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Effects of simultaneous inoculation of non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* jiangnan1# on overall quality, flavor compounds, and sensory analysis of *huangjiu*

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ABSTRACT

Non-*Saccharomyces* yeast (NSY) co-fermentation with *Saccharomyces cerevisiae* has been widely used to produce high-quality fermented alcoholic beverages. In this study, we evaluated the feasibility of 24 NSY strains belonging to 13 different genera co-fermentation with *S. cerevisiae* jiangnan1# in *huangjiu* brewing. Different fermentation trials were set up and the effects on *huangjiu* fermentation were also analyzed. Results indicated that simultaneous inoculation could realize the application of NSY, while the appropriate proportion of *S. cerevisiae* and NSY (1:1 or 1:10) showed differential effects. Finally, three types of NSY strains (*Zygosaccharomyces rouxii* Non-Sc11, *Kloeckera apiculata* Non-Sc17, and *Candida tropicalis* Non-Sc21) with high ethanol content (>16% vol), moderate concentration of higher alcohols (<400 mg/L, 14% vol), higher production of esters ($P < 0.05$), and lower organic acid ($P < 0.05$) were obtained ultimately. The sensory evaluation demonstrated that using no less than one kind of the three NSY strains in simultaneous fermentation with *S. cerevisiae* jiangnan1# (1:1) resulted in sensory diversity of new *huangjiu* styles. In addition, the method for evaluation and application of NSY in co-fermentation with *S. cerevisiae* jiangnan1# in *huangjiu* brewing had been established for the first time, which could be directly applied in industrial production of *huangjiu*.

1. Introduction

Yeasts have been widely ubiquitous in the fermentation process of fermented alcoholic beverages, and *Saccharomyces cerevisiae* is the dominant microbial player and has already a widespread industrial role (Barnett, 2000; Suárez-Lepe & Morata, 2012). *S. cerevisiae* actively participates in the production of aroma-active compounds during the alcoholic fermentation (Steensels, Meersman, Snoek, Saels, & Verstrepen, 2014; Van Wyk, Grossmann, Wendland, Von Wallbrunn, & Pretorius, 2019). To pursue more diverse products, a variety of new styles of fermented alcoholic beverages with complex flavor profiles occurred. Many other yeasts can also contribute to composition, flavor and aroma of fermented alcoholic beverages, which are not as widely studied as *S. cerevisiae* (Zhang et al., 2022). Non-*Saccharomyces* yeast (NSY) with high β-glucosidase activity can improve aroma complexity of fermented alcoholic beverages, which have been developed to create many styles and compositions in wine-making market (Nisiotou et al., 2018; Van Wyk et al., 2019; Varela, Sengler, Solomon, & Curtin, 2016). It has been found that there is predominance or prevalence NSY strains presenting in the early phases and finally dominated by the high

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alcohol-tolerant *S. cerevisiae* during the entire process of wine-making (Van Wyk et al., 2019). Therefore, appropriate inoculation ratio and inoculation time are the key factors for different NSY strains to play a role in the whole fermentation process.

Nowadays, it is well known that NSY can affect the final product due to some positive effects (Cai et al., 2018; Contreras et al., 2014). Several NSY have been found to possess different noteworthy enological outcomes (increasing desirable aroma compounds production, reducing ethanol and acetaldehyde content, and modulating acidity) during mixed-culture fermentations (Canonico, Agarbati, Comitini, & Ciani, 2016; Comitini et al., 2011; Gobbi et al., 2013; Hu, Jin, Mei, Li, & Tao, 2018; Kai Chen, Escott, et al., 2018; Wang et al., 2017). Many NSY strains have already been selected to test their enological potential by co-fermentation, but only a few species that provide some level of benefit to the point that commercial preparations have been developed (Van Wyk et al., 2019).

Huangjiu is one of the three oldest fermented alcoholic beverages (*huangjiu*, beer, and wine), which has more than 9, 000 years of history (Duan et al., 2018). Qu is a molded cereal prepared by the natural inoculation of molds, bacteria, and yeasts, and JIUYAO (fermentation starter) is a rich source of brewers' microbial resources, increasing the activity of yeast and the formation of aroma compounds (Chen, Liu, Tian, Ai, & Yu, 2020). Traditional *huangjiu* production uses fermentation starters (Qu and JIUYAO) to provide multiple brewing microorganism, while modern *huangjiu* usually employs pure cultures (*Aspergillus oryzae* and *S. cerevisiae*) instead of JIUYAO (Zhao, Liu, Han, Zhou, & Mao, 2022). Many studies have successfully used high throughput sequencing technologies (the bacterial 16S rRNA gene, fungal ITS2, and metagenomic) to analyze the microbial diversity of *huangjiu* fermentation starters and its influence on the fermentation processes (Huang et al., 2018; Liu et al., 2019). Also, a large number of brewing microorganisms have been screened out based on culture-dependent methods (Chen et al., 2020; Liu et al., 2019). However, most bacteria and fungi survive only in the early stage of fermentation due to faster yeast alcohol fermentation, while NSYs can present during the entire process with dominated numbers (Van Wyk et al., 2019).

Mixed-culture fermentations (co-culturing fermentation, multi-starter or mixed culture) refer to the co-fermentation with selected *S. cerevisiae* and other NSYs, which include some approaches such as simultaneous inoculation, sequential inoculation, immobilization of yeasts, active-dried yeasts, and artificial starters (Van Wyk, von Wallbrunn, Swiegers, & Pretorius, 2021, pp. 428–446). Nevertheless, mixed-culture fermentations still can not be accurately predicted and controlled due to the change of fermentation process, which may have both positive and negative impacts on the final products (Van Wyk et al., 2019). It should be noted that *huangjiu* is brewed with multiple species of microorganisms, endowing *huangjiu* unique flavor, taste, and style. "Parallel fermentation" of *huangjiu* refers to the combination of progressive saccharification of starch and alcoholic fermentation, which contributes to the high ethanol production of about 10% vol after 24 h and over 20% vol in the final fermentation mash (Zhao, Liu, Han, et al., 2022). Therefore, controlling mixed fermentation with selected *S. cerevisiae* and NSY strains to change the lack of flavor diversity in *huangjiu* remains a big challenge. To date, it is a shame that there are no studies employing NSY to improve the quality and styles of regional products in *huangjiu* industrial production. Hence, the urgent demand for NSY with specific phenotypes is still increasing to select and evaluate in *huangjiu* production.

In this work, we had evaluated the feasibility of 24 NSY strains belonging to 13 different genera co-fermentation with *S. cerevisiae* jiangnan1# in *huangjiu* brewing. The application possibility of NSY in *huangjiu* fermentation was studied for the first time. Different fermentation trials were set up and the effects on *huangjiu* fermentation were also analyzed. To our knowledge, there has been no report focused on these species of yeasts in *huangjiu* brewing. This research can improve flavor diversity of new *huangjiu* styles, which would be helpful in promoting potential industrial application of NSY in *huangjiu* brewing.

2. Materials and methods

2.1. Yeast strains and media

Twenty-four NSY strains (thirteen genera) were used in this study (Table 1). These strains were stored at −80 °C in the yeast extract peptone dextrose (YPD) medium with glycerol (15% v/v final concentration). *S. cerevisiae* jiangnan1# had been used for industrially production of *huangjiu* due to the excellent ability of low higher alcohols and high acetate esters. The rice hydrolysate medium was used as yeast starter culture, which was prepared according to the described methods (Zhao, Liu, Han, et al., 2022).

2.2. Preparation of yeast starter culture

Yeast starter cultures were firstly prepared, which were used for inoculation of yeast. Firstly, *S. cerevisiae* jiangnan1# and twenty-four NSY strains were spread on the YPD agar plates to obtain the monoclonal colonies. Then, each of them was pre-cultured in rice hydrolysate medium at 28 °C for 48 h, respectively. Finally, yeast starter culture was activated in another rice hydrolysate medium at 28 °C for 36 h–48 h, serial dilutions (10^{-4} to 10^{-6}) of each yeast starter culture sample was prepared in triplicate to determine yeast concentration by using the method of a Neubauer chamber. Different concentration of yeast starter cultures of different NSY strains were obtained, which could be used directly for *huangjiu* co-fermentation.

2.3. Huangjiu co-fermentation trials

Huangjiu co-fermentations trials were performed by simultaneous inoculation of NSYs and *S. cerevisiae* jiangnan1# (Fig. S1) and sequential inoculation of NSY strains followed by *S. cerevisiae* jiangnan1# after 24

Table 1
Information of yeast strains in this study.

ID	Names	Species (Strains)	Strain source
[a]jiangnan1#	CYY-661	*Saccharomyces cerevisiae*	Fermented mash of *huangjiu*
Non-Sc01	1056	*Schizosaccharomyces pombe*	China Center of Industrial Culture Collection, CICC
Non-Sc02	31215	*S. pombe*	CICC
Non-Sc03	NS7	*Pichia kudriavzevii*	Citric acid wastewater
Non-Sc04	NL3	*P. kudriavzevii*	Citric acid wastewater
Non-Sc05	NS2	*P. kudriavzevii*	Citric acid wastewater
[a]Non-Sc06	Y1	*P. kudriavzevii*	Raw wheat Qu
[a]Non-Sc07	S12	*P. kudriavzevii*	Cooked wheat Qu
[a]Non-Sc08	Y2	*Pichia fabianii*	Raw wheat Qu
[a]Non-Sc09	S16	*Pichia farinosa*	Cooked wheat Qu
Non-Sc10	MY01	*Torulaspora delbrueckii*	Fresh kiwi juice
Non-Sc11	MY03	*Zygosaccharomyces rouxii*	Fresh kiwi juice
Non-Sc12	MY04	*Z. rouxii*	Fresh kiwi juice
[a]Non-Sc13	YC01	*Wickerhamomyces anomalus*	JIUYAO
[a]Non-Sc14	YC22	*W. anomalus*	JIUYAO
[a]Non-Sc15	YC34	*W. anomalus*	JIUYAO
[a]Non-Sc16	Y11	*Candida rugosa*	Raw wheat Qu
Non-Sc17	2.711	*Kloeckera apiculata*	China General Microbiological Culture Collection Center, CGMCC
Non-Sc18	2.496	*K. apiculata*	CGMCC
[a]Non-Sc19	S21	*Trichosporon asahii*	Cooked wheat Qu
Non-Sc20	Sba	*Saccharomyces bayanus*	Raw wheat Qu
Non-Sc21	CS8	*Candida tropicalis*	Citric acid wastewater
Non-Sc22	NL2	*C. tropicalis*	Citric acid wastewater
[a]Non-Sc23	S4	*Clavispora lusitaniae*	Cooked wheat Qu
Non-Sc24	31693	*Issatchenkia orientalis*	CICC

[a] Strains were isolated from *huangjiu* brewing process.

h. Five inoculation ratio (Single, 1:1, 1:10, 1:100, and 1:1000) of each NSY were used to preliminary screen of superior strains and determine the co-fermentation style and inoculation ratio in simultaneous inoculation. The single inoculation of each NSY (7.0 or 8.0 Log CFU/mL, 110 mL) and *S. cerevisiae* jiangnan1# (8.0 Log CFU/mL, 110 mL) were set as the control fermentation trials. The inoculum ratio of NSY (8.0 Log CFU/mL) and S. *cerevisiae* jiangnan1# was 1:1 (55 mL + 55 mL), 10:1 (100 mL + 10 mL), 100:1 (109 mL + 1 mL), 1000:1 (110 mL + 0.1 mL), pre-cultured in rice hydrolysate medium. The inoculum ratio of NSY (7.0 Log CFU/mL) and S. *cerevisiae* jiangnan1# was 1:1 (100 mL + 10 mL), 10:1 (109 mL + 1 mL), 100:1 (110 mL + 0.1 mL), 1000:1 (110 mL + 0.01 mL). A total of 144 fermentation trials were performed in the preliminary screening.

Seven types of fermentation trials had been set up for the three NSY (SC11, SC17, and SC21) in simultaneous fermentation with *S. cerevisiae* jiangnan1#, containing jiangnan1# (100 mL), J-Non-Sc11 (jiangnan1# + Sc11, 55 mL + 55 mL), J-Non-Sc17 (jiangnan1# + Sc17, 55 mL + 55 mL), J-Non-Sc21 (jiangnan1# + Sc21, 55 mL + 55 mL), J-Non-Sc11-Sc17 (jiangnan1# + Sc11 + Sc17, 37 mL + 37 mL+ 37 mL), J-Non-Sc11-Sc21 (jiangnan1# + Sc11 + Sc21, 37 mL + 37 mL+ 37 mL), J-Non-Sc17-Sc21 (jiangnan1# + Sc17 + Sc21, 37 mL + 37 mL+ 37 mL), and J-Non-Sc11-Sc17-Sc21 (jiangnan1# + Sc11 + Sc17 + Sc21, 27.5 mL + 27.5 mL + 27.5 mL + 27.5 mL).

Each fermentation trail was mixed with the same steamed glutinous rice (1, 500 g), raw wheat Qu (117 g), cooked wheat Qu (18 g), water (1, 150 g), and differentiated yeast starter culture (100 mL) in a 5 L flask. Then, the above mixture was fermented at 30 °C for 5 days (primary fermentation) and at 15 °C for 15 days (post fermentation). All co-fermentation trials were performed in triplicate and finished after 20 days (the content of residual sugar after fermentation is no longer reduced).

2.4. Analysis of basic fermentation parameters

The alcohol, total acid, and amino acid nitrogen content were determined by the method used for *huangjiu* (Zhao, Liu, Han, et al., 2022). The residual sugar content was detected using the 3,5-dinitrosalicylic acid method (Miller, 1959).

2.5. Analysis of organic acids and volatile flavor compounds

The organic acids were determined by high performance liquid chromatograph (HPLC) according to the described methods of *huangjiu* (Zhao, Liu, Han, et al., 2022). The volatile flavor compounds in *huangjiu* were extracted and determined by headspace solid phase micro-extraction coupled with gas chromatography and mass spectrometry (HS-SPME-GC-MS) according to the 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 an internal standard. The dispersive liquid-liquid microextraction was used for determining the main HAs, 4-methyl-2-pentanol (0.4536 g/L in ethanol) was used as an internal standard to quantify the analytes using GC-MS (Zhou et al., 2020).

2.6. Sensory analysis

The *huangjiu* samples of simultaneous inoculation the final selected NSY (Non-Sc11, Non-Sc17, and Non-Sc21) followed by *S. cerevisiae* jiangnan1# were used for sensory analysis. A high-quality traditional *huangjiu* for more than 20 years and *huangjiu* sample fermented by *S. cerevisiae* jiangnan1# (the control) were also included. The descriptive sensory panel test was conducted at Research Center of Traditional Fermented Food (Wuxi, Jiangsu, China) by a tasting panel composed of 30 panelists. Finally, 10 trained panelists (five men and five women) aged between 22 and 45 years old with considerable tasting and substantial experience in different types of *huangjiu* products sensory analysis, specifically trained for this judging session. The panelists demonstrated better abilities in consistently, stably, and repeatedly, which were carrying out sensory analysis of the new styles of *huangjiu*. The description terminology included five kinds of taste attributes (sweet, bitter, astringent, acidic, and umami) and seven kinds of aroma attributes (alcoholic, smoky, honey, herb, caramel-like, fruity, and Qu aroma). Information of sensory attributes tested in this study had been shown in Table S1. The overall aroma intensity and overall taste intensity were also included for the determination of the degree of preference for the products, a general evaluation based on the scores of all attributes. The sensory attributes were scored on a five-point scale according to the methods (Chen, Xu, & Qian, 2013), ranging from 0 to 5 (0, not perceivable; 3, moderate intensity; 5, strongly perceivable).

2.7. Statistical analysis

Date analysis was performed using Origin 2022 64Bit (OriginLab Corporation, Northampton, MA, USA), and the results were expressed as mean ± standard deviations. Statistical analyses were performed with the IBM SPSS Statistical (V. 21.0, IBM Corp, NY, USA). One-way analysis of variance (ANOVA) test was used to identify significant differences ($P < 0.05$) between different *huangjiu* co-fermentation trials and the control. An ANOVA test was also carried out on the data obtained from the sensory descriptive analysis. The key flavor compounds difference of *huangjiu* samples by using different NSY strains were analyzed by heat maps made online (Metsalu & Vilo, 2015).

3. Results and discussion

3.1. Establishment of co-fermentation of NSY and S. cerevisiae jiangnan1# in huangjiu brewing

Sequential inoculations of NSY followed by *S. cerevisiae* jiangnan1# after 24 h of inoculation were first been considered. Unfortunately, the results (Total acids, >8.0 g/L) showed that all NSY strains did not finish fermentation, although the saccharification and liquefaction of starch (residual sugar, >50.0 g/L) were normal. The ethanol content produced by yeast in the early stage of fermentation was less, which could not inhibit the growth of other microbiota, while the excess concentration of acid produced by acid-producing microbiota may lead to rancidity. Although the sequential inoculation of NSY had been widely used in other fermented alcoholic beverage, it certainly had not been used in *huangjiu* fermentation because of its unique production process (Roullier-Gall, Bordet, David, Schmitt-Kopplin, & Alexandre, 2022; Tofalo et al., 2022). Another method of simultaneous inoculation with different inoculation proportion provided a possibility for the mixed-culture fermentations in *huangjiu* brewing. The basic parameters (alcohol and total acid) indicated that simultaneous inoculation could be used in *huangjiu* brewing (Table S2). Most co-fermentation trials at higher inoculation proportion (1:1 and 1:10) could perform fermentation completion, while it might occur sluggish or stuck fermentations at higher inoculation proportion (Table 2). Rancidity was occurred in all the co-fermentation trials at inoculation proportion (1:1000) after 24h. Although a higher inoculation proportion of NSY or single fermentation were able to produce a larger amount of volatile substances, the risks of rancidity and fermentation failure were also present (Table 2). Indeed, *S. cerevisiae* had stronger competition for nutrients than NSY at higher inoculation proportion (1:1 or 1:10), produced some metabolites (high concentration of alcohol), resulting in NSY disappearing soon and not playing a role in the co-fermentation process (Table 2). In-depth analysis was necessary for the application of NSY in *huangjiu* co-fermentation.

Table 2
Basic physicochemical indicators of *huangjiu* samples fermented by different NSY strains.

Strains (proportion)	Alcohol (% vol)	[a] Total acid (g/L)	Amino acid nitrogen (g/L)	Residual sugar (g/L)
jiangnan1# (Control)	16.2 ± 0.5	6.67 ± 0.25	1.12 ± 0.02	10.5 ± 2.5
J-Non-Sc03 (1:1)	16.2 ± 0.4^{ns}	7.13 ± 0.35^{ns}	1.18 ± 0.08^{ns}	12.5 ± 1.8^{ns}
J-Non-Sc03 (1:10)	15.2 ± 0.3*	6.44 ± 0.23^{ns}	1.09 ± 0.10^{ns}	16.5 ± 2.5^{ns}
J-Non-Sc04 (1:1)	14.3 ± 0.5**	6.41 ± 0.40^{ns}	0.92 ± 0.05**	25.2 ± 3.3**
J-Non-Sc04 (1:10)	16.2 ± 0.2^{ns}	6.56 ± 0.41^{ns}	0.91 ± 0.03**	11.5 ± 1.8^{ns}
J-Non-Sc08 (1:1)	16.5 ± 0.3^{ns}	6.23 ± 0.15^{ns}	1.02 ± 0.03*	10.5 ± 2.5^{ns}
J-Non-Sc08 (1:10)	16.2 ± 0.3^{ns}	6.01 ± 0.24*	0.91 ± 0.08*	12.5 ± 1.2 ^{ns}
J-Non-Sc11 (1:1)	15.5 ± 0.5^{ns}	7.68 ± 0.45**	1.15 ± 0.02^{ns}	15.5 ± 1.2*
J-Non-Sc11 (1:10)	17.1 ± 0.1*	7.20 ± 0.31^{ns}	1.05 ± 0.03*	10.2 ± 1.8^{ns}
J-Non-Sc12 (1:1)	16.2 ± 0.3^{ns}	7.09 ± 0.40^{ns}	1.13 ± 0.08^{ns}	9.6 ± 1.2^{ns}
J-Non-Sc12 (1:10)	15.2 ± 0.2*	7.11 ± 0.25^{ns}	1.12 ± 0.11^{ns}	18.5 ± 2.3*
J-Non-Sc13 (1:1)	15.5 ± 0.2*	6.53 ± 0.25^{ns}	1.12 ± 0.10^{ns}	19.8 ± 3.1*
J-Non-Sc13 (1:10)	16.2 ± 0.2^{ns}	6.71 ± 0.36^{ns}	0.98 ± 0.12^{ns}	12.4 ± 2.2^{ns}
J-Non-Sc14 (1:1)	16.2 ± 0.2^{ns}	7.91 ± 0.35**	1.11 ± 0.05^{ns}	14.5 ± 1.5^{ns}
J-Non-Sc14 (1:10)	16.2 ± 0.1^{ns}	7.85 ± 0.25**	1.02 ± 0.03^{ns}	12.2 ± 1.3^{ns}
J-Non-Sc17 (1:1)	17.1 ± 0.3*	6.04 ± 0.25*	1.10 ± 0.08^{ns}	10.3 ± 2.5^{ns}
J-Non-Sc17 (1:10)	16.7 ± 0.2^{ns}	6.22 ± 0.34^{ns}	1.13 ± 0.07^{ns}	11.5 ± 2.1^{ns}
J-Non-Sc20 (1:1)	16.2 ± 0.2^{ns}	7.36 ± 0.35*	1.13 ± 0.04^{ns}	10.3 ± 1.5^{ns}
J-Non-Sc20 (1:10)	15.2 ± 0.1*	6.12 ± 0.23*	1.02 ± 0.03^{ns}	18.5 ± 2.2*
J-Non-Sc21 (1:1)	17.1 ± 0.5^{ns}	6.84 ± 0.32^{ns}	1.08 ± 0.03^{ns}	6.5 ± 3.8^{ns}
J-Non-Sc21 (1:10)	15.2 ± 0.5^{ns}	6.57 ± 0.41^{ns}	1.15 ± 0.08^{ns}	15.8 ± 1.2*
J-Non-Sc23 (1:1)	16.2 ± 0.3^{ns}	6.29 ± 0.25^{ns}	1.12 ± 0.06^{ns}	11.6 ± 2.2^{ns}
J-Non-Sc23 (1:10)	15.2 ± 0.2*	6.20 ± 0.32^{ns}	1.08 ± 0.06^{ns}	12.3 ± 1.5^{ns}
J-Non-Sc24 (1:1)	16.5 ± 0.2^{ns}	7.92 ± 0.45**	1.12 ± 0.04^{ns}	10.7 ± 1.3^{ns}
J-Non-Sc24 (1:10)	14.3 ± 0.5**	7.13 ± 0.38^{ns}	1.06 ± 0.05^{ns}	15.7 ± 3.2^{ns}

a The content of the total acid represents for lactic acid calculation. Values are means ± standard deviations from three independent tests. Significance is conducted between the control and the other NSY strains, indicated at **, $P < 0.01$, *, $P < 0.05$, ns, no significance.

3.2. Evaluation of the inoculation proportion for different NSY in huangjiu co-fermentation

The simultaneous inoculation with appropriate inoculation proportion (1:1 and 1:10) in *huangjiu* co-fermentation had been established. In our current study, some co-fermentation trials reduced the production of ethanol (>1.0% vol) or produced higher total acid (>8.0 g/L) in the final product, which were not considered in the further study. To evaluate the positive effects of the inoculation proportion for different NSY on the final quality of *huangjiu*, 12 different NSY strains owning the potential fermentation characteristics were performed in triplicate. The production of ethanol were between 14% vol to 17% vol, while six co-fermentation trials (J-Non-Sc03, J-Non-Sc08, J-Non-Sc011, J-Non-Sc14, J-Non-Sc17, and J-Non-Sc21) produced no significantly or significantly ($P < 0.05$) higher amount of ethanol (Table 2). Other fermentation parameters in Table 2 (total acid, amino acid nitrogen, and residual sugar) were within adequate ranges, meeting the national standard "*Huangjiu*". The result showed that the inoculation proportion (1:1 or 1:10) of the six NSY strains did not influence the co-fermentation completion. Whether they had the positive effects on *huangjiu* needed the evaluation of the flavor substances and higher alcohols.

Higher alcohols and acetate esters produced by yeasts were two important volatile flavor substances in *huangjiu*, and it had been found the *huangjiu* fermented by *S. cerevisiae* jiangnan1# increased by 36.35% in acetate esters and decreased 24.99% in higher alcohols (Zhao, Liu, Yang, et al., 2022). Modulation of aroma and chemical composition could be implemented by using NSY of different *Saccharomyces* species (Pérez et al., 2022). *S. cerevisiae* jiangnan1# with low-yield higher alcohols and high-yield acetate esters had been found it can improve the quality, drinking comfort, and safety of *huangjiu* (Zhao, Liu, Yang, et al., 2022). To retain the excellent performance of *S. cerevisiae* jiangnan1#, the production of higher alcohols and acetate esters had been detected to evaluate the applicability of NSY strains for all the co-fermentations in our study. The results showed that J-Non-Sc14 (1:1) and J-Non-Sc21 (1:10) produced higher total higher alcohols than that of jiangnan1# (control) among the 12 NSY (Fig. 1A). Meanwhile, the production of the three types of acetate esters (ethyl acetate, isoamyl acetate, and 2-phenethyl acetate) were equivalent to the control strain *S. cerevisiae* jiangnan1# (Fig. 1B). The mixed cultures of some NSY also resulted in a

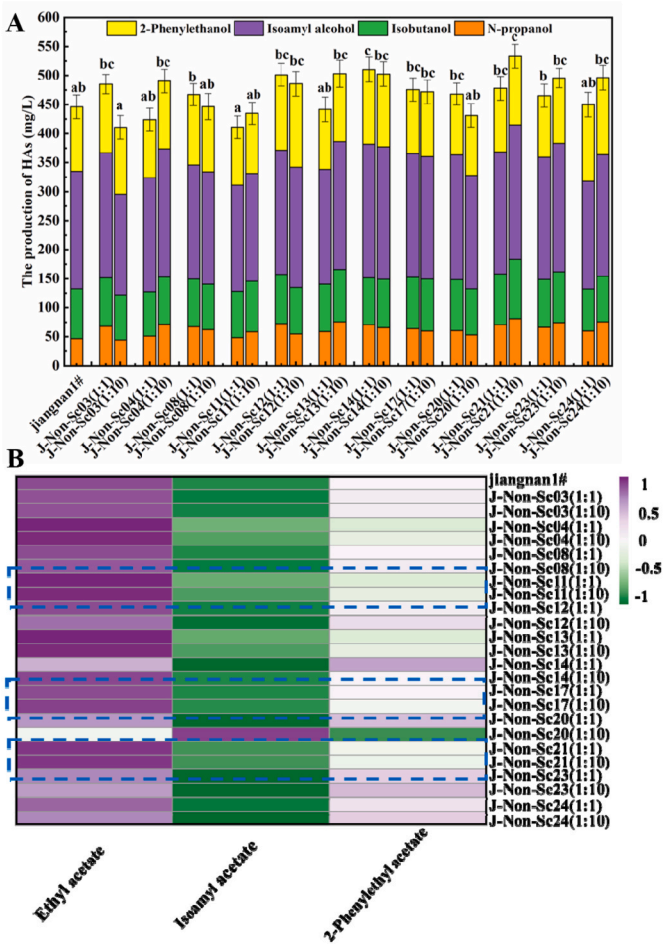

Fig. 1. The production of higher alcohols and acetate esters of *huangjiu* by using 12 different NSY and *S. cerevisiae* jiangnan1# in *huangjiu* co-fermentation. A: The production of higher alcohols. B: The production of acetate esters. Error bars represent the standard errors of the mean. Values with different letters are significantly different ($P < 0.05$) from each other according to Duncan tests.

reduction in ethanol, acidity, or acetate esters production, maybe the content of individual acetate ester was significantly increased in our study (Table 2 and Fig. 1), while the same results had been reported in some recent studies (Gobbi et al., 2013; Gonzalez, Quirós, & Morales, 2013; Li et al., 2022; Roullier-Gall et al., 2022; Tofalo et al., 2022). The purpose of this study was to find NSY strains suitable for *huangjiu* co-fermentation, which could not change the overall style as much as possible (alcohol did not significantly decrease, basic fermentation parameters were not significantly different), and improve the drinking comfort (higher alcohols did not increase, acetate esters did not decrease significantly). Therefore, three different strains of NSY (*Zygosaccharomyces rouxii* Non-Sc11, *Kloeckera apiculata* Non-Sc17, and *Candida tropicalis* Non-Sc21) were obtained and considered for further study by using a combined approach.

3.3. Evaluation of three different NSY strains/S. cerevisiae inoculations in huangjiu

Seven types of co-fermentation trials had been set up for the three NSY strains in this study. The basic physicochemical indicators and organic acid were shown in Table 3. All the enological parameters meet the national standard "*Huangjiu*", indicating the fermentation completion. Eight kinds of organic acids had been detected and shown in Table 4. The total content of organic acids were significant different between the co-fermentation trials and control ($P < 0.05$). The content of the two main organic acids (lactic acid and acetic acid) of co-fermentation trials were lower than that of the control, while the content of oxalic acid, tartaric acid, and citric acid were higher than the control ($P < 0.05$). The acetic acid in all co-fermentation trials showed a significantly decreased, which may be related to the enzyme-mediated esterification between acetic acid and ethanol. In previous research, more than two kinds of NSY and their pairwise combinations in co-fermentation with *S. cerevisiae* had been used to improve the aroma diversity and quality of wines (Contreras et al., 2014; Zhang et al., 2022). It had been reported that using NSY and *S. cerevisiae* in the production of wine could significantly decrease volatile acidity (lactic and acetic acid), while low volatile acidity content generally associated with negative aromas (Bely, Stoeckle, Masneuf-Pomarède, & Dubourdieu, 2008; Comitini et al., 2011). As had been reported, there were many NSY being used to modulate the final aroma profile in both positive and negative ways, whereas esters and higher alcohols were usually improved (Contreras et al., 2014; Renault, Coulon, de Revel, Barbe, & Bely, 2015; Van Wyk et al., 2019). The production of ethanol, higher alcohols, and flavor substance had been comprehensively analyzed in this study. Compared to the control, the co-fermentation trials of J-Non-Sc-17-Sc-21 and J-Non-Sc-11-Sc-17-Sc-21 had significant decrease in ethanol (<1% vol), whereas the concentration of the other co-fermentation trials was not significantly different (Fig. 2A). The content of total higher alcohols showed that all the co-fermentation trials were not significantly different (Fig. 2), the moderate concentrations of which typically contributed positively to sensory properties and drinking comfort of *huangjiu*.

To analyze the contribution of using different NSY strains to flavor components, a total of 43 aromatic compounds (14 alcohols, 3 acetate esters, 16 ethyl esters, 5 acids, and 5 others) were detected and shown in Fig. 2. The heat map plot showed that NSY significantly affected *huangjiu* aromatic compounds, highlighting the most ethyl esters that showed greater variability (Fig. 2). Two co-fermentation trials of J-Non-Sc17-Sc21 and J-Non-Sc11-Sc17-Sc21 showed weaker production than that of jiangnan1#, related to the decrease of ethanol. Amino acid was one of the rich indispensable components in *huangjiu*, which was flavor precursor substances, providing various tastes and nutritive value (Wang et al., 2014). Although the three NSYs (*Z. rouxii* Non-Sc11, *K. apiculata* Non-Sc17, and *C. tropicalis* Non-Sc21) had been used in *huangjiu* fermentation for the first time, the above results indicated the potential significance of their ability to significantly modulate the final aroma profile and improve *huangjiu* quality in terms of its sensorial appeal. The osmotolerant yeasts *Z. rouxii* and *C. tropicalis* (high sugar tolerance) had been widely applied in wine production (Gonzalez et al., 2013; Xiang et al., 2018). *Zygosaccharomyces* spp. had been suggested because of their ability to produce aroma profile and proteins with killing activities rather than produce acidity, while *C. tropicalis* could be used to positively affect the taste and flavor of wine and produce new fermentation products (Magyar & Tóth, 2011; Rojo et al., 2014; Soden, Francis, Oakey, & Henschke, 2000). *K. apiculata* strains had been reported they could increase in esters and relative decrease in alcohols and acids, expanding the flavor diversity with producing enzymes and aroma compounds (Martin, Valera, Medina, Boido, & Carrau, 2018; Matraxia et al., 2021). It should note that the simultaneous inoculation of three NSY strains together was not suitable for *huangjiu* according to the current results in our study, although the alcohol content could meet the requirements.

Table 3
Basic physicochemical indicators of *huangjiu* samples fermented by three different NSY strains.

Strains	Alcohol (% vol)	*Total acid (g/L)	Amino acid nitrogen (g/L)	Residual sugar (g/L)
jiangnan1#	17.80 ± 0.00^{b}	6.64 ± 0.24^{bc}	0.87 ± 0.04^{a}	4.5 ± 0.8^{a}
J-Non-Sc11	16.90 ± 0.85^{ab}	6.23 ± 0.21^{ab}	0.80 ± 0.04^{a}	6.8 ± 0.8^{b}
J-Non-Sc17	17.50 ± 0.85^{ab}	6.96 ± 0.32^{c}	0.86 ± 0.02^{a}	4.7 ± 0.6^{a}
J-Non-Sc21	17.50 ± 0.85^{ab}	6.64 ± 0.22^{bc}	0.80 ± 0.03^{a}	4.1 ± 0.7^{a}
J-Non-Sc11-Sc17	17.10 ± 0.28^{ab}	6.28 ± 0.14^{ab}	0.86 ± 0.02^{a}	4.2 ± 0.2^{a}
J-Non-Sc11-Sc21	17.50 ± 0.42^{ab}	6.29 ± 0.20^{ab}	0.82 ± 0.02^{a}	4.4 ± 0.5^{a}
J-Non-Sc17-Sc21	16.30 ± 0.48^{a}	6.27 ± 0.24^{ab}	0.82 ± 0.04^{a}	4.5 ± 0.7^{a}
J-Non-Sc11-Sc17-Sc21	16.00 ± 0.55^{a}	6.02 ± 0.34^{a}	0.83 ± 0.04^{a}	3.0 ± 1.2^{a}

* The content of the total acid represents for lactic acid calculation. Values are means ± standard deviations from three independent tests. Different letters in the same column indicate significantly differences ($P < 0.05$) from each other using Duncan's multiple comparison test.

3.4. Sensory evalution

The co-fermentation of NSY showed significant positive effects on *huangjiu* composition and flavor substances, which definitely affected the sensory of new fermentation *huangjiu* products. Fig. 3 showed that the mean scores of each attribute of the *huangjiu* samples, as averaged across the relative replicates. Statistically significant differences between the two *huangjiu* samples were found for 10 of the 14 sensorial attributes evaluated in Fig. 3A. Considering moderate intensity (black line) as an evaluation standard, the overall taste intensity showed significant differences between the two *huangjiu* samples for the positive contribution of sweet and umami taste ($P < 0.01$) and negative contribution of astringent taste($P < 0.01$). The overall aroma intensity was no significant differences between the two samples, which may the combined results of the positive contribution of fruity and honey aromas for jiangnan1# ($P < 0.01$), while other aroma intensity (alcoholic, smoky, Qu, and caramel-like aromas) were scored higher for traditional *huangjiu* ($P < 0.01$). It had been revealed that traditional *huangjiu* was significantly higher intensity of the Qu aroma, smoky, and caramel-like aroma than modern *huangjiu*, resulting in the traditional *huangjiu* a higher overall aroma intensity (Chen, Xu, & Qian, 2018). It showed that each NSY strain in simultaneous fermentation with *S. cerevisiae* jiangnan1# owed a differential sensory style, the overall taste intensity and overall aroma intensity were significant differences between the co-fermentation trials (J-Sc11, J-Sc17, and J-Sc21) and the control

Table 4
Eight kinds of organic acid of *huangjiu* samples fermented by three different NSY strains.

Types (mg/L)	Oxalic acid	Tartaric acid	Pyruvic acid	Malic acid	Lactic acid	Acetic acid	Citric acid	Succinic acid
jiangnan1#	122.2 ± 4.5^{a}	98.5 ± 8.2^{a}	186.2 ± 16.2^{ab}	400.5 ± 24.2^{ab}	6060.5 ± 80.5^{e}	2200.2 ± 20.8^{f}	152.3 ± 12.5^{a}	224.3 ± 8.5^{d}
J-Non-Sc11	160.5 ± 5.2^{b}	284.1 ± 15.6^{b}	172.3 ± 15.8^{ab}	388.2 ± 18.8^{a}	5700.4 ± 76.8^{d}	1744.4 ± 18.5^{d}	332.4 ± 10.5^{c}	220.5 ± 9.6^{d}
J-Non-Sc17	158.3 ± 4.6^{b}	308.2 ± 18.5^{bc}	200.5 ± 14.6^{b}	500.3 ± 21.3^{cd}	6080.4 ± 68.6^{e}	1840.5 ± 17.7^{e}	356.5 ± 25.3^{c}	280.5 ± 7.8^{e}
J-Non-Sc21	124.1 ± 2.5^{a}	328.0 ± 17.5^{c}	172.1 ± 13.5^{ab}	416.4 ± 20.2^{ab}	4100.5 ± 69.5^{a}	980.5 ± 21.5^{a}	324.6 ± 21.3^{c}	124.3 ± 8.8^{b}
J-Non-Sc11-Sc17	160.5 ± 4.8^{b}	312.5 ± 13.2^{bc}	170.8 ± 12.3^{a}	429.2 ± 18.5^{b}	4700.4 ± 71.2^{b}	1480.4 ± 20.6^{b}	332.4 ± 20.5^{c}	160.8 ± 8.6^{c}
J-Non-Sc11-Sc21	161.2 ± 4.7^{b}	309.2 ± 15.4^{bc}	169.2 ± 14.5^{a}	482.8 ± 16.8^{cd}	5166.3 ± 70.5^{c}	1560.4 ± 23.4^{c}	320.4 ± 24.1^{c}	120.8 ± 9.3^{b}
J-Non-Sc17-Sc21	176.3 ± 5.1^{c}	332.4 ± 14.9^{c}	172.2 ± 10.6^{a}	508.5 ± 17.5^{d}	5160.7 ± 69.5^{c}	1556.2 ± 22.3^{c}	252.6 ± 23.3^{b}	92.4 ± 9.1^{a}
J-Non-Sc11-Sc17-Sc21	160.2 ± 3.9^{b}	312.5 ± 13.8^{bc}	160.5 ± 15.5^{a}	468.5 ± 15.5^{c}	5000.7 ± 58.8^{c}	1552.4 ± 21.5^{c}	324.4 ± 22.1^{c}	112.4 ± 8.5^{b}

Values are means ± standard deviations from three independent tests. Different letters in the same column indicate significantly differences ($P < 0.05$) from each other using Duncan's multiple comparison tests.

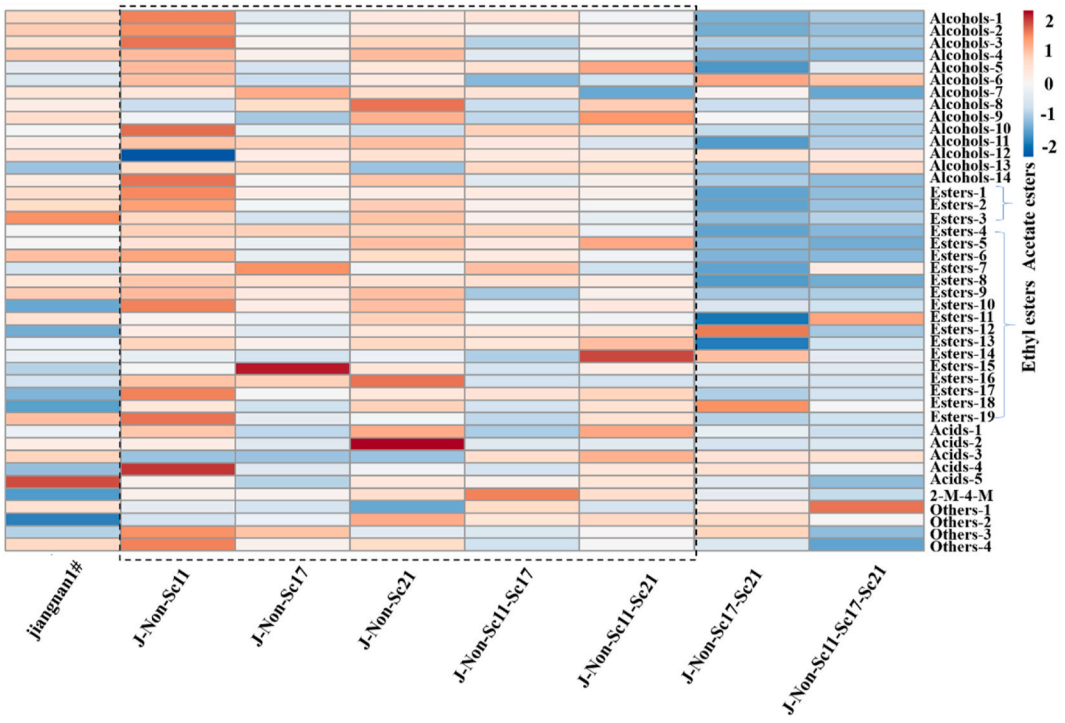

Fig. 2. The production of major volatile compounds of *huangjiu* co-fermentation trails by using 3 different NSY and *S. cerevisiae* jiangnan1#. Alcohols-1: 2-methyl-1-propanol; Alcohols-2: 3-methyl-1-propanol; Alcohols-3: 2-phenylethanol; Alcohols-4: 3-methylthiopropanol; Alcohols-5: 3-ethoxy-1-propanol; Alcohols-6: 4-Hydroxyphenethyl alcohol; Alcohols-7: 1-octen-3-ol; Alcohols-8: 2-nonanol; Alcohols-9: farnesol; Alcohols-10: 2, 3-butanediol; Alcohols-11: 1-butanol; Alcohols-12: 2-ethyl-1-hexanol; Alcohols-13: D-citronellol; Alcohols-14: 1-pentanol; Esters-1: ethyl acetate; Esters-2: isoamyl acetate; Esters-3: phenylethyl acetate; Esters-4: ethyl caprate; Esters-5: ethyl heptanoate; Esters-6: ethyl isovalerate; Esters-7: ethyl caprylate; Esters-8: ethyl valerate; Esters-9: ethyl nonanoate; Esters-10: ethyl hydrogen succinate; Esters-11: ethyl crotonate; Esters-12: ethyl benzoate; Esters-13: ethyl palmitate; Esters-14: ethyl caproate; Esters-15: ethyl phenylacetate; Esters-16: ethyl myristate; Esters-17: ethyl laurate; Esters-18: ethyl butanoate; Esters-19: phenethyl butyrate; Acids-1: dodecanoic acid; Acids-2: octanoic acid; Acids-3: hexanoic acid; Acids-4: n-hexadecanoic acid; Acids-5: nonanoic acid; 2-M-4-M: 2-methoxy-4-vinylphenol; Other-1: benzaldehyde; Other-2: 2,3-dihydrobenzofuran; Other-3: 4-ethylphenol; Other-4: 2,4-ditertiary butylphenol.

jiangnan1# ($P < 0.01$). The sensory evalution of two co-fermentation trials (J-Sc11 and J-Sc17) preferred the light type *huangjiu* (mellow, soft, delicate, and fresh), containing more balanced sensory attributes, while J-Sc21 had too higher acidic taste (Fig. 3B). Interestingly, three co-fermentation trials (J-Sc11-Sc17, J-Sc11-Sc21, and J-Sc17-Sc21) of two different NSY in simultaneous fermentation with *S. cerevisiae* jiangnan1 showed differential sensory style (Fig. 3C), owing a higher overall taste intensity due to the significant differences in all the taste attributes (weakening in astringent, bitter, and acidic; strengthening in sweet and umami). Also, the overall aroma intensity was no significant differences, the aroma attributes (weakening in honey, fruity, and alcoholic aromas; strengthening in Qu and herb aromas) were a differential style between jiangnan1# and co-fermentation trails, indicating that the *huangjiu* samples of co-fermentation trails were more closer to traditional *huangjiu* (Fig. 3A and C). Indeed, using no less than one kind of the three NSY strains in simultaneous fermentation with *S. cerevisiae* jiangnan1# resulted in sensory diversity of new *huangjiu* styles.

4. Conclusion

A method for evaluation and application of NSY in co-fermentation with *S. cerevisiae* jiangnan1# in *huangjiu* brewing has been established for the first time. Simultaneous inoculation with appropriate proportion of *S. cerevisiae* and NSY can achieve the application of NSY in *huangjiu* fermentation, while different strains had differentiated effects on the final products. Three NSY strains belonging to different genus are final obtained, seven types of fermentation trials have been presented for evaluating their effects on overall quality, flavor substances, and sensory properties of *huangjiu*. Some consistent results have shown that the total content of organic acids is significant increase ($p < 0.05$), the

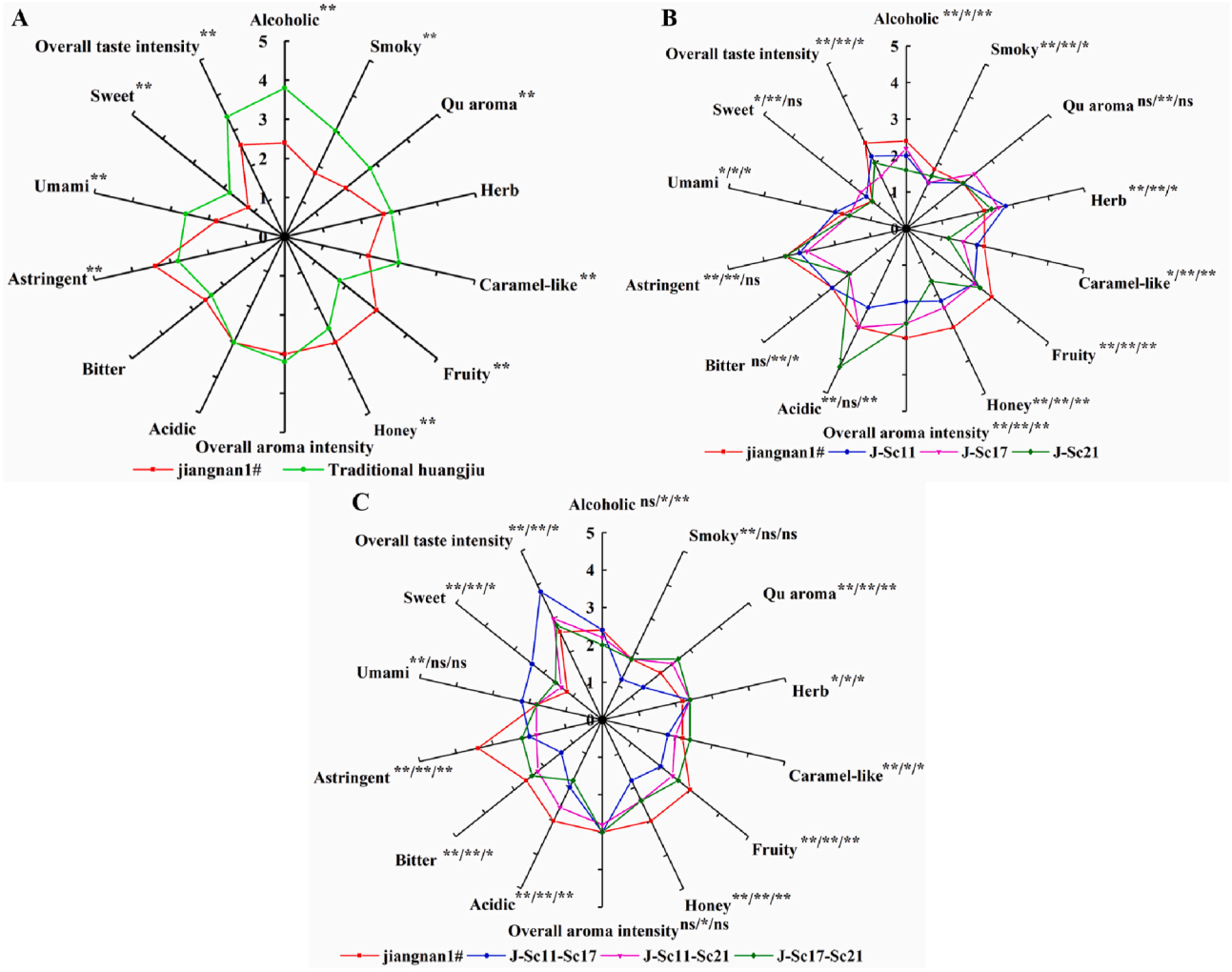

Fig. 3. The aroma and taste profiles of *huangjiu* co-fermentation trails by using 3 different NSY and *S. cerevisiae* jiangnan1#. An ANOVA test was also carried out on the data obtained from the sensory (aroma and taste) descriptive analysis. Significance was indicated at **, $P < 0.01$, *, $P < 0.05$ between the fermentation trials and the control. A: The *huangjiu* samples fermented by jiangnan1# and traditional *huangjiu*; B: The *huangjiu* co-fermentation trails by using *S. cerevisiae* jiangnan1# and single NSY strain; C: The *huangjiu* co-fermentation trails by using *S. cerevisiae* jiangnan1# and two different NSY strains.

concentration of higher alcohols is moderate, and the content of ethyl esters are improved in final *huangjiu* products by the use of NSY compared with that fermented by *S. cerevisiae* jiangnan1# alone. Our research provided comprehensive fermentation characters for the different species of NSY strains in order to improve overall quality, increase the variety of product flavor and sensory styles, and meanwhile improve drinking comfort of *huangjiu*. Moreover, using three types of NSY resulted in sensory diversity of *huangjiu*. Further research is needed to study the fermentation characteristics of other NSY strains separated from the traditional *huangjiu* brewing, which could improve drinking comfort and promote the quality of modern *huangjiu*.

Author statement

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102539.

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