LWT - Food Science and Technology 132 (2020) 109839

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Biomass suppression of *Hanseniaspora uvarum* by killer *Saccharomyces cerevisiae* highly increased fruity esters in mixed culture fermentation

Yue-Qi Li [a,1], Kai Hu [a,1], Yin-Hu Xu [b], Wen-Chao Mei [a], Yong-Sheng Tao [a,c,*]

[a] College of Enology, Northwest A&F University, Yangling, Shaanxi, 712100, China
[b] Angel Yeast Co., LTD, Yichang, Hubei, 443003, China
[c] Shaanxi Engineering Research Center for Viti-viniculture, Yangling, Shaanxi, 712100, China

ARTICLE INFO

Keywords:
Wine yeast
Antagonistic activity
Winemaking
Aroma compound
Fatty acid

Chemical compounds studied in this article:
Ethyl acetate (PubChem CID: 8857)
Isobutyl acetate (PubChem CID: 8038)
Isoamyl acetate (PubChem CID: 31276)
Ethyl butyrate (PubChem CID: 77632)
Ethyl hexanoate (PubChem CID: 31265)
Ethyl octanoate (PubChem CID: 7799)
Ethyl decanoate (PubChem CID: 8048)

ABSTRACT

Hanseniaspora uvarum strain Yun268 was inoculated with *Saccharomyces cerevisiae* strains of different antagonistic abilities (RV002 and RV171) to evaluate fruity ester production in mixed fermentation. Co-inoculation (CI) and sequential inoculation (SI) of two yeast species were performed in synthetic must and in Granoir winemaking. Fermentation kinetics, the formation of yeast biomass and fruity esters were monitored during fermentation, and the aroma attributes of the final wines were quantified by trained panelists. Results showed that killer *S. cerevisiae* suppressed biomass of Yun268 more than common *S. cerevisiae*, especially in CI treatment. Higher concentration of medium chain fatty acids was associated with the higher biomass suppression of *H. uvarum* in CI with killer *S. cerevisiae*, which resulted in the increased formation of fruity esters, but effectively restricted the production of ethyl acetate. However, more biomass and longer survival time of *H. uvarum* in SI considerably increased volatile acidity (3-fold higher than that in single fermentation), which delayed the reproduction of *S. cerevisiae* temporarily, and elevated the levels of ethyl acetate. Winemaking confirmed that the co-culture of *H. uvarum* and killer *S. cerevisiae* highly improved fruity aromas, which was due to the increase in ester production, especially medium chain fatty acid ethyl esters.

1. Introduction

Mixed fermentation involving non-*Saccharomyces* (NS) yeast can shape the chemical profile of wine aromatic compounds and improve the diversity of wine aroma (Johnson, 2013; Padilla, Gil, & Manzanares, 2016; Wyk, Grossmann, Jürgen, Wallbrunn, & Pretorius, 2019). Currently, there is increasing interest toward mixed fermentation with different NS yeasts due to their positive effects on enological performance (Ciani & Comitini, 2015). Esters significantly affect the formation of desired fruity and floral aromas (Poivet et al., 2018; Saenz-Navajas et al., 2016). In particular, acetate esters and fatty acid ethyl esters are formed via the reaction of alcohol and corresponding fatty acids during fermentation (Ebeler, 2001). Ma, Yan, Wang, Zhang, and Tao (2017) observed that the mixed fermentation of selected *Pichia fermentans* and *Saccharomyces cerevisiae* increased the acetate and ethyl ester content in Ecolly dry white wine. In addition, a study on mixed fermentation of *Torulaspora delbrueckii* and *S. cerevisiae* showed that concentrations of isobutyl acetate and isoamyl acetate were systematically increased (Renault, Coulon, de Revel, Barbe, & Bely, 2015). The different growth rates of yeasts affect ester production. Among the studied NS species, *Hanseniaspora uvarum* showed high ability to produce fruity esters in mixed fermentation with *S. cerevisiae*. However, over metabolism of *H. uvarum* may yield more ethyl acetate, resulting in unpleasant off-flavor similar to that of nail polish (Hu, Jin, Mei, Li, & Tao, 2018; Hu, Jin, Xu, & Tao, 2018). Therefore, it is necessary to regulate the performance of *H. uvarum* in ester production during mixed fermentation.

The killer ability of yeasts was first observed in certain strains of *Saccharomyces* and was related to yeast interactions (Marquina, Santos, & Peinado, 2002). Certain *S. cerevisiae* strains may release toxins that inhibit or regulate the growth of other yeasts (Orentaite, Poranen, Oksanen, Daugelavicius, & Bamford, 2016). Generally, killer *S. cerevisiae* is used to prevent the infection of grape natural yeasts and bacteria and effectively guarantee the purity of fermentation. One study showed that killer yeast can affect the microbial biomass balance of two *S. cerevisiae* strains during mixed culture (Ramon-Portugal, Delia, Strehaiano, & Riba, 1997). In winemaking, the co-inoculation of *S. cerevisiae*

* Corresponding author. College of Enology, Northwest A&F University, 22 Xinong Road, Yangling, Shaanxi, 712100, China.
E-mail address: taoyongsheng@nwsuaf.edu.cn (Y.-S. Tao).
[1] These authors equally contributed to this work.

https://doi.org/10.1016/j.lwt.2020.109839
Received 26 March 2020; Received in revised form 2 July 2020; Accepted 4 July 2020
Available online 13 July 2020

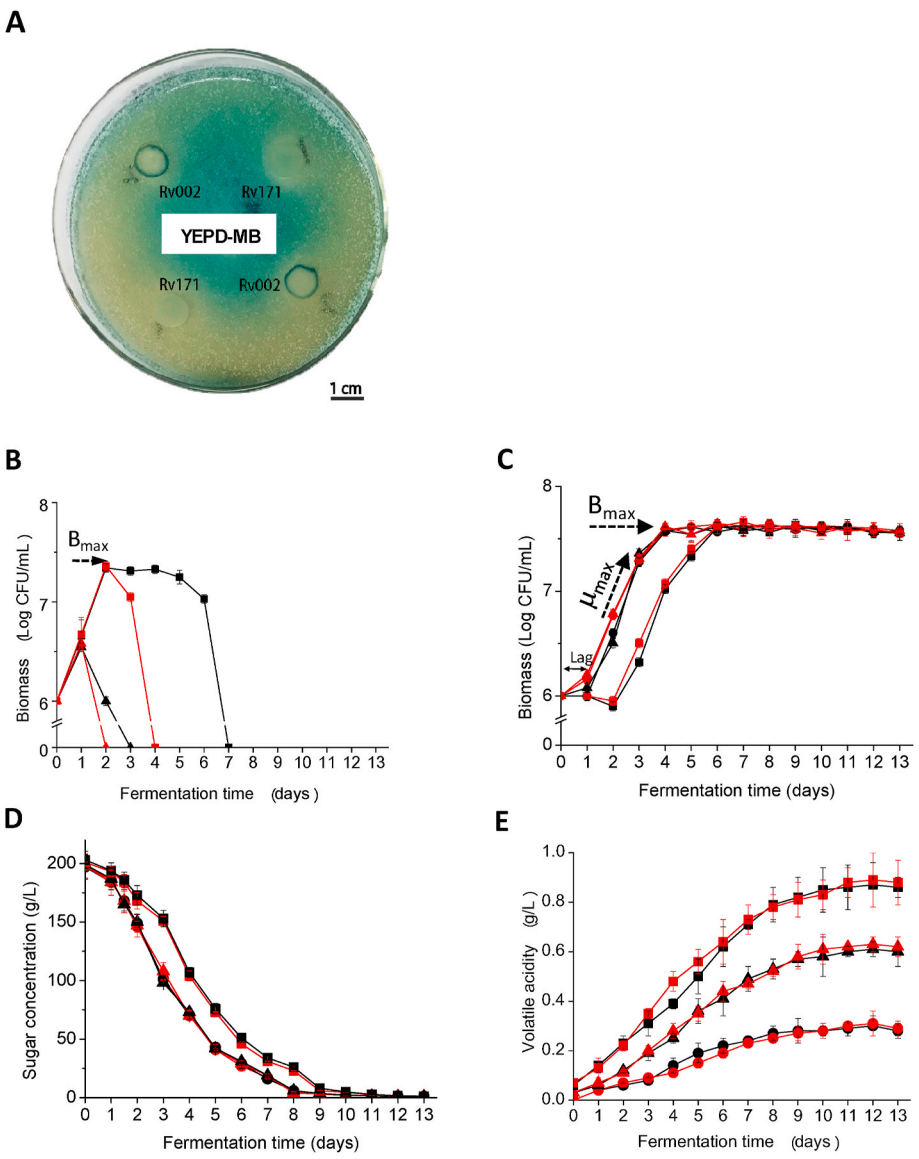


Fig. 1. Fermentation kinetics and enological parameters in SGM fermentations: (A) Identification of yeast antagonistic ability, (B) biomass of *H. uvarum*, (C) biomass of *S. cerevisiae*, (D) sugar consumption of fermentation, and (E) production dynamics of volatile acid during fermentation. RV171 (black circle), RV002 (red circle), CI-RV171 (black triangle), CI-RV002 (red triangle), SI-RV171 (black square), and CI-RV002 (red square). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

strains with different antagonistic abilities enhanced the quality of sparkling wines (Lombardi, De Leonardis, Lustrato, Testa, & Iorizzo, 2015). A few studies have investigated the suppressive effect of *S. cerevisiae* strains on the physiological properties of NS yeast during fermentation (Branco, Viana, Albergaria, & Arneborg, 2015; Kemsawasd et al., 2015). However, very little is known about the suppressive effect of *S. cerevisiae* on the production of wine aroma compounds during mixed culture fermentation with NS strains.

In this study, *H. uvarum* strain Yun268 was inoculated with *S. cerevisiae* strains RV002 (high antagonistic ability, killer) and RV171 (low antagonistic ability, non-killer) using different inoculation strategies for Granoir winemaking. The response of ester production and aroma profiles to the suppression of *H. uvarum* by *S. cerevisiae* was evaluated. The results may provide knowledge regarding the regulation of NS yeast, and contribute to the efficient modulation of aroma in wine fermentation.

2. Materials and methods

2.1. Chemicals and reagents

Glucose, fructose, peptone, yeast extract powder, agar, citric acid, L-tartaric acid, L-malic acid, methylene blue, NaCl, NaOH, SO_2, $(NH_4)_2SO_4$, $MgSO_4$, $MnSO_4$, KCl, Na_2HPO_4, and KH_2PO_4 were obtained from Aoboxing Bio-Tech Co. Ltd (Beijing, China). p-Nitrophenyl acetate (C2, 99%), p-nitrophenyl butyrate (C4, 99%), p-nitrophenyl hexanoate (C6, 98%), p-nitrophenyl octanoate (C8, 97%) (J&K, Beijing, China), and p-nitrophenyl decanoate (C10, 98%) were purchased from Sigma-Aldrich, Shanghai, China. Chemical standards used for identification and quantification of volatiles were at least 97% pure (Sigma-Aldrich, Shanghai, China). Water was purified using a Milli-Q system (Millipore, Bedford, USA).

2.2. Strains and media

S. cerevisiae strains RV002 (high antagonistic ability) and RV171 (low antagonistic ability) were supplied by Angel Yeast Co., Ltd. (Yichang, China). These two strains were chosen to create different suppression levels for NS yeast during mixed fermentation. *H. uvarum* Yun268 was selected from the Yun-Nan Hong vineyard (Yunnan Province, China), and preserved in China Center for Type Culture Collection (CCTCC M2013658).

The killer activity of *S. cerevisiae* against *H. uvarum* (Yun268) was tested using YEPD-MB agar (YEPD containing 0.03 g/L methylene blue

Table 1
Characteristics of yeast growth in single and mixed culture fermentations.

Characteristics	RV171	RV002	CI-RV171	CI-RV002	SI-RV171	SI-RV002
Lag (d)						
S. cerevisiae	1.20 ± 0.06a	0.42 ± 0.60bc	1.49 ± 0.04a	0.23 ± 0.32c	1.35 ± 0.06a	1.11 ± 0.07 ab
μ_{max} (1/h)						
S. cerevisiae	0.75 ± 0.01b	0.54 ± 0.10cd	0.90 ± 0.01a	0.47 ± 0.01d	0.60 ± 0.01c	0.58 ± 0.01c
B_{max} (Log CFU/mL)						
S. cerevisiae	7.63 ± 0.05a	7.64 ± 0.05a	7.62 ± 0.06a	7.64 ± 0.04a	7.64 ± 0.05a	7.67 ± 0.05a
H. uvarum	–	–	6.54 ± 0.04b	6.58 ± 0.04b	7.33 ± 0.03a	7.36 ± 0.04a

Values followed by different letters in a row indicate significant differences ($P < 0.05$) by Duncan test.

and 20 g/L agar) according to the procedure reported by Santo, Galego, Goncalves, and Quintas (2012). Approximately 10^5 cells/mL of *H. uvarum* (48 h old) were plated as a lawn onto YEPD-MB agar. After 2 h, 10^8 cells/mL of RV002 and RV171 (48 h old) were inoculated in a concentrated area (1 cm diameter) and cultivated at 28 °C for 2 d. *S. cerevisiae* was classified as a killer isolate when it inhibited *H. uvarum* growth on the agar surfaces and the inhibition zone was surrounded by a dark blue boundary.

2.3. Fermentation experiments

Mixed culture fermentation included two modalities: co-inoculation (CI) in which *H. uvarum* Yun268 and *S. cerevisiae* (either RV171 or RV002) were co-inoculated, and sequential inoculation (SI) where *H. uvarum* Yun268 was inoculated 24 h prior to *S. cerevisiae*. The initial concentration of each yeast strain was 1×10^6 cells/mL. Single fermentation with RV002 or RV171 served as the control. Alcohol fermentation was considered completed when the residual sugar content dropped below 2 g/L according to Fehling titration method (GB/T 15038-2006, 2006). Wine samples were stored at 4 °C for further analysis.

A synthetic grape must (SGM) (pH 3.2) was used to simulate wine fermentation. The SGM contained 100 g/L glucose, 100 g/L fructose, 1 g/L yeast extract (Aoboxing, Beijing, China), 0.3 g/L citric acid, 5 g/L L-tartaric acid, 5 g/L L-malic acid, 2 g/L $(NH_4)_2SO_4$, 5 g/L KH_2PO_4, 0.4 g/L $MgSO_4$, 0.2 g/L NaCl, and 0.05 g/L $MnSO_4$ (Rossouw, Du Toit, & Bauer, 2012). Fermentation was performed in 1-L Erlenmeyer flask at 22 °C. Fermentation kinetics was monitored via sugar consumption every 24 h. Biomasses of the strains were counted using Wallerstein (WL) nutrient agar medium and volatile acidity of must (expressed as acetic acid content) was determined using distillation/titration method every 24 h (GB/T 15038-2006, 2006). The dynamic changes in fruity ester production and esterase activity were also determined every 24 h.

Granoir grapes (*V. vinifera* L.), introduced from Switzerland, were harvested manually from a vineyard in Heyang (Shaanxi Province, China). The contents of sugar and acid (expressed as tartaric acid) were 178 g/L and 7.96 g/L, respectively. Sugar and acid contents were determined using Fehling and NaOH titration methods, respectively (GB/T 15038-2006, 2006). Grapes were destemmed, crushed, treated with 60 mg/L SO_2, and divided into 10-L glass jars. After that, each must was treated with 20 mg/L pectinase (Optizym®, Laffort Co. Bordeaux, France) at room temperature, followed by maceration with skins at 4 °C for 24 h. Before yeast inoculation, 22 g/L sucrose was added into the must to achieve the same level of final ethanol content as SGM. Fermentation was performed at 22 °C. After fermentation, the wine samples were treated with 60 mg/L SO_2 and stored at 4 °C for further analysis.

2.4. Esterase activity assay

Esterase activity of fermentation yeasts was assayed using the *p*-NP method according to Hu, Jin, Mei, et al. (2018). Substrate specificity of the esterase was determined for the following five *p*–nitro-phenyl (*p*–NP) esters with different chain lengths: C2 (*p*–NP acetate), C4 (*p*–NP butyrate), C6 (*p*–NP hexanoate), C8 (*p*–NP octanoate), and C10 (*p*–NP decanoate). The absorbance of the released *p*–NP was determined at 400 nm using an ultraviolet–visible spectrophotometer (UV1780, Shimadzu, Shanghai, China) against a blank solution with the inactivated enzyme.

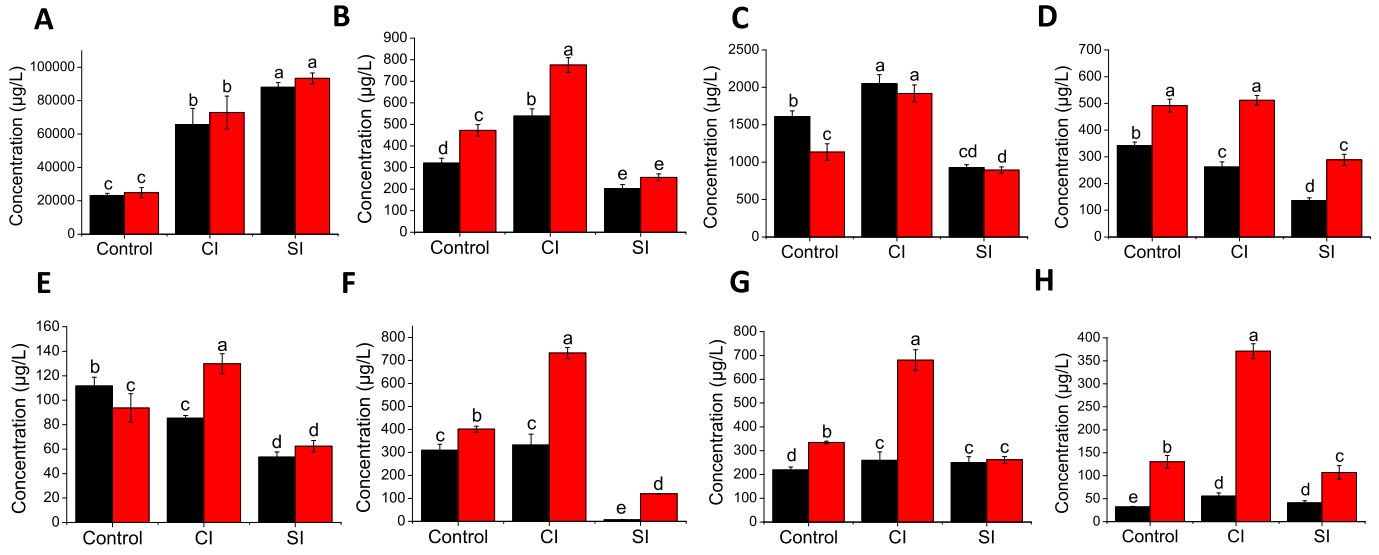


Fig. 2. The levels of fruity esters in SGM fermentations: (A) Ethyl acetate, (B) isobutyl acetate (C) isoamyl acetate, (D) 2-phenylethyl acetate, (E) ethyl butyrate, (F) ethyl hexanoate, (G) ethyl octanoate, and (H) ethyl decanoate. Values with different letters indicate significant differences among all the treatments ($P < 0.05$) by Duncan test. RV171 (black bar), and RV002 (red bar). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Two-way ANOVA regarding the effects of *S. cerevisiae* type, inoculation modality, and their interaction on the production of fruity esters.

Compounds	Factors		
	S. cerevisiae type	Inoculation modality	*Interaction*
Ethyl acetate	ns	***	ns
Isobutyl acetate	***	***	**
Isoamyl acetate	**	***	*
2-Phenethyl acetate	***	***	*
Ethyl butyrate	*	***	**
Ethyl hexanoate	***	***	***
Ethyl octanoate	***	***	***
Ethyl decanoate	***	***	***

*, **, *** Indicate significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$; ns indicates not significant. *S. cerevisiae* type: RV002 and RV171. Inoculation modality: Sc, CI, and SI. Interaction: interaction effect between *S. cerevisiae* type and inoculation modality.

Unit of esterase activity (U) was expressed as micromoles of *p*–NP released per minute per gram of dry cells at 40 °C.

2.5. Gas chromatography-mass spectrometry (GC-MS) analysis

Volatile compounds were analyzed using headspace solid-phase microextraction-gas chromatography/mass spectrometry (HS-SPME-GC/MS) (Hu et al., 2019). Wine sample (8 mL), internal standard (40 μg/L 2-octanol), and 2 g NaCl were added in a 15-mL headspace bottle with stirrer magnets. The mixture was placed in a 40 °C water bath with stirring for 15 min at 600 rpm. After that, the volatiles were extracted using a DVB/CAR/PDMS fiber (50/30 μm film thickness) for 30 min with continuous heating and stirring. Then, the extracted volatiles were desorbed in the GC injector at 250 °C for 5 min. Volatile analysis was conducted using GC-MS-QP2020 (Shimadzu Corporation, Japan) equipped with a DB-WAXETER capillary column (60 m × 0.25 mm × 0.25 μm, Agilent J & W, USA). The carrier gas was helium (purity ≥ 99.999%) at 1.5 mL/min. The GC program started at 40 °C, after which the temperature was raised to 160 °C at 4 °C/min, then to 220 °C at 7 °C/min, and held at 220 °C for 8 min. MS transfer line and ion source temperatures were 220 °C and 200 °C, respectively. Ion-electron impact spectra at 70 eV were scanned in the range m/z 35–350 amu with 0.2 s interval.

Volatile compounds were identified by comparing their retention time and mass spectra with those of pure standards using the NIST17 mass spectral library. The identified compounds were quantified by interpolating the relative areas versus the area of the internal standard (2-octanol) using calibration graphs established for pure standards.

2.6. Sensory analysis

Wine sensory analysis was performed according to the method reported by Kong, Li, Jin, Zhu, and Tao (2019). Panelists (18 females and 12 males) were trained with a 54-aroma kit (Le Nez du Vin®, France) until their identification accuracy for each aroma exceeded 95%. The 54-aroma kit was divided into several aroma groups, such as flower (rose, lily, and violet), sweet fruit (peach and plum), and acid fruit (apple and pear). Samples were presented in black wine glasses labeled with three-digit codes and were distributed randomly. Each panelist was asked to describe aromas using 4–6 terms from the aroma kit and grade the intensity using a five-point scale. The modified frequency (*MF* %) of each aroma characteristic was calculated using the formula:

$$MF\% = \sqrt{F(\%)I(\%)}$$

where *F*% is the average detection frequency of the described referred terms in an aroma group by the panel, and *I*% is the average intensity of the described referred terms in the group expressed as the percentage of maximum intensity.

2.7. Statistical analysis

All the experiments were conducted in triplicate. One-way analysis of variance (ANOVA) with Duncan test ($\alpha = 0.05$) was applied to compare the data and two-way ANOVA test was conducted to assess the effects of the two tested factors (*S. cerevisiae* type and inoculation modality) and their interaction on ester production. Pearson correlation analysis was used to establish the correlation between esters and three factors (higher alcohols, fatty acids, and esterase activities). Principal component analysis (PCA) was used to measure the variance among fermentation modalities (data were normalized by Z-score) using SPSS statistical package version 20.0 (SPSS Inc. Chicago, IL, USA). ComBase (https://www.combase.cc/index.php/en) was used to calculate yeast growth parameters, including maximum growth rate (μ_{max}, 1/h), lag phase (Lag, d), and maximum biomass (B_{max}, Log CFU/mL).

3. Results and discussion

3.1. Biomass suppression of H. uvarum during mixed fermentation with S. cerevisiae

It is well-established that, during the co-inoculation, NS yeasts generally exist only in the early stage of fermentation while *S. cerevisiae* continues to survive till completion of alcohol fermentation (Domizio et al., 2011). Based on the color of the colony boundary (Fig. 1A), the killer *S. cerevisiae* RV002 showed higher antagonistic ability against *H. uvarum*. Results showed that the biomass suppression of *H. uvarum* had different responses to inoculation strategies (CI and SI) and *S. cerevisiae* types. The killer *S. cerevisiae* and CI suppressed *H. uvarum* more than others (Fig. 1B and C). Yeasts in mixed fermentation preferentially use easily assimilable nitrogen sources, expand biomass rapidly, and produce toxic substances for competitors. However, *S. cerevisiae,* particularly killer *S. cerevisiae*, triggers the early death of non-*Saccharomyces* cells (Albergaria, Francisco, Gori, Arneborg, & Girio, 2010; Perez-Nevado, Albergaria, Hogg, & Girio, 2006). Ciani and Comitini (2015) also proposed that cell-to-cell contact mechanisms and the competition for nutrients might be involved in the early death of NS yeasts and the dominance of *S. cerevisiae*. *H. uvarum* cells in SI-RV002 reached B_{max} on day 2, and then began to die once *S. cerevisiae* was inoculated. Furthermore, B_{max} of *H. uvarum* increased by 12% in SI compared to that in CI (Table 1). During SI, the rapid consumption of nutrients by *H. uvarum* temporarily prevented the implantation of *S. cerevisiae*. Hence, the biomass of *H. uvarum* increased with longer survival time. The lowest biomass and shortest survival time of *H. uvarum* in CI-RV002 fermentation can be explained by the stronger competitiveness of RV002. For *S. cerevisiae* in both single and co-cultures, the growth of RV002 showed lower values of lag (d) and μ_{max} (1/h) than RV171. The B_{max} of *S. cerevisiae* showed no significant difference in any treatment. It was obvious that the suppression of *H. uvarum* was improved by CI and the killer activity of *S. cerevisiae*. Although the maximum biomass of *S. cerevisiae* was delayed for several days in mixed fermentation kinetics, the fermentations of SGM were all completed successfully (Fig. 1D).

3.2. Increase in production of fruity esters in mixed fermentation of H. uvarum and S. cerevisiae

Studies have shown that NS yeasts in mixed fermentation can promote the production of esters, which increases the aroma complexity of wine (Johnson, 2013; Carrau, Gaggero, & Aguilar, 2015; Padilla et al., 2016). In this work, ethyl acetate level did not vary significantly between RV002 and RV171 in either single or mixed cultures, but it was the highest in sequential cultures (Fig. 2). This result was consistent with the growth of *H. uvarum* (Fig. 1B) and the production of volatile acids

Table 3
Concentrations of fermentative compounds quantified using SPME-GC-MS in wine samples (μg/L).

RT[A]	Compounds	Threshold[B]	RV002	CI-RV002	SI-RV002	RV171	CI-RV171	SI-RV171	OAV[D]	Odor description[C]
Higher alcohols (μg/L)			388686 ± 30779a	398369 ± 9711a	384119 ± 15341a	380574 ± 30269a	364043 ± 31577a	394424 ± 24627a		
12.80	Isobutyl alcohol	40000[1]	121644 ± 5991 ab	130470 ± 7980a	111733 ± 967bc	103908 ± 4950cd	100983 ± 1313d	122675 ± 7323 ab	>1	alcohol, nail polish
16.77	Isoamyl alcohol	30000[1]	267012 ± 24786a	267876 ± 1730a	272365 ± 14373a	276637 ± 25316a	263037 ± 30262a	271730 ± 17303a	>1	whisky, nail polish
25.72	1-Heptanol	1000[1]	8±0a	8±0a	6±1b	7±0 ab	7±0 ab	7±0 ab	<0.1	green
29.21	1-Octanol	120[1]	11±1a	6±0b	5±0b	11±1a	5±1b	4±1b	<0.1	orange, rose
32.53	1-Nonanol	600[1]	6±0a	4±0b	5±0 ab	6±1a	4±1b	5±0 ab	<0.1	mushroom, sweet
35.52	1-Decanol	400[1]	5±1b	5±1b	5±0b	5±1b	7±0a	3±0c	<0.1	orange flowery
Acetate esters (μg/L)			70320 ± 3009c	81203 ± 2901bc	92275 ± 683a	72180 ± 4014c	86530 ± 3291b	95463 ± 704a		
6.51	Ethyl acetate	7500[1]	70320 ± 3009c	81203 ± 2901bc	92275 ± 683a	72180 ± 4014c	86530 ± 3291b	95463 ± 704a	>1	pineapple, fruity
Acetates of higher alcohols (μg/L)			480 ± 24b	449 ± 29cd	491 ± 36b	473 ± 41bc	416 ± 16d	538 ± 29a		
9.72	Isobutyl acetate	1600[1]	317 ± 13 ab	298 ± 20bc	356 ± 26a	284 ± 24c	281±7c	360 ± 17a	0.1–1	flowery
13.42	Isoamyl acetate	30[1]	163 ± 11bc	151±9cd	135 ± 10d	189 ± 17a	135±9d	178 ± 12 ab	>1	banana, fruity
Phenylethyls (μg/L)			39241 ± 3318 ab	41056 ± 2892 ab	49294 ± 4463a	44978 ± 3697 ab	38667 ± 4290b	45522 ± 4297 ab		
37.25	2-Phenylethyl acetate	250[2]	35±4b	43±2 ab	47±4a	40±4 ab	44±4 ab	38±3 ab	0.1–1	floral
39.42	2-Phenylethanol	10000[2]	39206 ± 3314 ab	41013 ± 2890 ab	49247 ± 4459a	44938 ± 3693 ab	38623 ± 4286b	45484 ± 4294 ab	>1	rose, pollen
Fatty acids ethyl esters (μg/L)			871 ± 49bc	1134 ± 92a	830 ± 73bc	727 ± 53c	987 ± 158 ab	849 ± 78bc		
Short chain fatty acid ethyl esters			270 ± 26 ab	298 ± 22a	235 ± 14b	254 ± 18 ab	296 ± 13a	230 ± 22b		
8.20	Ethyl isobutyrate	15[2]	42±2a	43±1a	41±1a	40±1a	44±2a	33±2b	>1	strawberry
10.49	Ethyl butyrate	20[1]	204 ± 22 abc	233 ± 21a	167 ± 11c	189 ± 14bc	223 ± 11 ab	172 ± 19c	>1	strawberry, apple
10.82	Ethyl 2-methylbutyrate	18[1]	24±2bc	22±0c	27±2 ab	25±3 abc	29±0a	25±1 abc	>1	sweet fruit
Medium chain fatty acid ethyl esters			601 ± 23bc	836 ± 70a	595 ± 59bc	473 ± 35c	691 ± 145 ab	619 ± 56bc		
17.71	Ethyl hexanoate	5[1]	296 ± 17bc	396 ± 39a	282 ± 28bc	226 ± 11c	323 ± 36b	279 ± 23bc	>1	floral, fruity
25.34	Ethyl octanoate	5[2]	261±2bc	380 ± 26a	261 ± 28bc	215 ± 21c	274 ± 105bc	260 ± 26bc	>1	pineapple, floral
32.08	Ethyl decanoate	200[1]	44±4b	60±5a	52±3 ab	32±3c	48±4b	42±5b	0.1–1	fruity
Fatty acids (μg/L)			5099 ± 457 ab	5555 ± 333a	4793 ± 214 abc	4289 ± 365bc	4696 ± 150bc	4122 ± 378c		
29.63	Isobutyric acid	2300[3]	2653 ± 239a	2394 ± 152a	2350 ± 20a	2212 ± 243 ab	2469 ± 88a	1907 ± 183b	>1	cheese, rancid
37.63	Hexanoic acid	420[2]	1323 ± 126b	1709 ± 17a	1280 ± 141b	1120 ± 62b	1259 ± 13b	1280 ± 112b	>1	cheese, fatty
42.08	Octanoic acid	500[1]	919 ± 84b	1178 ± 140a	933 ± 35b	778 ± 44b	755 ± 37b	804 ± 81b	>1	rancid, creamy
46.48	Decanoic acid	1000[1]	204±8bc	274 ± 24a	230 ± 18b	179 ± 16c	213 ± 12bc	131±2d	0.1–1	fatty, unpleasant
Volatile acidity (g/L)[E]			0.29 ± 0.02c	0.65 ± 0.05b	0.90 ± 0.10a	0.28 ± 0.02c	0.63 ± 0.04b	0.88 ± 0.09a		

Note: [A] RT, retention time on a DB-WAX column. [B, C] NF, Not found. Reference numbers are provided in brackets following the odor thresholds. [1] Peng, Wen, Tao, and Lan (2013); [2] Hu, Jin, Xu, and Tao (2018); [3] Mayr et al. (2014). [D] OAV = concentration/threshold.
[E] Volatile acid (expressed as acetic acid content) was determined using the steam distillation method. Values followed by different letters in a row indicate significant differences ($P < 0.05$) by Duncan test.

(Fig. 1E). For fruity esters, SI decreased the content of most of the esters, while CI improved the production of most esters. Compared to the single fermentation, short and medium chain fatty acid ethyl esters (FAEEs) in SI were decreased by 43.6% and 44.9%, respectively. However, medium chain FAEEs in CI were increased by 49.7%. In contrast, the levels of isobutyl acetate, 2-phenylethyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate in CI-RV002 were higher than those in CI-RV171 ($P < 0.05$). It is now known that interaction between *S. cerevisiae* and NS yeasts plays a fundamental role in their growth and the formation of flavor substances (Cheraiti, Guezenec, & Salmon, 2005; Renault, Albertin, & Bely, 2013; Taillandier, Quoc, Julien-Ortiz, & Brandam, 2014). To further investigate the effect of *S. cerevisiae* type and inoculation modality on ester profiles, two-way ANOVA test was applied (Table 2). With the exception of ethyl acetate, which only depended on inoculation modality, the levels of other esters were affected by both factors. CI was the most effective modality to increase the amount of fruity esters. The killer *S. cerevisiae* was more detrimental for *H. uvarum*, and the latter only survived for two days in CI. This led to the highest levels of fruity esters in CI-RV002, especially medium chain FAEEs. However, SI decreased the content of fruity esters, with the exception of ethyl acetate. This might be caused by the higher survival time and biomass (2×10^7 CFU/ml) of *H. uvarum* at the early stage of fermentation in SI, which led to increased consumption of special nutrients and limited the production of fruity esters, but considerably improved the content of ethyl acetate. In practice, the participation of NS yeasts always increases the levels of acetic acids and their esters. The level of ethyl acetate was the highest among fruity esters. However, excess ethyl acetate (˃ 100 mg/L) is likely to impart off-flavor odor (Hu, Jin, Mei, et al., 2018; Sumby, Grbin, & Jiranek, 2010). In this study, the decrease in both B_{max} and *H. uvarum* survival time suppressed the formation of ethyl acetate in the presence of killer *S. cerevisiae* (CI).

Real wine samples were obtained using the above yeast inoculation strategies to verify the findings in SGM fermentations. Twenty-one fermentative volatiles were quantified in wine samples (Table 3).

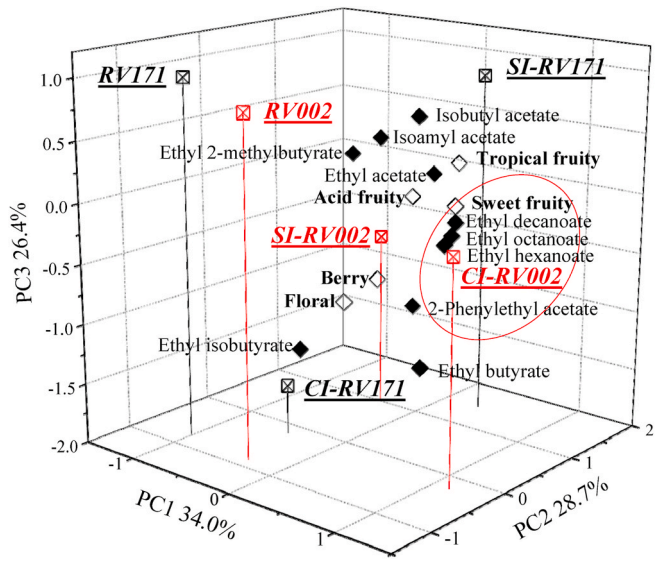
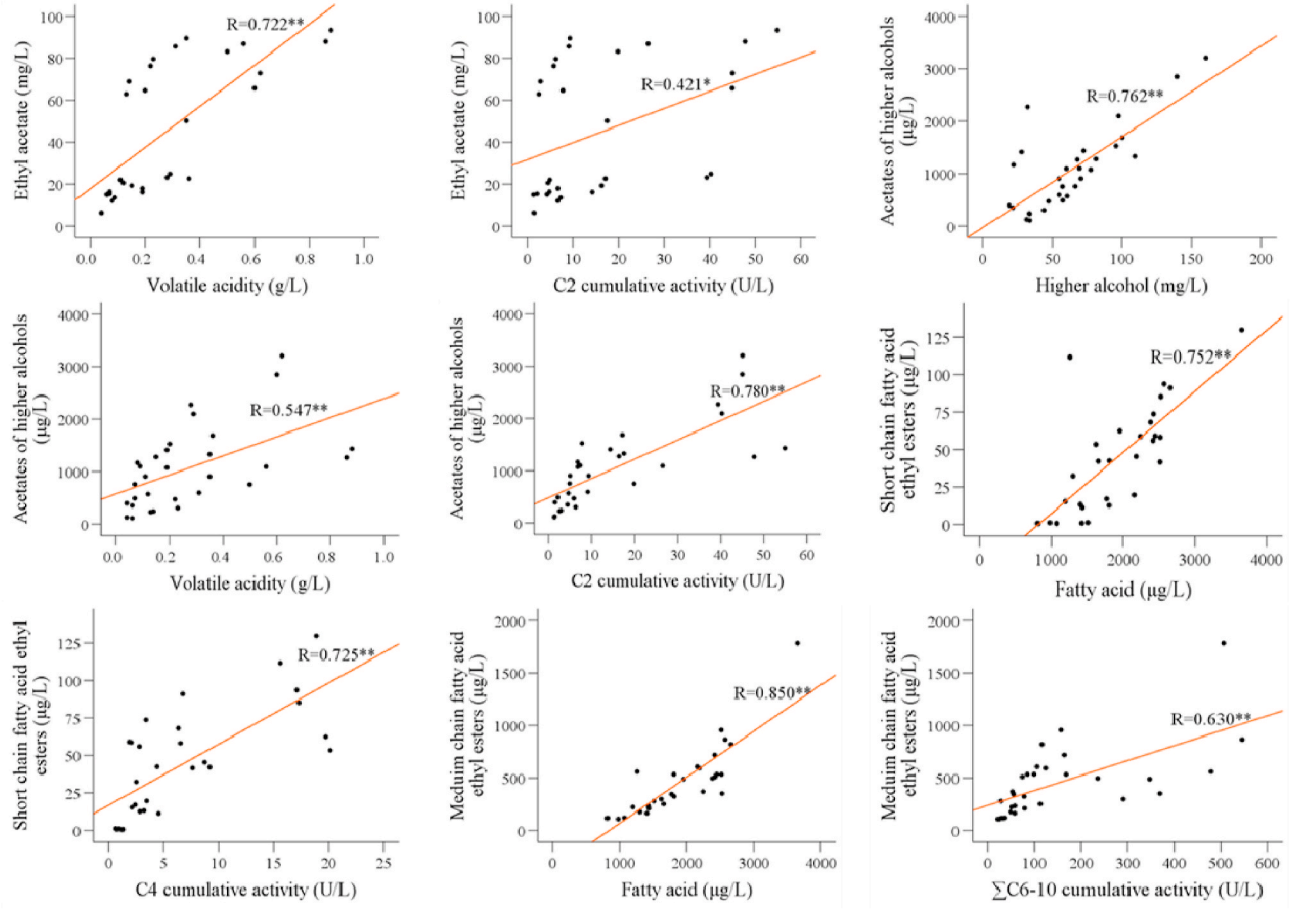


Fig. 3. Principal component analysis (PCA) of fruity esters and aroma attributes of wine. Data are mean values of the replicate fermentations. Wine sample (black/red square), esters (solid diamond), and aroma traits (open diamond). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

These fermentative volatiles included ethyl acetate, acetates of higher alcohols (AHAs), FAEEs, higher alcohols, fatty acids and phenylethyls. The results for production of ethyl acetate and volatile acidity were consistent with those obtained using SGM. The average content of AHAs and FAEEs in the inoculations with RV002 was higher than that with RV171. Five aroma attributes, namely tropical fruity, sweet fruity, acid fruity, berry, and floral, were selected to describe the aroma profile of wine samples. The modified frequencies (*MF* %) of the five aroma attributes in wine samples are shown in Fig. S1. Except for tropical fruity, CI-RV002 wine had high *MF* values of other aroma traits, especially sweet fruity (*MF* = 58%) and floral (*MF* = 46%) aroma. This enhancement can be attributed to the increase in ester level in CI-RV002 wine, since these esters generally contribute to fruity and floral aromas (Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017; Sumby et al., 2010). To understand the effect of each treatment on ester and aroma attributes, PCA of fruity esters and aroma attributes was performed (Fig. 3). Compared with the single fermentation, CI promoted the production of FAEEs while SI increased the levels of AHAs and ethyl acetate. CI-RV002 wine had the highest levels of fruity esters (especially medium chain FAEEs) with controlled level of ethyl acetate.

3.3. Factors affecting the formation of fruity esters

It is believed that both yeasts in mixed fermentation preferentially use easily assimilable nitrogen sources, expand biomass rapidly, and produce toxic substances for competitors (Perez-Nevado et al., 2006;



27

Fig. 4. Correlation analysis between fruity esters and three impact factors (higher alcohol, fatty acid and esterase activity) in SGM fermentations. Higher alcohol = ∑ (isobutyl alcohol + isoamyl alcohol + 2-phenylethanol). Fatty acid = ∑ (butyric acid + hexanoic acid + octanoic acid + decanoic acid). * Indicates significant differences at $P < 0.05$; ** indicates significant differences at $P < 0.01$.

Albergaria et al., 2010). Amino acids in grape juice are the assimilable nitrogen sources commonly available for rapid metabolism of yeast. Higher alcohols are produced via the Ehrlich pathway, which involves the degradation of amino acids (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). In this study, the contents of three higher alcohols and four fatty acids were compared, as shown in Fig. S2. CI (especially CI-RV002) possibly accelerated the rate of amino acid consumption, which increased the levels of higher alcohols in SGM fermentations, although the acceleration phenomenon of CI in real wine fermentation was not significant (Table 3). This may be the reason that real grape juice contains assimilable nitrogen sources other than amino acids, such as ammonium and short chain peptides (Kevvai, Kütt, Nisamedtinov, & Paalme, 2016). Together with ethanol, the production of medium chain fatty acids and high amounts of acetic acid can negatively affect the growth of a co-fermenting yeast species (Ciani & Comitini, 2015). In this work, CI significantly improved the production of fatty acids, while SI elevated the levels of acetic acids and ethyl acetate, in both SGM and real wine fermentations (Fig. S2, Table 3). The use of *H. uvarum* in mixed fermentation can contribute to the production of medium chain fatty acids and corresponding ethyl esters (Hu, Jin, Mei, et al., 2018). This study further found that killer rather than non-killer *S. cerevisiae* was capable of inducing the formation of fatty acids and esters when used in mixed fermentation with *H. uvarum*. This finding highlighted the remarkable role of killer *S. cerevisiae* in regulating aroma production during mixed fermentation.

In addition, yeasts form esters via the reaction of alcohol and fatty acids during fermentation, which may be affected by esterase that balances the synthesis and hydrolysis of esters (Ugliano & Moio, 2005). The participation of NS yeasts in mixed fermentation can increase esterase activity, which might be the reason for stronger and diversified wine aroma (Johnson, 2013; Ma et al., 2017). In this work, the accumulation of C2 esterase activities during SI fermentations was higher than that of others, especially SI-RV002. While the sum of C2–C10 esterase activities in CI was higher than that of other treatments, especially CI-RV002 (Fig. S3). A correlation analysis of fruity esters between higher alcohols, fatty acids, and esterase activities is shown in Fig. 4. Pearson correlation coefficient (r) between ethyl acetate and cumulative C2 esterase activities was 0.421 ($P < 0.05$), while r between ethyl acetate and volatile acidity was 0.722 ($P < 0.01$). The r values between AHAs and higher alcohols, volatile acidity, and cumulative C2 esterase activities were 0.762 ($P < 0.01$), 0.547 ($P < 0.01$), and 0.780 ($P < 0.01$), respectively. Furthermore, short and medium chain FAEEs were significantly correlated with fatty acids and cumulative esterase activities ($P < 0.01$). In particular, the r between medium chain FAEEs and fatty acid was 0.850. As esterase balances the synthesis and hydrolysis of esters, its activity mostly depends on the content of the substrates. The phenotypes observed in this study indicated that fatty acid was the most critical factor in the production of FAEEs (particularly medium chain FAEEs). The suppression of *H. uvarum* biomass by killer *S. cerevisiae* and inoculation modality changed the levels of fatty acids, including acetic acid, which modified the production of fruity esters in mixed fermentations. In particular, CI-RV002 mainly promoted the content of medium chain FAEEs, which enhanced the fruity aroma of wine, likely due to the positive interaction between the two yeast species.

4. Conclusions

In conclusion, this study showed that the growth of co-fermenting yeast species depended on the inoculation modality and the antagonistic activity of *S. cerevisiae*. The biomass and survival time of *H. uvarum* reflected the production levels of fatty acids and acetic acid, which influenced the contents of fruity esters and ethyl acetate in mixed fermentations. The biomass suppression of *H. uvarum* during co-inoculation with the killer *S. cerevisiae* increased the production of fruity esters, with the exception of ethyl acetate, which contributed to the enhancement of fruity aroma trait of final wine. However, more biomass and longer survival time of *H. uvarum* in SI considerably increased the content of ethyl acetate. These findings indicate that the co-inoculation of *H. uvarum* and killer *S. cerevisiae* is an effective way to improve wine aroma.

Declaration of competing interest

The authors declare no competing financial interest.

CRediT authorship contribution statement

Yue-Qi Li: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Kai Hu:** Conceptualization, Investigation, Formal analysis, Writing - original draft. **Yin-Hu Xu:** Formal analysis, Writing - original draft. **Wen-Chao Mei:** Investigation, Formal analysis. **Yong-Sheng Tao:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Acknowledgments

This work was supported by the Shaanxi Science Fund for Distinguished Young Scholars (2020JC-22), the National Natural Science Foundation of China (31972199), the Scientific and Technological Key Projects of Xinjiang production and Construction Corps (2019AB025) and the National Key Research and Development Program of China (2019YFD1002504). The authors would like to thank Ai-Hua Li from College of Food Science and Engineering, Northwest A&F University for the excellent technical assistance of instrumental analysis. We thank the tasting panelists for aroma evaluation and the vineyard in Heyang for providing grapes.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109839.

References

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., & Girio, F. (2010). *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Applied Microbiology and Biotechnology, 86*, 965–972.

Branco, P., Viana, T., Albergaria, H., & Arneborg, N. (2015). Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells. *International Journal of Food Microbiology, 205*, 112–118.

Carrau, F., Gaggero, C., & Aguilar, P. S. (2015). Yeast diversity and native vigor for flavor phenotypes. *Trends in Biotechnology, 33*, 148–154.

Cheraiti, N., Guezenec, S., & Salmon, J. M. (2005). Redox interactions between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in mixed culture under enological conditions. *Applied and Environmental Microbiology, 71*, 255–260.

Ciani, M., & Comitini, F. (2015). Yeast interactions in multi-starter wine fermentation. *Current Opinion in Food Science, 1*, 1–6.

Domizio, P., Romani, C., Comitini, F., Gobbi, M., Lencioni, L., Mannazzu, I., et al. (2011). Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Annals of Microbiology, 61*, 137–144.

Dzialo, M. C., Park, R., Steensels, J., Lievens, B., & Verstrepen, K. J. (2017). Physiology, ecology and industrial applications of aroma formation in yeast. *FEMS Microbiology Reviews, 41*, S95–S128.

Ebeler, S. E. (2001). Analytical chemistry: Unlocking the secrets of wine flavor. *Food Reviews International, 17*, 45–64.

GB/T 15038-2006. (2006). *Analytical methods of wine and fruit wine*. Beijing: China Standard Press.

Hazelwood, L. A., Daran, J. M., van Maris, A. J. A., Pronk, J. T., & Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: A century of Research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology, 74*, 2259–2266.

Hu, K., Jin, G. J., Mei, W. C., Li, T., & Tao, Y. S. (2018). Increase of medium-chain fatty acid ethyl ester content in mixed *H. uvarum/S. cerevisiae* fermentation leads to wine fruity aroma enhancement. *Food Chemistry, 239*, 495–501.

Hu, K., Jin, G. J., Xu, Y. H., & Tao, Y. S. (2018). Wine aroma response to different participation of selected *Hanseniaspora uvarum* in mixed fermentation with *Saccharomyces cerevisiae*. *Food Research International, 108*, 119–127.

Hu, K., Jin, G. J., Xu, Y. H., Xue, S. J., Qiao, S. J., Teng, Y. X., et al. (2019). Enhancing wine ester biosynthesis in mixed *Hanseniaspora uvarum/Saccharomyces cerevisiae* fermentation by nitrogen nutrient addition. *Food Research International, 123*, 559–566.

Johnson, E. A. (2013). Biotechnology of non-*Saccharomyces* yeasts—the ascomycetes. *Applied Microbiology and Biotechnology, 97*, 503–517.

Kemsawasd, V., Branco, P., Almeida, M. G., Caldeira, J., Albergaria, H., & Arneborg, N. (2015). Cell-to-cell contact and antimicrobial peptides play a combined role in the death of *Lachanchea thermotolerans* during mixed-culture alcoholic fermentation with *Saccharomyces cerevisiae*. *FEMS Microbiology Letters, 362*, fnv103.

Kevvai, K., Kütt, M. L., Nisamedtinov, I., & Paalme, T. (2016). Simultaneous utilization of ammonia, free amino acids and peptides during fermentative growth of *Saccharomyces cerevisiae*: Simultaneous utilization of ammonia, free amino acids and peptides. *Journal of the Institute of Brewing, 122*, 110–115.

Kong, C. L., Li, A. H., Jin, G. J., Zhu, X. L., & Tao, Y. S. (2019). Evolution of volatile compounds treated with selected non-*Saccharomyces* extracellular extract during pinot noir winemaking in monsoon climate. *Food Research International, 119*, 177–186.

Lombardi, S. J., De Leonardis, A., Lustrato, G., Testa, B., & Iorizzo, M. (2015). Yeast autolysis in sparkling wine aging: Use of killer and sensitive *Saccharomyces cerevisiae* strains in Co-culture. *Recent Patents on Biotechnology, 9*, 223–230.

Marquina, D., Santos, A., & Peinado, J. M. (2002). Biology of killer yeasts. *International Microbiology the Official Journal of the Spanish Society for Microbiology, 5*, 65–71.

Ma, D. C., Yan, X., Wang, Q. Q., Zhang, Y. N., & Tao, Y. S. (2017). Performance of selected *P. fermentans* and its excellular enzyme in co-inoculation with *S. cerevisiae* for wine aroma enhancement. *LWT - Food Science Technology, 86*, 361–370.

Mayr, C. M., Geue, J. P., Holt, H. E., Pearson, W. P., Jeffery, D. W., & Francis, I. L. (2014). Characterization of the key aroma compounds in Shiraz wine by quantitation, aroma reconstitution, and omission studies. *Journal of Agricultural and Food Chemistry, 62*, 4528–4536.

Orentaite, I., Poranen, M. M., Oksanen, H. M., Daugelavicius, R., & Bamford, D. H. (2016). K2 killer toxin-induced physiological changes in the yeast *Saccharomyces cerevisiae*. *FEMS Yeast Research, 16*, fow003.

Padilla, B., Gil, J., & Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology, 7*, 411.

Peng, C. T., Wen, Y., Tao, Y. S., & Lan, Y. Y. (2013). Modulating the formation of Meili wine aroma by prefermentative freezing process. *Journal of Agricultural and Food Chemistry, 61*, 1542–1553.

Perez-Nevado, F., Albergaria, H., Hogg, T., & Girio, F. (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *International Journal of Food Microbiology, 108*, 336–345.

Poivet, E., Tahirova, N., Peterlin, Z., Xu, L., Zou, D. J., Acree, T., et al. (2018). Functional odor classification through a medicinal chemistry approach. *Science Advances, 4*, Article eaao6086.

Ramon-Portugal, F., Delia, M. L., Strehaiano, P., & Riba, J. P. (1997). Mixed culture of killer and sensitive *Saccharomyces cerevisiae* strains in batch and continuous fermentations. *World Journal of Microbiology and Biotechnology, 14*(1), 83–87.

Renault, P. E., Albertin, W., & Bely, M. (2013). An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Applied Microbiology and Biotechnology, 97*, 4105–4119.

Renault, P., Coulon, J., de Revel, G., Barbe, J. C., & Bely, M. (2015). Increase of fruity aroma during mixed *T. delbrueckii/S. cerevisiae* wine fermentation is linked to specific esters enhancement. *International Journal of Food Microbiology, 207*, 40–48.

Rossouw, D., Du Toit, M., & Bauer, F. F. (2012). The impact of co-inoculation with Oenococcus oeni on the trancriptome of *Saccharomyces cerevisiae* and on the flavour-active metabolite profiles during fermentation in synthetic must. *Food Microbiology, 29*, 121–131.

Saenz-Navajas, M. P., Alegre, Y., de-la-Fuente, A., Ferreira, V., Garcia, D., Eizaguirre, S., et al. (2016). Rapid sensory-directed methodology for the selection of high-quality aroma wines. *Journal of the Science of Food and Agriculture, 96*, 4250–4262.

Santo, D. E., Galego, L., Gonçalves, T., & Quintas, C. (2012). Yeast diversity in the Mediterranean strawberry tree (Arbutus unedo L.) fruits' fermentations. *Food Research International, 47*, 45–50.

Sumby, K. M., Grbin, P. R., & Jiranek, V. (2010). Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. *Food Chemistry, 121*, 1–16.

Taillandier, P., Quoc Phong, L., Julien-Ortiz, A., & Brandam, C. (2014). Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. *World Journal of Microbiology and Biotechnology, 30*, 1959–1967.

Ugliano, M., & Moio, L. (2005). Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of Oenococcus oeni. *Journal of Agricultural and Food Chemistry, 53*, 10134–10139.

Wyk, N. V., Grossmann, M., Jürgen, W., Wallbrunn, C. V., & Pretorius, I. S. (2019). The whiff of wine yeast innovation: Strategies for enhancing aroma production by yeast during wine fermentation. *Journal of Agricultural and Food Chemistry, 67*, 13496–13505.