 3.14[b]	104.72 ± 3.28[ab]	103.39 ± 1.67[ab]	100.85 ± 2.45[a]	Solvent	<0.05
Isoamyl alcohol	302.18 ± 2.29[b]	258.55 ± 1.41[a]	266.71 ± 7.44[a]	256.45 ± 2.45[a]	254.56 ± 3.45[a]	Alcoholic, banana	<0.01
2-Phenylethyanol	146.57 ± 5.03[c]	110.83 ± 3.10[b]	108.63 ± 4.57[ab]	112.25 ± 2.15[b]	103.00 ± 3.58[a]	Rose	<0.05

The unit of isoamyl acetate and 2-phenylethy acetate is μg/L, the other compounds are mg/L. [1]The final two strains preserved in the glycerol tube were successively passaged on the plate (6 generations). [2]The yeast were separated after the fermentation of the sample with the target strain as the starter. Values are means ± standard deviations from at least three independent tests. Values with different letters in the same row are significantly different ($P < 0.05$) from each other using Duncan's multiple comparison tests.

but the ethanol content was 1–2 % vol lower since then, which might be associated with the generation of more acetic acid and AEs (Fig. 2). Determining the key aroma compounds of the pilot strain after the fermentation, indicated that *huangjiu* produced by jiangnan1# had 24.99 % decrease in the HA content (20.97 % isobutanol, 27.66 % iso-amyl alcohol, and 32.73 % 2-phenylethanol), 36.35 % increase in the AE content (36.31 % EA, 14.31 % IA, and 54.32 % 2-PA). However, HAs (12.24 % isobutanol, 10.91 % isoamyl alcohol and 28.06 % 2-phenyle-thanol) decreased by 14.87 %, AEs (45.37 % EA, 19.78 % IA, and 65.52 % 2-PA) increased by 22.85 % in final product of jiangnan3# (Fig. 3A, B). Briefly, *S. cerevisiae* jiangnan1# and jiangnan3# produced high amounts of esters and acids, but low amounts of alcohols and aldehydes.

OAs, AAs, BAs, urea, and EC as well the volatile compounds were also determined to evaluate and analyze the applicability of these strains. The most common harmful component, EC, is formed by the spontaneous reaction between urea and ethanol, and its average con-centration is 160 µg/kg in *huangjiu* (Zhao et al., 2013). In the present study, the concentrations of EC and urea produced by pilot strains in *huangjiu* fermentation were measured, and the results (Fig. 4A, 4B) showed that the jiangnan1# strain degraded 13.89 % of urea and 45.51 % of EC, while the concentrations of urea and EC decreased by 10.19 % and 41.4 % in jiangnan3#, respectively. It is clear that the presence of EC in *huangjiu* would affect its quality and drinking safety, although there is no legislation about the maximum allowed level for EC in *huangjiu* to date. EC concentrations in *huangjiu* range from 8 to 515 µg/kg, it can reach up to 242.2 µg/kg in some products, as detected using high performance liquid chromatography-fluorescence detection (HPLC-FLD) method (Fu et al., 2010; Wu et al., 2011). However, the EC concentration of jiangnan1# (73.01 µg/kg) and jiangnan3# (67.91 µg/kg) were below 100 µg/kg, lower the maximum level of EC in sake (another kind of rice wine similar to *huangjiu*) in America (Zhang et al., 2018; Zhao et al., 2013). As one of the rich indispensable components and flavor precursor substances of *huangjiu*, AAs provide various tastes such as umami, acerbity, sweet, bitter, and briny. The total AA con-centrations of jiangnan1# and jiangnan3# were significantly different (*P* < 0.05) but did not affect the overall taste (Figs. S5 and 5). The content and type of OAs can influence the final quality of *huangjiu*. Furthermore, OAs affect the color, flavor and biological stability of *huangjiu*, and an appropriate amount of OAs can reduce the sweetness, enhance the strong flavor, and promote the formation of esters during storage (Wang et al., 2014). The total OA concentrations of jiangnan1# and jiangnan3# showed significant differences (*P* < 0.05) compared

with those of 85# owing to the different acetic acid contents (Fig. S6). BAs are formed mainly by microbial decarboxylation of AAs, which are low molecular organic nitrogenous compounds widely present in FABs (Gardini, Özogul, Suzzi, Tabanelli, & Özogul, 2016). Excessive BAs can cause adverse physiological effects in the human body and pose risks to the drinking safety of FABs. Although seven types of BAs were detected in *huangjiu*, only four were present in < 5 mg/L; others were present in < 1 mg/L (Fig. S7). Meanwhile, the total content of BAs was relatively low owing to the short rice soaking time, and *S. cerevisiae* in *huangjiu* brewing contributed less to BAs (Liu et al., 2021b). However, the *huangjiu* sample fermented by *S. cerevisiae* jiangnan1# had higher safety and better quality. A higher ratio of ester content to HAs is known to influence sensory properties, especially the enhanced fruity flavor of FABs (Steensels, Meersman, Snoek, Saels, & Verstrepen, 2014).

3.5. Sensory evaluation

To determine the sensory differences between *huangjiu* fermented by *S. cerevisiae* 85# and jiangnan1#, aroma and taste profiles were deter-mined by well-trained panelists. The results (Fig. 5) showed that the honey and acidic attributes for jiangnan1# were significantly weaker (*p* < 0.01) but fruity attribute was higher (*p* < 0.01) compared with those of the control 85#. This might be because of the lower contents of HAs (*p* < 0.05) and 2-phenylethanol (rose aroma, *p* < 0.01) and the higher content of ethyl acetate (fruity, *p* < 0.01) (Tables 2 and, S6). The overall aroma profile of the young *huangjiu* is relatively simple, resulting in the higher intensity of the fruity aroma in young *huangjiu* than in aged *huangjiu* (Chen, Wang, Qian, Li, & Xu, 2019). A more fruity aroma is better for enhancing the theme aroma of aged *huangjiu*. However, wines with increased eater contents possess an enhanced fruity flavor (Valero, Moyano, Millan, Medina, & Ortega, 2002). The higher AEs content is another important factor that enhances the aroma of aged *huangjiu*. The *huangjiu* samples fermented by jiangnan1# had a higher content of AEs and lower content of HAs, which had positive effects on improving the quality of *huangjiu*. To more intuitively evaluate the drinking comfort of the two types of *huangjiu*, several notable physical symptoms of hang-overs with clearly perceived characteristics were considered. Ques-tionnaires, animal behavior evaluation systems, and biomarker evaluation systems have been applied to evaluate the drinking comfort of alcoholic beverages. However, there is a lack of population-based studies to explain the mechanism of hangovers caused by FABs, which influences the drinking comfort. In our previous studies, we found that the key substances HAs, BAs, aldehydes, and ethanol in alcoholic bev-erages affect drinking comfort, and we analyzed their metabolic rates in the blood through animal experiments (Cryprinus carpio intoxication and Sprague-Dawley models) (Sun et al., 2020). Moreover, many studies have also found that HAs have negligible effects on the intoxicating degree under an optimal proportion of alcohols/acids/esters, while some acids, esters and other substances in FABs may have a relieving effect. Other physical and psychological symptoms such as headache, thirst, and cognitive ability are related to ethanol, BAs, acidity, intake dose, and other profiles in *huangjiu*. New product tasting han been performed at National *Huangjiu* Engineering Technology Research Center by specialized sommelier. The jiangnan1# product showed bet-ter drinking comfort with the same intake dose as 85# product. The most basic symptoms of hangover, headache, was caused due to ethanol rather thanother congeners and ingredients (Hori, Fujii, Hatanaka, & Suwa, 2003; J Rohsenow & Howland, 2010). BAs are substances that are difficult to decompose and cause headaches (Husnik et al., 2006; Sun et al., 2020). The content of ethanol and BAs showed no significant difference in two *huangjiu* (Table S6, Fig. S7). BAs are mainly produced by bacteria such as lactic acid bacteria during the rice soaking process in *huangjiu* brewing (Liu, Sun, et al., 2021). Scientific methods that comprehensively assess symptoms of hangovers have not been estab-lished to represent the symptoms with an objective numerical value, while only single or partial ingredients have been individually assessed
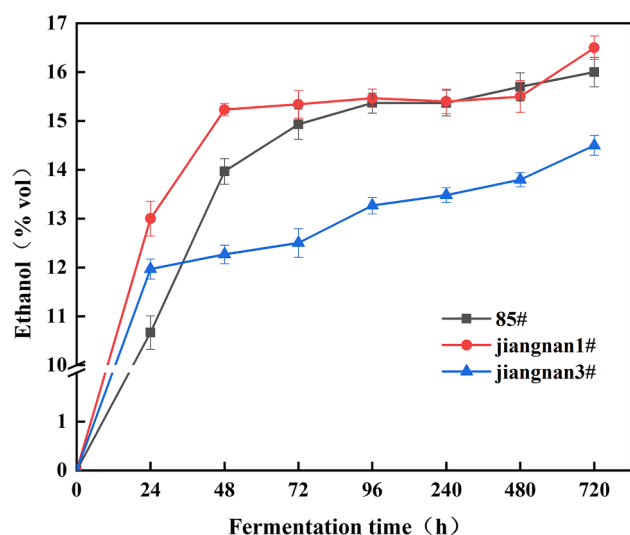
Fig. 2. The ethanol content of the strains *S. cerevisiae* 85#, jiangnan1# and jiangnan3# in *huangjiu* fermentation.
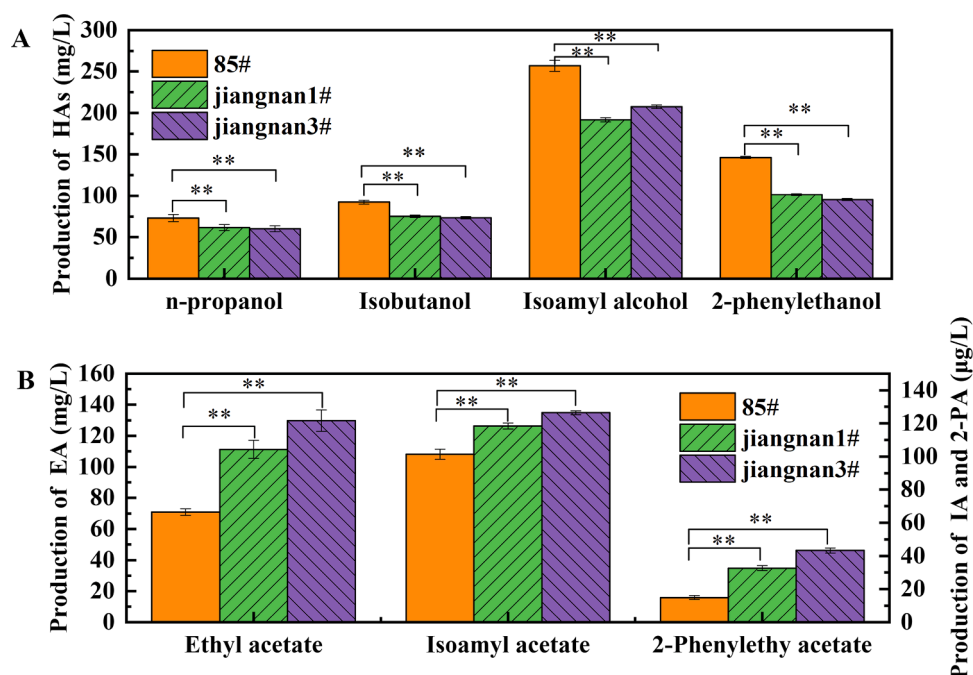
Fig. 3. The production of higher alcohols and acetate esters of *S. cerevisiae* 85# and jiangnan1# in *huangjiu* fermentation. A: Higher alcohol production of 85#, jiangnan1# and jiangnan3#. B: Acetate ester production by 85#, jiangnan1# and jiangnan3#. **, $P < 0.01$, *, $P < 0.05$. Error bars represent the standard deviations of the mean.
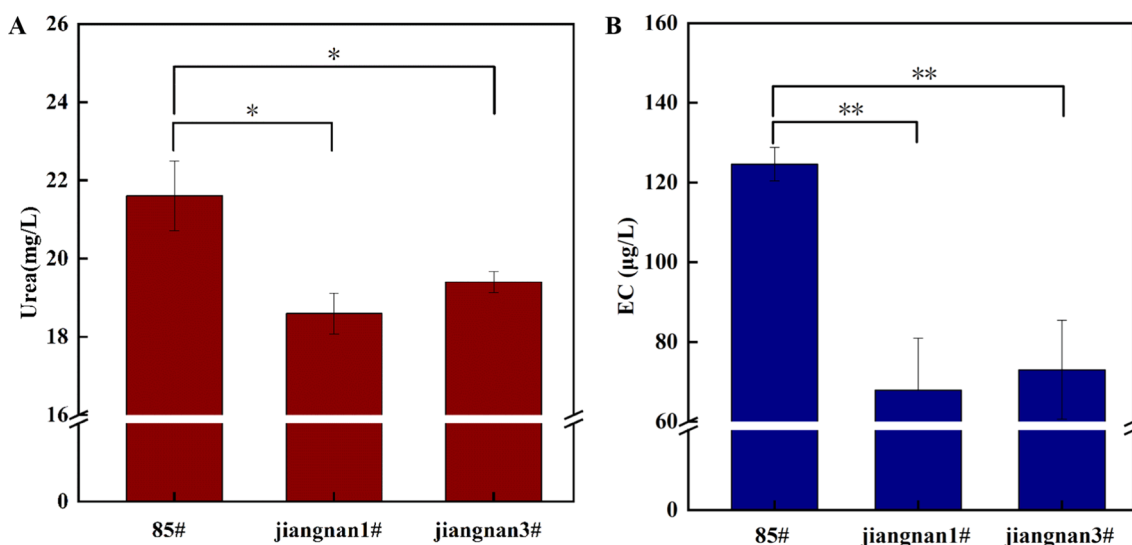

Fig. 4. The production of urea and EC of *S. cerevisiae* 85# and jiangnan1# in *huangjiu* fermentation. A: Production of urea in *huangjiu* fermentation with *S. cerevisiae* 85# and jiangnan1#. B: Production of EC in *huangjiu* fermentation with *S. cerevisiae* 85# and jiangnan1#. **, $P < 0.01$, *, $P < 0.05$. Error bars represent the standard deviations of the mean.

(Hori, Fujii, Hatanaka, & Suwa, 2003). As popular low-degree FABs, a more scientific and systematic evaluation system for *huangjiu* drinking comfort should be established in the future. Meanwhile, the key core microorganism *S. cerevisiae* should be further analyzed to obtain a better quality *huangjiu*, which could also help improve the quality and drinking comfort of *huangjiu* and other FABs.

4. Conclusions

In the present study, we first established a non-GMO approach for determining superior *S. cerevisiae* strains to regulate and control AE and HA production based on lab-scale *huangjiu* brewing. Two newly obtained

S. cerevisiae strains (jiangnan1# and jiangnan3#) produced safer, more comfortable drinking, and higher-quality *huangjiu*. Non-volatile substances (AAs and OAs) and sensory characteristics were also been compared and analyzed. The overall sensory evaluation results showed that the *huangjiu* products fermented by jiangnan1# were similar to the *Shaoxing-jiu* flavor and taste produced by the factory strain 85#. Meanwhile, the EC content in jiangnan1# *huangjiu* was low. The *S. cerevisiae* strains jiangnan1# and jiangnan3# may be suitable and have the significant potential for industrial applications for producing other FABs as well. This is because of the ability of the strains to produce HAs and AEs and evaluation based on the content produced per unit of alcohol (1 % vol).
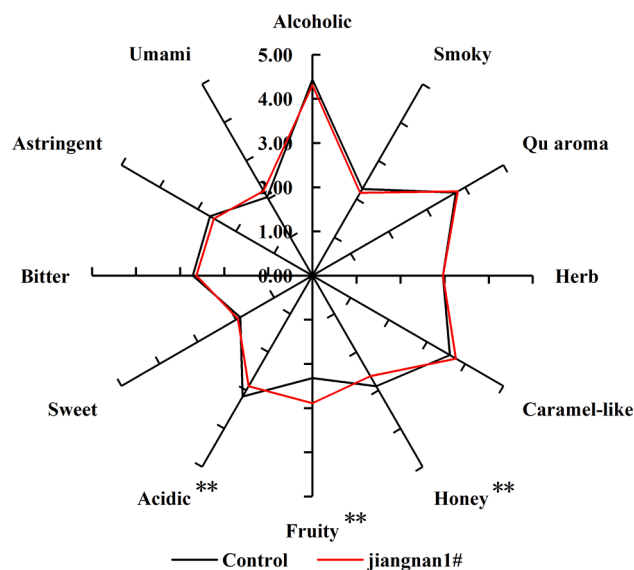
Fig. 5. The aroma and taste profiles of *huangjiu* fermented by 85# (control) and jiangnan1#. Significance was indicated at **, $P < 0.01$, *, $P < 0.05$. Error bars represent the standard deviations of the mean.

CRediT authorship contribution statement

Yuzong Zhao: Conceptualization, Investigation, Methodology, Project administration, Writing – original draft. **Shuangping Liu:** Data curation, Formal analysis, Funding acquisition, Writing – review & editing. **Qilin Yang:** Data curation, Formal analysis. **Xiao Han:** Supervision, Project administration. **Zhilei Zhou:** Investigation, Methodology, Visualization. **Jian Mao:** Resources, Project administration, Funding acquisition, 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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111763.

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