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Wine aroma response to different participation of selected *Hanseniaspora uvarum* in mixed fermentation with *Saccharomyces cerevisiae*

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Abstract: Wine aroma response to a selected *Hanseniaspora uvarum* Yun268 strain was investigated using different inoculation strategies with commercial *Saccharomyces cerevisiae* yeast, namely, simultaneous fermentation (SiF), sequential fermentation (SeF), *S. cerevisiae* fermentation treated with extracellular extract of *H. uvarum* (EE), and pure *S. cerevisiae* fermentation (PF). Contributive volatiles in the perception of enhanced aroma traits were uncovered by partial least-squares regression. Results showed that controlled inoculation resulted into different amounts of *H. uvarum* Yun268, which distinctively affected the chemical and sensory profiles of wines. The concentration of aromatic compounds could be increased by *H. uvarum* Yun268 yeasts via high levels of β-glucosidase activity and fatty acids. Terpenes, C_{13}-norisoprenoids, acetate esters, ethyl esters, and fatty acids served as the impact volatiles that contributed to the enhanced aroma traits. SiF specifically increased the contents of C_{13}-norisoprenoids, terpenes, and ethyl esters, while EE enhanced varietal volatile content rather than those of fermentative ones. However, excessive *H. uvarum* Yun268 in sequential inoculation elevated the concentrations of acetate esters and volatile phenols, triggering nail polish odor in Cabernet Sauvignon red wines.

Keywords: wine aroma; *Hanseniaspora uvarum*; extracellular extract; mixed fermentation; partial least-squares regression

1. Introduction

Balanced and complex aroma is one of the most striking factors that determine consumer preference of wine. In recent years, non-*Saccharomyces* yeasts have gained popularity in enology owing to their ability to reduce ethanol content, enhance acidity, increase colour stability, and modify specific chemical or aromatic compounds of wines (Jolly, Augustyn, & Pretorius, 2003, 2006; Ciani, Comitini, Mannazzu, & Domizio, 2010; Gobbi et al., 2013; Loira et al., 2015; Varela, Sengler, Solomon, & Curtin, 2016). Several novel non-*Saccharomyces* strains have been released commercially, including *Torulaspora delbrueckii*, *Zygosaccharomyces*, and *Kluyveromyces thermotolerans* (Jolly, Varela, & Pretorius, 2014).

One particular interest in current research is using non-*Saccharomyces* yeasts to shape wine sensory profile (Varela, 2016). Diverse wine aroma profiles are generated by different non-*Saccharomyces* isolates, showing a strongly strain-dependent process. Isolates from *Starmerella bacillaris* can encourage typical aromas of Touriga Nacional red wines, such as bergamot, violet and rock-rose (Teixeira, Caldeira, & Duarte, 2015). Sauvignon Blanc wines produced with *Candida zemplinina* are characterized by fermented apple, dried peach, and stewed fruit aromas (Whitener et al., 2016). By combining different non-*Saccharomyces* strains with *S. cerevisiae* in a Shiraz winemaking trial, Du Plessis et al. (2017) found that yeast selection displayed a significant impact on berry aroma.

Selected non-*Saccharomyces* strains contribute to wine aroma via production of desirable metabolites or interaction with *S. cerevisiae*. Owing to low ethanol tolerance, non-*Saccharomyces* yeasts are commonly used in mixed fermentation with *S. cerevisiae* to

enhance wine aroma while achieving complete alcoholic fermentation. However, mixed fermentations improve wine aroma unstably, and even contribute to the off-flavour. Anfang, Brajkovich, and Goddard (2009) reported that the most significant increase in varietal thiol content is only achieved with a 9:1 ratio of *Pichia kluyveri*/*S. cerevisiae* inoculum. Studies on mixed fermentation of *T. delbrueckii* and *S. cerevisiae* showed that the content of major ethyl esters is slightly increased in simultaneous fermentation while it is decreased by 44% in sequential fermentation (Renault, Coulon, de Revel, Barbe, & Bely, 2015). In addition, sequential inoculation of *Wickerhamomyces anomalus* and *S. cerevisiae* could elevate the concentration of ethyl acetate to an unacceptable level (200-350 mg/dm^3) (Ye, Yue, & Yuan, 2014). Hence, the enological potential of non-*Saccharomyces* yeast varies with different fermentation strategies involving *S. cerevisiae*. Improved knowledge regarding the impact of mixed fermentation strategies on wine chemical and sensory responses is necessary.

The apiculate yeast *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) is one of the most predominant non-*Saccharomyces* species found on grapes (Fleet, 2003), which can contribute to natural wine aroma when used in mixed fermentations. For instance, low frequency of *H. uvarum* during fermentation leads to the lack of aroma complexity of Folle Blanche wines from the Basque region in Spain (Rementeria et al., 2003). Following studies also indicated that selected *H. uvarum* strains can be used in mixed fermentation to improve the typical concept of wine aroma, such as in Sauvignon Blanc white wine from South Africa and Negroamaro red wine from Southern Italy (Jolly et al., 2003; Tristezza, Tufariello, Capozzi, Spano, Mita, & Grieco, 2016). In addition to the using of yeast cells, non-*Saccharomyces* yeasts can also produce flavour enzymes during mixed fermentation

(Basso, Alcarde, & Portugal, 2016), and hence, the exploratory utilization of their extracellular extract may possess certain desirable enological interests. Recently, we isolated a novel *H. uvarum* strain with high β-glucosidase activity from a local wine region in China (Hu et al., 2016). This strain was identified able to increase medium-chain fatty acid ethyl ester content through a synergistic interaction with *S. cerevisiae* (Hu, Jin, Mei, Li, & Tao, 2018).

In the present work, we addressed the chemical and sensory responses in local white and red wines (Ecolly and Cabernet Sauvignon) to simultaneous fermentation (SiF), sequential fermentation (SeF), extracellular extract treatment (EE) and pure fermentation (PF). In addition to the assessment of the modulation of aromatic compound levels, β-glucosidase and esterase activities were also assayed. Moreover, the underlying relationship between volatiles and aroma traits were regressed using partial least-squares regression (PLSR).

2. Materials and methods

2.1. Grapes

Ecolly (*Vitis vinifera* L.) is a local white grape cultivar hybridized with Chardonnay, Riesling, Chenin Blanc, and their two intermediate hybrids (Li, Zhang, Wang, & Liu, 2000; Wang et al., 2017). Ecolly grapes were obtained from the Caoxingzhuang vineyard (Yangling, Shannxi Province, China), with sugar content of 185 g/dm^3, acidity of 4.2 g/dm^3 (as tartaric acid), yeast assimilable nitrogen (YAN) of 177 mg N/dm^3 and pH 3.58. Cabernet Sauvignon (CS, *V. vinifera* L.) red grapes were collected from a vineyard in Jingyang County (Shaanxi Province, China), with 190 g/dm^3 sugar, 6.1 g/dm^3 acid (as tartaric acid), 263 mg N/dm^3 YAN

and pH 3.73. Sugar content and acidity were determined using titration methods according to the National Standard of China (GB/T 15038-2006, 2006). YAN levels were assayed using the formaldehyde method (Zoecklein et al., 1995).

2.2. Yeasts and extracellular extract

H. uvarum strain Yun268 was isolated from a vineyard in Mile County (Yunnan Province, China). This isolate has been identified by sequence analysis of the 26S rDNA D1/D2 domain (Hu et al., 2016), and preserved in China Center for Type Culture Collection (CCTCC M2013658). The commercial *S. cerevisiae* wine yeast was CEREVISIAE (Actiflore®, Laffort, France).Yeast cells were maintained in yeast extract-peptone-dextrose medium (YPD) (2% glucose, 2% peptone, 1% yeast extract) containing 40% (v/v) glycerol at −20 °C.

To obtain *H. uvarum* extracellular extract, yeasts were cultured at 28 °C with shaking at 170 rpm for 72 h (1% yeast extract, 2% peptone, 2% glucose, 0.3% NH_4NO_3, 0.4% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 1% Tween 80), followed by centrifugation at 4 °C and 6790 × g for 15 min. The supernatant was filtered with a 0.45 μm membrane, and concentrated 10 times with polyethylene glycol (PEG 20000, Merck, Germany) at 4 °C.

2.3. Fermentation strategies

Fermentations were conducted in duplicate in 20 dm^3 laboratory-scale fermenters where total yeast inoculation was controlled at 6×10^6 CFU/mL. In mixed fermentation, the inoculum ratio of *H. uvarum* and *S. cerevisiae* was 1:2 in two forms: SiF where they were simultaneously inoculated, and SeF where *H. uvarum* was inoculated 48 h before *S. cerevisiae*. For EE fermentation, 0.35% (v/v) of extract preparation was added along with *S. cerevisiae* to

adjust the additive activity of β-glucosidase to 1 U/dm^3. PF with *S. cerevisiae* used as the control. Sampling was carried out every 24 h to analyze ethanol accumulation and yeast biomass. Ethanol content was assayed using the distillation/densitometry method (GB/T 15038-2006, 2006).Yeast biomass evolution was monitored using Wallerstein nutrient (WL) agar medium. WL is a differential medium that allows the identification of yeasts according to colour and morphology of the colonies (Domizio et al., 2011). The colony of *H. uvarum* Yun268 was "dark green flat", whereas the colony of *S. cerevisiae* CEREVISIAE was "creamy convex", which could be significantly differentiated from those of native predominant yeasts (Supplementary Fig. 1).

Grapes were crushed using a commercial de-stemmer/crusher, treated with 55 mg/dm^3 of SO_2, and macerated at 4 °C for 16 h. CS must was inoculated with the yeasts, fermented with the skins at 23 °C, and subjected to maceration three times a day by pushing the skins into the juice. Ecolly juice has been separated from the skins, clarified using 1 g/dm^3 bentonite, inoculated with yeasts using the same method, and fermented at 20 °C. To compensate for the insufficient sugar content of grapes because of rainy climate during the ripening season, 20 g/dm^3 and 25 g/dm^3 sucrose were added to Ecolly and CS juice 12 h after the yeast inoculation, respectively, to obtain 12% alcohol content. Sulfur dioxide (60 mg/dm^3) was added when the sugar content dropped below 2 g/dm^3 (GB/T 15038-2006, 2006). Meanwhile, CS juice has been separated from the skins, and clarified using 1 g/dm^3 bentonite. Wine samples were stored at 4 °C for six months before analysis. This process was used to prevent malolactic fermentation, and to achieve the clarification and stabilization of wines. During the storage period, three rackings were performed by siphoning the samples into clean jars.

2.4. Enzyme activity assay

The method of determining β-glucosidase activity was adapted from Rodríguez, Lopes, Broock, Valles, Ramón, and Caballero (2004). The reaction mixture contained 0.2 mL enzyme extract and 0.25 mL of 1 mmol/dm^3 *p*-NP-β-D-glucopyranoside (Sigma, Shanghai, China) in 0.75 mL citrate/phosphate buffer (pH 5.0). Reaction was incubated at 40 °C for 30 min and terminated with 1 mL of 0.5 M Na_2CO_3. Esterase activity was determined using different substrates that attached to *p*-NP (Pérez-Martín, Seseña, Izquierdo, & Palop, 2013), including *p*-NP-acetate (Sigma, Shanghai, China), *p*-NP-butyrate (J&K, Beijing, China), and *p*-NP-octanoate (J&K, Beijing, China). Enzyme solution (0.2 mL) was mixed with 0.08 mL of 25 mM *p*-NP ester in 1.72 mL of 0.1 M citrate/phosphate buffer (pH 5.0) and reacted at 37 °C for 60 min. Subsequently, 0.2 mL of 0.5 M NaOH was added to stop the reaction.

The generated *p*-NP was measured at 400 nm using an ultraviolet-visible spectrophotometer (Cary 60, Agilent Technologies) against a blank without substrates. Units of activity (U) were defined as the micromoles of *p*-NP liberated per minute per milliliter of enzyme solution.

2.5. Volatile analysis

Volatiles were analyzed using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME/GC-MS) according to Wang et al. (2017). Briefly, volatiles were extracted with a 50/30 μm DVB/CAR/PDMS fiber (Supelco, Bellefonte PA, USA). Wine (8 mL), 2-octanol (400 μg/dm^3), and 1 g NaCl were held in a gas-tight vial, equilibrated in a 40 °C water bath with stirring for 15 min, extracted for 30 min,

and then desorbed in the GC injector at 250 °C for 8 min using a TRACE 1310 GC coupled with an ISQ LT MS (Thermo Scientific, USA). A DB-WAX column (60 m × 0.25 mm × 0.25 μm, Agilent J & W, USA) was used. Injection was splitless with 0.8 min relay time. Helium was the carrier gas at the flow rate of 1 mL/min. GC program: initial temperature 40 °C, raised to 130 °C at 3 °C/min, raised to 250 °C at 4 °C/min, and maintained 250 °C for 8 min. MS program: mass range 25-350 amu, scanned at 0.2 s intervals, ion source temperature 250 °C. Injector and transfer line temperature was 250 °C. Mass spectra were recorded in electron impact (EI) ionization mode.

Chemical standard solutions were prepared using model wine (11% v/v ethanol, 6 g/dm^3 tartaric acid, and pH adjusted to 3.4 with 1 M NaOH) according to Tao, Li, Wang, and Zhang (2008). Volatile identification was conducted by comparing the retention times and mass spectra with those of pure standards in the Wiley 275.L library (Agilent Technologies Inc.), followed by quantitative analysis by interpolating the relative areas versus the area of internal standard using the calibration graphs established for pure standards (method characteristics are shown in Supplementary Table 1). The concentrations of volatiles without pure standards were obtained using the same calibration graphs as that of one of the compounds with the most similar chemical structure (Perestrelo, Fernandes, Albuquerque, Marques, & Câmara, 2006).

2.6. Sensory analysis

Wine aroma was evaluated in duplicate by a tasting panel consisting of eight females and nine males trained with a 54-aroma kit (Le Nez du Vin®, France) for one month. During the training, their performances were evaluated by an aroma identification test every six days

until their identification accuracy for each aroma reached above 95%. The analysis was conducted in a tasting room at 23 °C. Approximately 30 mL wine (15 °C) was held in a black wine glass and was distributed in a completely random order. Each test session included two groups, and four samples were analyzed in each group. The interval between two samples was 1-2 min. The panelists evaluated wine aroma according to the following procedure: they smelled the aroma of static wine sample for approximately 5-8 s, then shook the wine to smell the aroma for 5-10 s, defined aroma using 4–6 terms from the aroma kit, and scored their intensity using a five-point scale, where "1" – weak, "2" – slightly weak, "3" – medium, "4" – slightly intense, "5" – intense. Additional descriptor, such as nail polish odor, was generated by the panelists. Final aroma traits were quantified by the MF% value, which was the mixture of detection frequency and aroma intensity (Dravnieks, 1982).

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

where F is the detection frequency in the percentage of the total number of panelists and I is the average intensity in the percentage of five (the possible maximum intensity).

2.7. Statistical analysis

Aroma data were analyzed using one-way analysis of variance (ANOVA) with Duncan test, and those with significant differences were subjected to principal component analysis (PCA) using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Enzyme activities were compared using the t-test. Regression of aroma traits by volatiles was conducted using partial least-squares regression (PLSR) on PLSR 1 with Unscrambler 9.7 (Camo, Trondheim, Norway).

3. Results and discussion

3.1. Ethanol accumulation during wine fermentation

As shown in Fig. 1, all the fermentation regimens achieved the final ethanol concentration of 11.6 % in Ecolly wines and 12.1% in CS wines (general parameters of wines are shown in Supplementary Table 2). Both PF and EE showed similar dynamics in these two wines, suggesting that the addition of *H. uvarum* extracellular extract did not affect ethanol production. In contrast, the participation of *H. uvarum* yeast in SeF decreased the fermentation rate of Ecolly and CS wines. Indeed, an obvious lag phase (0-2 d) was observed in SeF prior to *S. cerevisiae* inoculation.

It has recently been found that ethanol accumulation is the main factor responsible for the death of *H. uvarum* during mixed fermentation (Wang, Mas, & Esteve-Zarzoso, 2015). With the slow accumulation rate of ethanol at 0-2 d, biomass of *H. uvarum* Yun268 was enhanced more in SeF than in SiF (Fig. 2 A1, B1). Especially for SeF Ecolly wine, considerable proportion of *H. uvarum* Yun268 were observed till the end of fermentation (Supplementary Fig. 2 A2). Ethanol tolerance of *H. uvarum* may be enhanced in sequential inoculation. Unlike the inhibitory effect of ethanol on *H. uvarum*, the addition of extracellular extract influenced *S. cerevisiae* yeasts negligibly (Fig. 2 A2, B2). However, the involvement of the *S. cerevisiae* biomass in SiF and SeF was reduced. Besides, the reduction of *S. cerevisiae* proportion in SeF was more pronounced compared to that in SiF (Supplementary Fig. 2), which was in agreement with Medina, Boido, Dellacassa, and Carrau (2012). They found that the nutrient pre-consumption of *H. vineae* could reduce the ability of *S. cerevisiae* to grow after it was

sequentially inoculated. The competition of non-*Saccharomyces* yeasts with *S. cerevisiae* has also been reported by Canonico, Agarbati, Comitini, and Ciani (2016), who observed that *T. delbrueckii* could reduce the biomass of *S. cerevisiae* at an inoculum ratio of 1:1 and even dominate the process at higher ratios. Our results showed that *H. uvarum* Yun268 was a weak competitor when fermented with *S. cerevisiae*. The controlled inoculations induced their different participation levels, which may impact the aroma profiles of wine.

3.2. Effect of fermentation strategies on aromatic compound content of wines

According to different sources, volatile compounds in young wines can be classified into varietal and fermentative components. The varietal components are mainly stored as odorless glycosides in grapes, and can be hydrolyzed to free volatile compounds by β-glucosidase. Fermentative volatiles are formed when yeasts metabolize sugar and amino acid (Robinson et al., 2014). In this study, these two compounds were discussed separately to highlight the modulation of each component in different inoculation strategies.

3.2.1. Varietal and prefermentative aroma compounds

Nineteen varietal compounds were quantitated, including C_6 alcohols (also prefermentative compounds), terpenes, C_{13}-norisoprenoids, and volatile phenols (Table 1). Their profiles varied between Ecolly and CS wines, however, both *H. uvarum* yeasts and its extracellular extract significantly enhanced the content of these compounds in Ecolly and CS wines compared to that in pure *S. cerevisiae* fermentation.

Varietal volatiles were analyzed using PCA to distinguish wines obtained from different fermentation strategies in the first two PCs. Wines from SeF, SiF, and EE tightly surrounded

most aroma compounds in the positive parts of PC1 and PC2 for both Ecolly and CS wines (Fig. 3 A and B). Among them, SeF wines associated with most terpenes, C_{13}-norisoprenoids, and volatile phenols. SiF were closely related to terpineol, and linalool oxide in Ecolly and CS wines. In contrast, EE clustered with (E, E)-farnesol and β-ionone in Ecolly wines, as well as C_6 alcohols, and linalool in CS wines.

Previous studies showed that the presence of non-*Saccharomyces* yeasts during mixed fermentation increased varietal compound content, such as those of terpenes and C_{13}-norisoprenoids (Sadoudi et al., 2012; Liu, Lu, Duan, & Yan, 2016). In this study, we investigated whether β-glucosidase from *H. uvarum* Yun268 was responsible for the increase in varietal compound content. Indeed, both *H. uvarum* Yun268 (0.09 U/mL) and its concentrated extracellular extract (0.28 U/mL) exhibited β-glucosidase activity (Fig. 4 A and B, respectively). Especially, the β-glucosidase activity of *H. uvarum* yeast was 6.6-fold higher than that of *S. cerevisiae*, which explained the mechanism via which participation of *H. uvaum* yeasts contributed to the improvement in terpene and C_{13}-norisoprenoid content. Furthermore, this effect was additive in SeF than in SiF since the biomass of *H. uvarum* Yun268 was higher in the former. However, higher levels of β-glucosidase activity in SeF also increased the volatile phenol content in CS wine by 53%. Although the effect of non-*Saccharomyces* yeast on phenol levels has rarely been reported, their enhancement may generate unpleasant phenolic or medicinal odor traits in wines (Loscos, Hernandez-Orte, Cacho, & Ferreira, 2007).

3.2.2. Fermentative aroma compounds

Table 2 shows the quantification of 30 fermentative volatiles, including acetate esters,

ethyl esters, fatty acids, higher alcohols and carbonyls in Ecolly and CS wines. Unlike the varietal volatiles, the amounts, and not the kinds, of fermentative volatiles varied between two mono-varietal wines. As shown by PCA (Fig. 5 A and B), acetate esters and phenyls (2-phenylethyl acetate, 2-phenylethanol, and benzyl alcohol) were generated in higher amounts in both Ecolly and CS wines obtained from SeF. However, major ethyl esters and their corresponding fatty acids clustered with SeF Ecolly wines rather than with SeF CS wines, which may be induced by lower initial YAN level in Ecolly grape must (Carrau et al., 2008). Instead, ethyl esters were specifically related to SiF wines of both varieties. In contrast, EE and PF wines clustered with few ethyl esters, ethyl lactate, and diethyl succinate. Therefore, compared to the general increase in varietal compound content, modulation of fermentative aromas, typically those of acetate esters and ethyl esters, appeared to be more specific for *H. uvarum* Yun268 yeasts.

Esters are generated by lipid and acetyl-CoA metabolism of yeasts in alcoholic fermentation, which is regulated by fatty acids and biosynthetic enzymes (Saerens et al., 2008). Although *H. uvarum* extracellular extract possessed 0.11-0.35 U/mL esterase activity towards C2, C4 and C8 esters (Fig. 4B), its addition increased the content of only few ethyl esters in wine. Thus, the *H. uvarum* extracellular extract contributed significantly to varietal volatile content.

Hanseniaspora yeast is a known high acetate ester producer (Viana, Gil, Genovés, Vallés, & Manzanares, 2008; Liu, Arneborg, Toldam-Andersen, Petersen, & Bredie, 2017). Similar to volatile phenols, the increased acetate ester levels were only observed in SeF Ecolly (62%) and SeF CS (153%) wines, demonstrating that their enhancement could be induced by high

population proportion of *H. uvarum* to *S. cerevisiae*. The higher increment of acetate ester content in SeF CS wine may be attributed to high YAN level in CS must that encouraged yeasts to express *ATF1* gene responsible for acetate ester production (Saerens et al., 2008). Since *H. uvarum* Yun268 did not exhibit higher level of esterase activity (C2, C4 and C8) than *S. cerevisiae* (Fig. 4A), high concentrations of acetate esters and ethyl esters may result from the increase in the corresponding fatty acid levels (Table 2). It is noteworthy that SiF wines with the high content of ethyl esters did not contain elevated levels of acetate esters. However, ethyl ester content increased in SeF Ecolly wine by 25%, whereas it decreased in SeF CS wine by 16%, suggesting that the differences in nutrient composition between the two grape varieties may induce different aroma production of the yeasts. Further investigations are required to explain this effect.

3.3. Sensory response to fermentation strategies

Following sensory analysis suggested that mixed fermentations could enhance wine aroma in comparison with pure *S. cerevisiae* fermentation (Fig. 6). Briefly, tropical fruity and floral traits were supremely encouraged by SeF, followed by EE and SiF. Temperate fruity odor, such as apple and pear, was strongly enhanced in SeF Ecolly wine and SiF CS wine. Vegetal odor was not influenced in Ecolly wines, whereas it decreased in CS wines. Unfortunately, nail polish odor was triggered in CS wine from SeF, in agreement with the strong production of such off-flavor in red wines obtained from *Hanseniaspora* species (Teixeira et al., 2015).

To explore the underlying relationships between these volatiles and enhanced aroma traits, their correlation models were built using PLSR (Table 3). Volatiles with coefficients > 0 or < 0 indicated that the perception of an aroma is determined not only by few components

delivering the trait, but also by the presence of other negative odorants. Terpenes, C_{13}-norisoprenoids, and acetate esters acted as the most contributive odorants offering tropical fruity and floral odor, irrespective of wine types. This finding supported the results of a previous study where fruity odor was shown to be the result of a synergetic interaction between norisoprenoids and esters (Escudero, Campo, Fariña, Cacho, & Ferreira, 2007). In contrast, ethyl esters and their corresponding fatty acids were responsible for the enhancement of temperate fruity aroma.

Interestingly, the contribution of terpenes, C_{13}-norisoprenoids, and acetate esters to temperate fruity trait were opposite in Ecolly (positive) and CS wines (negative). Similar observations were made regarding the effect of ethyl esters and fatty acids on tropical fruity aromas in these two wines. This suggested that wine types affected aroma perception from the same odorants. In fact, only floral trait from these two wines could be well predicted with PLSR (R^2 cal/val = 0.74/0.51), where C_{13}-norisoprenoids (+0.447), acetate esters (+0.299), and terpenes (+0.277) played the most positive roles (Fig. 7). We also observed that the presence of volatile phenols in CS wines provoked a higher aroma release of tropical fruity (+0.221) and floral trait (+0.195) than Ecolly wines (Table 3), although they were not capable of presenting such odors (Loscos et al., 2007). Unfortunately, high contents of volatile phenols (+0.207) as well as acetate esters (+0.223) also contributed to the formation of nail polish odor in SeF CS wines. From the perspective of aroma characteristics among acetate esters (Sumby, Grbin, & Jiranek, 2010), ethyl acetate may act as the backbone odorant related to such off-flavor.

4. Conclusion

In conclusion, the fruity and floral traits in Ecolly and Cabernet Sauvignon wines were distinctively modulated by different participation levels of *H. uvaum* Yun268, which affected the concentrations of specific varietal and fermentative volatiles. The persistence of *H. uvarum* Yun268 yeasts was improved in SeF, thus supremely enhancing tropical fruity and floral aromas. However, excessive *H. uvarum* yeasts in SeF slowed down fermentation rate, and triggered nail polish-like odor in CS wines through increasing the contents of acetate esters and volatile phenols. A combination of instrumental and sensory analysis suggested that simultaneous fermentation and the use of *H. uvarum* extracellular extract could be utilized to improve the overall quality of wine aromas.

Author Contributions

Hu K. and Jin G.-J. are joint first authors. Tao Y.-S. and Hu K. conceived and designed the experiments; Hu K., Jin G.-J., and Xu Y.-H. performed the experiments; Hu K., and Jin. G.-J. analyzed the data and prepared manuscript. Tao Y.-S, Jin. G.-J. and Hu K. reviewed the paper.

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Conflict of interest

The authors declare no competing financial interest.

Supplementary Data

Supplementary Fig. 1. Colony morphologies on WL medium (72-h-old, 28 °C) of inoculated strains (A) and native predominant yeasts isolated from Ecolly (B) and CS (C) musts.

Supplementary Fig. 2. Relative proportion of yeasts during fermentation of Ecolly (A) and CS (B) wines obtained from SiF (1) and SeF (2).

Supplementary Table 1. Linearity, detection limit and precision of the method.

Supplementary Table 2. Physicochemical parameters of wines obtained from different fermentation strategies.

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Figure Captions

Fig. 1. Ethanol accumulation in Ecolly (A) and CS (B) wines obtained from PF (●), EE (○), SiF (■), and SeF (▲).

Fig. 2. Yeast biomass evolution of *H. uvarum* Yun268 (1) and *S. cerevisiae* CEREVISIAE (2) during fermentation of Ecolly (A) and CS (B) wines: PF (●), EE (○), SiF (■), and SeF (▲).

Fig. 3. Principal component analysis of varietal volatiles obtained from Ecolly (A) and CS (B) wines.

Fig. 4. Activity of β-glucosidase and esterase of yeasts (A) and concentrated *H. uvarum* extracellular extract (B). * Significant level 95%. ns, not significant.

Fig. 5. Principal component analysis of fermentative volatiles obtained from Ecolly (A) and CS (B) wines.

Fig. 6. Aroma MF% values of Ecolly (A) and CS (B) wines obtained from PF (●), EE (○), SiF (■), and SeF (▲): * Difference significant at 95% confidence level; ns, not significant.

Fig. 7. PLS regression of floral trait from volatiles in Ecolly and CS wines.

Tables

Table 1 Concentrations of varietal and prefermentative volatiles in Ecolly and CS wines (μg/dm^3).

Compounds	RI*	OT[#]	Ecolly					CS			
			PF	EE	SiF	SeF		PF	EE	SiF	SeF
C_6 alcohols											
1-Hexanol[†]	1366	8000[1]	483 ± 38^{a}	570 ± 28^{ab}	489 ± 34^{a}	610 ± 32^{b}		6609 ±421^{A}	7587 ± 377^{B}	6254 ±591^{A}	6567 ±316^{A}
(E)-3-hexen-1-ol	1401	400[1]	17 ± 4^{a}	32 ± 7^{b}	31 ± 2^{b}	35 ± 4^{b}		92 ± 3	71 ± 6	81 ± 16	88 ± 11
(Z)-3-hexen-1-ol[†]	1415	400[1]	35 ± 4	44 ± 5	38 ± 2	40 ± 10		81 ±3^{AB}	98 ± 18^{B}	76 ± 3^{AB}	71 ± 5^{A}
Subtotal			535 ± 46^{a}	646 ± 40^{ab}	558 ± 38^{a}	685 ± 46^{b}		6782 ± 427^{A}	7756 ± 401^{B}	6411 ± 610^{A}	6726 ± 332^{A}
Terpenes											
α-terpinene	1183	nf	5 ± 0^{a}	7 ± 0^{c}	6 ± 1^{b}	9 ± 1^{d}		4 ± 0^{A}	4 ± 0^{A}	4 ± 0^{A}	5 ± 0^{B}
linalool oxide	1464	500[1]	9 ± 0^{a}	13 ± 1^{b}	23 ± 0^{c}	24 ± 1^{c}		3 ± 0^{A}	4 ± 0^{A}	6 ± 1^{B}	5 ± 1^{B}
linalool[†]	1600	25[1]	nd	nd	nd	nd		1 ± 0^{A}	3 ± 0^{B}	1 ± 0^{A}	1 ± 0^{A}
hotrienol	1620	nf	32 ± 3^{a}	32 ± 2^{a}	32 ± 2^{a}	39 ± 1^{b}		nd	nd	nd	nd
4-terpineol[†]	1633	11-400[1]	nd	nd	nd	nd		2 ± 0^{A}	3 ± 0^{B}	4 ± 1^{B}	3 ± 0^{B}
α-terpineol	1706	250[3]	31 ± 1^{a}	31 ± 3^{a}	38 ± 2^{b}	38 ± 3^{b}		nd	nd	nd	nd
β-citronellol	1786	100[4]	nd	nd	nd	nd		31 ± 3^{A}	43 ± 3^{B}	30 ± 3^{A}	44 ± 7^{B}
(E)-geraniol[†]	1856	30[4]	nd	nd	nd	nd		2 ± 0^{A}	3 ± 0^{B}	2 ± 0^{A}	3 ± 0^{B}
(E,E)-farnesol	2373	20[2]	5 ± 0^{a}	6 ± 0^{b}	5 ± 0^{a}	5 ± 0^{a}		nd	nd	nd	nd

Subtotal			82 ± 4[a]	89 ± 6[a]	104 ± 5[b]	115 ± 6[b]		41 ± 3[A]	60 ± 3[B]	47 ± 5[AB]	61 ± 8[B]
C_{13}-norisoprenoids											
β-ionone	1524	0.09[1]	3 ± 1[a]	7 ± 1[b]	4 ± 0[a]	5 ± 1[ab]		nd	nd	nd	nd
α-ionone†	1528	0.09[1]	2 ±0	2 ±0	2 ± 0	2 ± 1		1 ± 0[A]	2 ± 0[B]	1 ± 0[A]	2 ± 0[B]
TDN	1751	20[5]	2 ± 0	3 ± 1	3 ± 0	3 ± 0		nd	nd	nd	nd
β-damascenone†	1832	0.05[1]	4 ± 0[a]	4 ± 0[a]	4 ± 0[a]	6 ± 0[b]		10 ± 0[A]	15 ± 1[B]	18 ± 2[B]	19 ± 2[B]
Subtotal			11 ± 1[a]	16 ± 2[b]	13 ± 0[ab]	16 ± 2[b]		11 ± 0[A]	17± 1[B]	19 ± 2[B]	21 ± 2[B]
Volatile phenols											
4-ethyl-2-methoxy phenol	2034	33[1]	nd	nd	nd	nd		21 ± 3	24 ± 1	24 ± 2	25 ± 2
eugenol†	2176	6[1]	nd	nd	nd	nd		3 ± 0[A]	6 ± 0[B]	3 ± 0[A]	9 ± 0[C]
2,4-di-tert-butyl-phenol	2330	200[1]	nd	nd	nd	nd		147 ± 12[A]	135 ± 10[A]	158 ± 15[A]	228 ± 17[B]
Subtotal			0	0	0	0		171 ± 15[A]	165 ± 11[A]	185 ± 17[A]	262 ± 19[B]

*RI, retention index on a DB-WAX column. nf: not found; nd: not detected by GC-MS; TDN: 1,1,6-trimethyl-1,2-dihydronaphthalene.

†Compounds quantified with pure standards.

PF, pure *S. cerevisiae* fermentation; EE, fermentation with *H. uvarum* extracellular extract and *S. cerevisiae*; SiF, simultaneous fermentation with *H. uvarum* Yun268 and *S. cerevisiae*; SeF, sequential fermentation with *H. uvarum* Yun268 and *S. cerevisiae*.

Mean values displaying different letters within each row were significantly different according to the Duncan test at 95% confidence level, while mean values without letters were not significantly different.

[#]Odor threshold were taken from literature: [1] Tao and Zhang, (2010); [2] Coelho, Coimbra, Nogueira, and Rocha (2009); [3] Mayr, Geue, Holt, Pearson, Jeffery, and Francis (2014); [4] Peng, Wen, Tao, and Lan (2013); [5] Sacks, Gates, Ferry, Lavin, Kurtz, and Acree (2012).

Table 2 Concentrations of fermentative volatiles in Ecolly and CS wines (μg/dm^3).

Compounds	RI*	OT#	Ecolly					CS			
			PF	EE	SiF	SeF		PF	EE	SiF	SeF
Acetate esters											
ethyl acetate†	885	7500[1]	12686 ± 882^{a}	13322 ± 746^{a}	12502 ± 864^{a}	**20806 ± 2071**^{b}		25636 ± 2081^{A}	27898 ± 3461^{A}	27772 ± 1744^{A}	**65706 ± 3790**^{B}
isobutyl acetate†	999	1600[1]	17 ± 2^{a}	**35 ± 4**^{c}	20 ± 2^{ab}	21 ± 1^{b}		58 ± 2^{A}	56 ± 3^{A}	55 ± 3^{A}	**79 ± 7**^{B}
isoamyl acetate†	1132	30[1]	718 ± 34^{b}	494 ± 28^{a}	**930 ± 45**^{c}	**924 ± 61**^{c}		441 ± 26^{A}	461 ± 18^{A}	**519 ± 34**^{B}	**566 ± 40**^{B}
hexyl acetate†	1287	670[1]	40 ± 2^{b}	33 ± 2^{a}	40 ± 2^{b}	**48 ± 3**^{c}		79 ± 4^{A}	87 ± 2^{AB}	78 ± 5^{A}	**95 ± 5**^{C}
2-phenylethyl acetate†	1829	250[1]	237 ± 49^{b}	144 ± 10^{a}	**318 ± 26**^{c}	**326 ± 32**^{c}		606 ± 56^{B}	323 ± 24^{A}	580 ± 31^{B}	**1406 ± 184**^{C}
∑			13698 ± 969^{a}	14028 ± 790^{a}	13810 ± 939^{a}	**22125 ± 2168**^{b}		26820 ± 2169^{A}	28825 ± 3508^{A}	29004 ± 1817^{A}	**67852 ± 4026**^{B}
Ethyl esters											
ethyl isobutyrate	909	15[1]	111 ± 10	124 ± 10	121 ± 17	98 ± 5		67 ± 7^{A}	**116 ± 12**^{B}	83 ± 9^{AB}	78 ± 8^{A}
ethyl propanoate	951	1800[1]	223 ± 14^{ab}	**282 ± 29**^{b}	238 ± 17^{ab}	217 ± 23^{a}		210 ± 29^{A}	**305 ± 20**^{B}	201 ± 22^{A}	214 ± 15^{A}
ethyl butyrate†	1026	20[1]	468 ± 16^{a}	**601 ± 18**^{b}	**552 ± 30**^{b}	**558 ± 34**^{b}		335 ± 24	349 ± 29	340 ± 28	334 ± 25
ethyl 2-methylbutyrate	1044	18[1]	72 ± 3^{a}	87 ± 6^{ab}	**123 ± 9**^{c}	90 ± 5^{b}		**27 ± 2**^{C}	17 ± 1^{A}	**29 ± 1**^{C}	23 ± 2^{B}
ethyl isovalerate	1062	3[2]	**184 ± 9**^{c}	**191 ± 10**^{c}	161 ± 9^{b}	91 ± 7^{a}		**87 ± 3**^{C}	61 ± 4^{B}	**89 ± 4**^{C}	52 ± 2^{A}
ethyl hexanoate†	1244	14[2]	830 ± 35^{a}	912 ± 47^{a}	935 ± 44^{ab}	**1047 ± 64**^{b}		263 ± 8^{C}	215 ± 11^{B}	**330 ± 47**^{D}	159 ± 12^{A}
ethyl octanoate†	1446	5[2]	1215 ±	1156 ±	**1600 ±**	1522 ±		121 ±	84 ± 3^{A}	**163 ±**	63 ± 4^{A}

Compounds	RI*	OT[#]	Ecolly					CS			
			PF	EE	SiF	SeF		PF	EE	SiF	SeF
			142^{ab}	74^{a}	**73**^{c}	142^{bc}		7^{B}		**14**^{C}	
ethyl decanoate†	1651	200[2]	443 ± 49^{b}	230 ± 21^{a}	**743 ± 63**^{c}	**792 ± 50**^{c}		12 ± 2^{A}	**17 ± 1**^{B}	**16 ± 1**^{B}	**17 ±2**^{B}
∑			3546 ± 278^{a}	3583 ± 215^{a}	**4473 ± 262**^{b}	**4415 ± 330**^{b}		1122 ± 82^{AB}	1164 ± 81^{AB}	**1251 ± 126**^{B}	940 ± 70^{A}
Other esters											
ethyl lactate†	1363	14000[2]	**4386 ± 490**^{b}	**4456 ± 370**^{b}	3599 ± 248^{a}	3725 ± 197^{a}		3087 ± 220^{B}	**3920 ± 210**^{C}	3048 ± 187^{B}	2380 ± 118^{A}
diethyl succinate†	1681	6000[1]	8848 ± 505^{c}	**12479 ± 752**^{d}	7234 ± 400^{b}	4412 ± 280^{a}		**6976 ± 463**^{C}	5504 ± 375^{B}	**6623 ± 394**^{C}	4632 ± 221^{A}
∑			13234 ± 995^{c}	**16935 ± 1122**^{d}	10833 ± 648^{b}	8137 ± 477^{a}		**10063 ± 683**^{B}	**9424 ± 585**^{B}	**9671 ± 581**^{B}	7012 ± 339^{A}
Volatile fatty acids											
hexanoic acid†	1863	420[2]	1394 ± 64^{a}	**1838 ± 82**^{b}	1384 ± 46^{a}	**2050 ± 108**^{b}		602 ± 57^{B}	653 ± 36^{BC}	**749 ± 28**^{C}	397 ± 25^{A}
octanoic acid†	2083	500[2]	6347 ± 565^{a}	6408 ± 333^{a}	6657 ± 492^{a}	**10823 ± 562**^{b}		982 ± 83^{B}	996 ± 76^{B}	**1224 ± 133**^{C}	497 ± 34^{A}
decanoic acid†	2296	1000[2]	2073 ± 101^{b}	1215 ± 143^{a}	2121 ± 160^{b}	**4019 ± 294**^{c}		179 ± 14^{B}	140 ± 6^{A}	**235 ± 22**^{C}	147 ± 7^{AB}
∑			9814 ± 730^{a}	9461 ± 558^{a}	10162 ± 698^{a}	**16892 ± 964**^{b}		1763 ± 154^{B}	1789 ± 118^{B}	**2208 ± 183**^{C}	1041 ± 66^{A}
Higher alcohols											
isobutyl alcohol†	1108	40000[2]	3494 ± 179^{a}	**4153 ± 136**^{b}	3496 ± 191^{a}	**4499 ± 205**^{b}		8335 ± 347^{A}	**10771 ± 737**^{B}	8282 ± 394^{A}	8188 ± 228^{A}
1-butanol†	1165	150000[2]	1081 ± 124^{a}	1020 ± 77^{a}	**1274 ± 112**^{b}	868 ± 65^{a}		726 ± 60^{A}	950 ± 31^{B}	**1366 ± 84**^{C}	828 ± 78^{AB}
isoamyl alcohol†	1230	30000[1]	107081	117357	111935	100177		103010	113801	104483	99404 ±

Compounds	RI*	OT[#]	Ecolly					CS			
			PF	EE	SiF	SeF		PF	EE	SiF	SeF
			± 5576	± 8815	± 6150	± 5605		± 4255	± 4798	± 6326	4440
3-methyl-1-pentanol	1247	500[2]	**193 ± 9**^{b}	**178 ± 6**^{b}	**189 ± 16**^{b}	97 ± 7^{a}		53 ± 3^{A}	**77 ± 4**^{B}	56 ± 3^{A}	52 ± 2^{A}
1-heptanol	1450	200-300[2]	**118 ± 5**^{c}	35 ± 2^{a}	**124 ± 9**^{c}	45 ± 3^{b}		238 ± 5^{C}	35 ± 2^{A}	**252 ± 4**^{D}	145 ± 7^{B}
1-octanol	1605	900[2]	16 ± 1^{b}	**19 ±1**^{c}	15 ± 0^{b}	12 ± 0^{a}		13 ± 0^{B}	**29 ± 1**^{C}	12 ± 1^{B}	9 ± 0^{A}
1-nonanol	1676	600[1]	8 ± 1^{b}	**14 ± 1**^{c}	7 ± 0 ^{a}	7 ± 0^{a}		**20 ± 2**^{B}	15 ± 1^{A}	**21 ± 1**^{B}	**20 ± 1**^{B}
1-decanol†	1781	400[1]	9 ± 1	8 ± 1	8 ± 1	10 ± 1		4 ± 0^{B}	3 ± 0^{A}	**5 ± 0**^{C}	3 ± 0^{A}
benzyl alcohol	1896	200000[2]	1535 ± 128^{a}	**2246 ± 157**^{b}	1640 ± 104^{a}	**2269 ± 92**^{b}		616 ± 30^{A}	684 ± 48^{A}	622 ± 41^{A}	**782 ± 33**^{B}
2-phenylethanol†	1931	14000[1]	21942 ±1342	22828 ± 1548	18405 ± 1883	17339 ±3592		34704 ± 1649^{AB}	34145 ± 2500^{A}	34811 ± 1745^{AB}	**45793 ± 7346**^{B}
∑			135477 ± 7366	147858 ± 10744	137094 ± 8466	125324 ± 9570		147719 ± 6351	160510 ± 8122	149910 ± 8599	155224 ± 12135
Carbonyls											
1-nonanal†	1390	15[1]	3 ± 1	4 ± 0	3 ± 0	3 ± 0		3 ± 0^{B}	2 ± 0^{A}	**4 ± 0**^{C}	3 ± 0^{B}
furfural	1476	14100[3]	942 ± 55	1083 ± 63	1200 ± 127	1257 ± 168		540 ± 33^{A}	**742 ± 58**^{B}	509 ± 42^{A}	486 ± 27^{A}
∑			945 ± 56	1087 ± 63	1203 ± 127	1260 ± 168		543 ± 33^{A}	**744 ± 58**^{B}	513 ± 42^{A}	489 ± 27^{A}

*RI, retention index on a DB-WAX column. †Compounds quantified with pure standards.

PF, pure *S. cerevisiae* fermentation; EE, fermentation with *H. uvarum* extracellular extract and *S. cerevisiae*; SiF, simultaneous fermentation with *H. uvarum* Yun268 and *S. cerevisiae*; SeF, sequential fermentation with *H. uvarum* Yun268 and *S. cerevisiae*.

Mean values displaying different letters within each row were significantly different according to the Duncan test at 95% confidence level, while mean values without letters were

not significantly different.

[#]Odor threshold were taken from: [1] Peng et al. (2013); [2] Tao and Zhang (2010); [3] Mayr et al. (2014).

Table 3. Regression coefficients* of aroma traits and modified compounds from Ecolly and CS wines.

Compounds	Ecolly				CS			
	Tropical fruity	Temperate fruity	Floral		Tropical fruity	Temperate fruity	Floral	Nail polish
Terpenes	0.210	**0.262**	**0.249**		0.123	-0.122	0.170	0.105
C_{13}-norisoprenoids	**0.231**	0.096	0.162		0.156	-0.077	**0.215**	0.130
Acetate esters	**0.230**	0.205	**0.243**		**0.231**	-0.215	**0.201**	**0.223**
Ethyl esters	0.129	**0.243**	0.146		-0.175	**0.221**	-0.119	-0.194
Fatty acids	0.226	0.223	0.219		-0.196	**0.242**	-0.141	-0.207
Volatile phenols	N/A	N/A	N/A		**0.221**	-0.185	**0.195**	**0.207**
Carbonyls	N/A	N/A	N/A		-0.109	0.053	-0.059	-0.111
C_6 alcohols	N/A	N/A	N/A		-0.049	-0.007	-0.016	-0.055
B0W	1.336	-1.710	-1.892		4.198	4.824	4.586	1.509
R^2 (Validation)	0.692	0.754	0.650		0.835	0.697	0.517	0.655
R^2 (Calibration)	0.787	0.829	0.798		0.946	0.848	0.770	0.908

*Standard coefficients the high positive value of which is presented in bold form. N/A, not applicable.

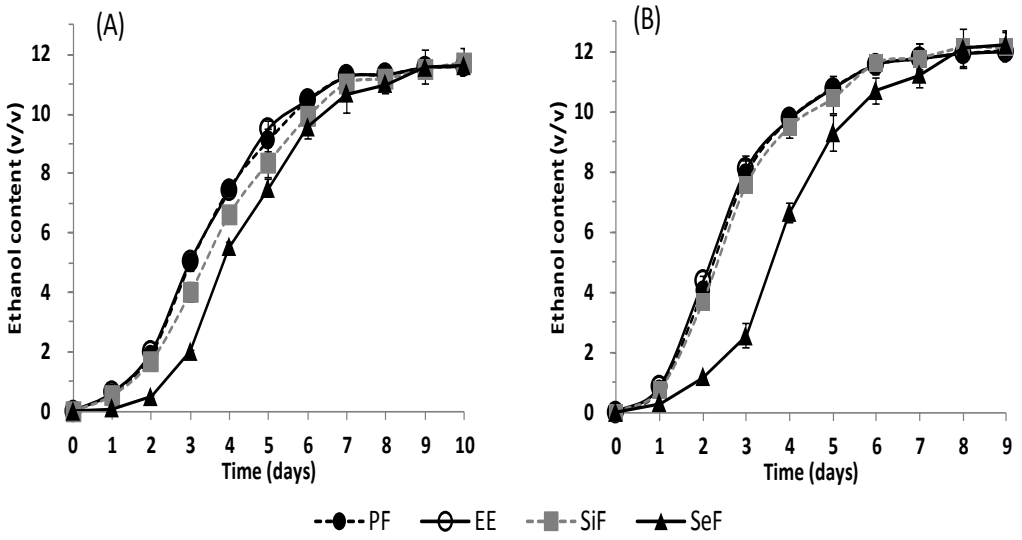


Fig. 1. Ethanol accumulation in Ecolly (A) and CS (B) wines obtained from PF (●), EE (○), SiF (■), and SeF (▲).

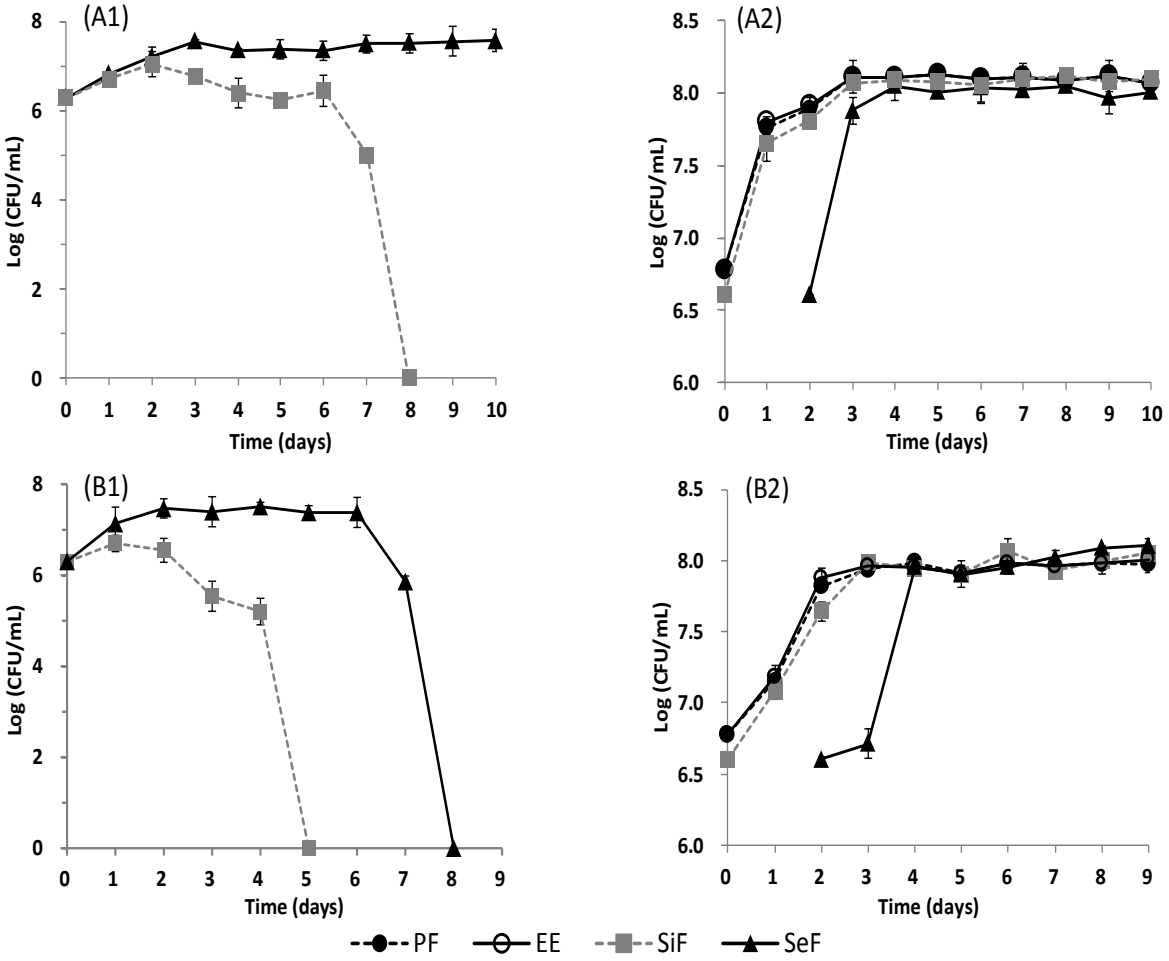


Fig. 2. Yeast biomass evolution of *H. uvarum* Yun268 (1) and *S. cerevisiae* CEREVISIAE (2) during fermentation of Ecolly (A) and CS (B) wines: PF (●), EE (○), SiF (■), and SeF (▲).

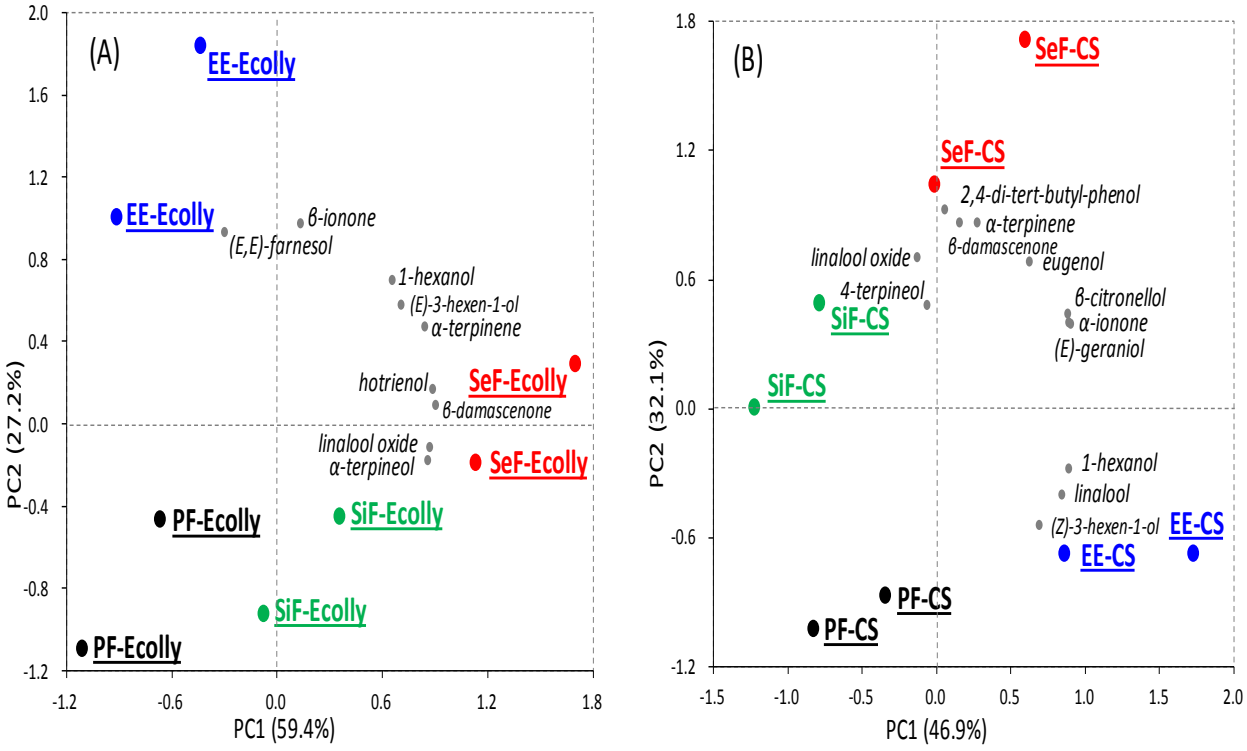


Fig. 3. Principal component analysis of varietal volatiles obtained from Ecolly (A) and CS (B) wines.

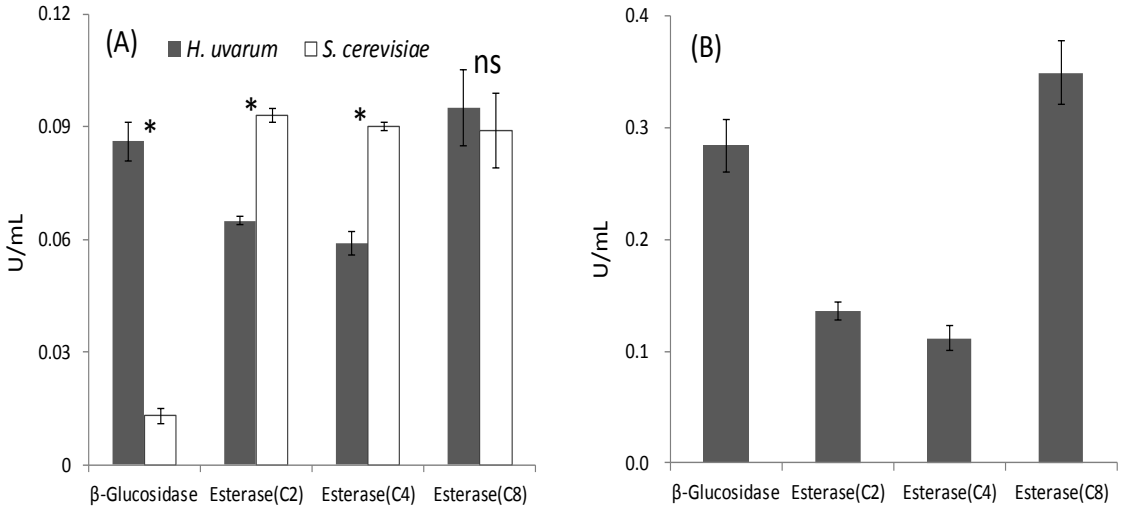


Fig. 4. Activity of β-glucosidase and esterase of yeasts (A) and concentrated *H. uvarum* extracellular extract (B): * Difference significant at 95% confidence level; ns, not significant.

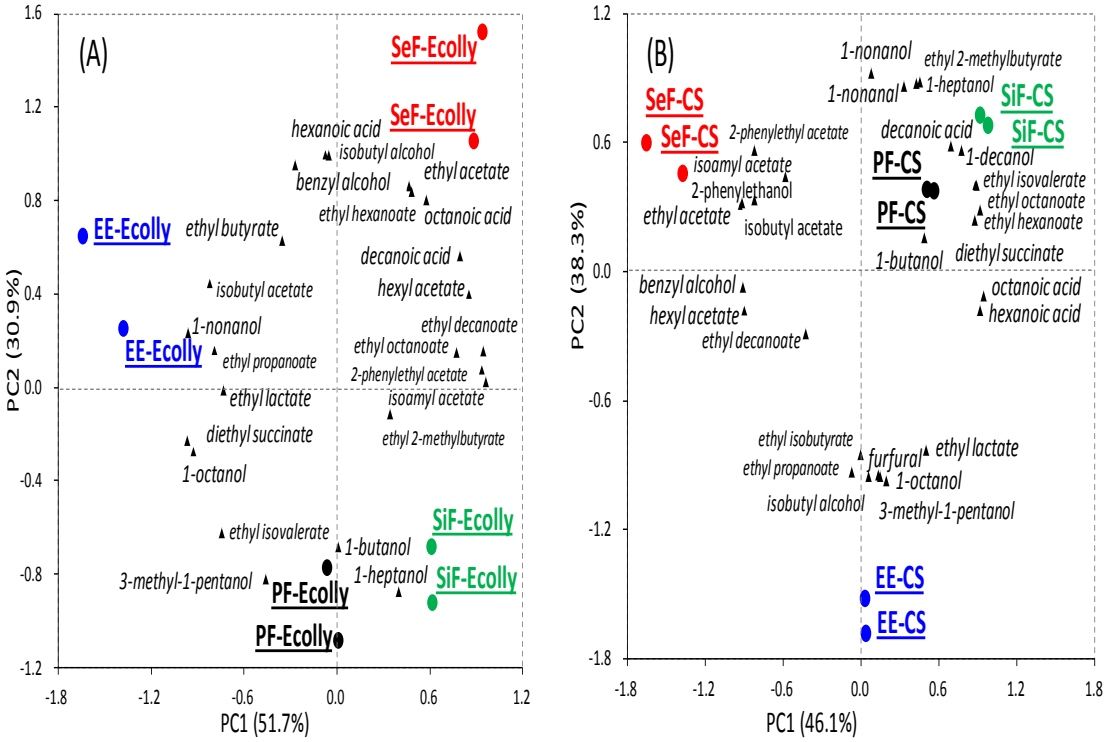


Fig. 5. Principal component analysis of fermentative volatiles obtained from Ecolly (A) and CS (B) wines.

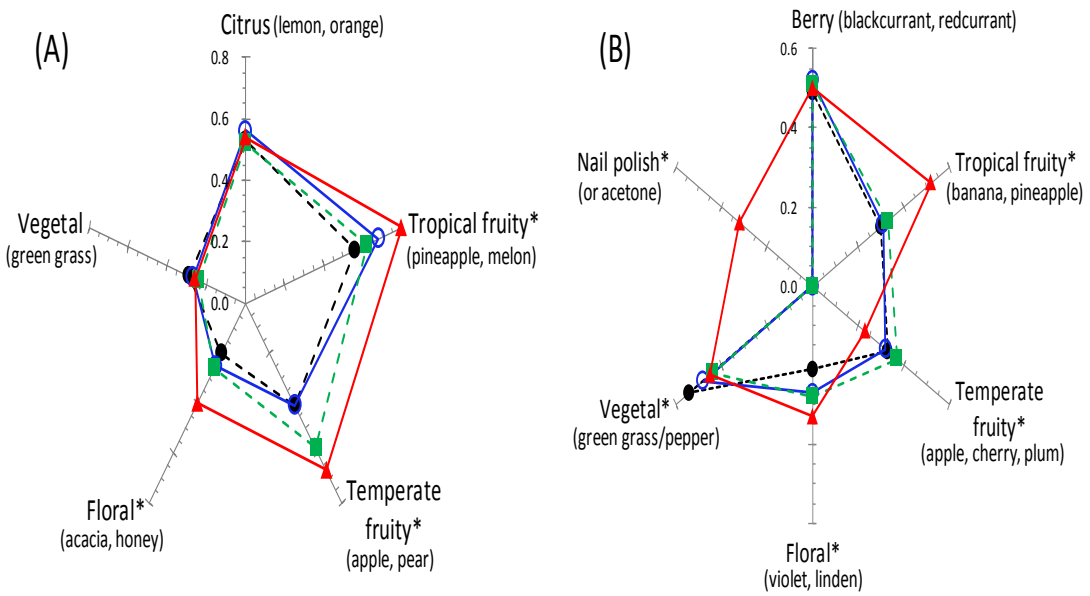


Fig. 6. Aroma MF% values of Ecolly (A) and CS (B) wines obtained from PF (●), EE (○), SiF (■), and SeF (▲): * Difference significant at 95% confidence level; ns, not significant.

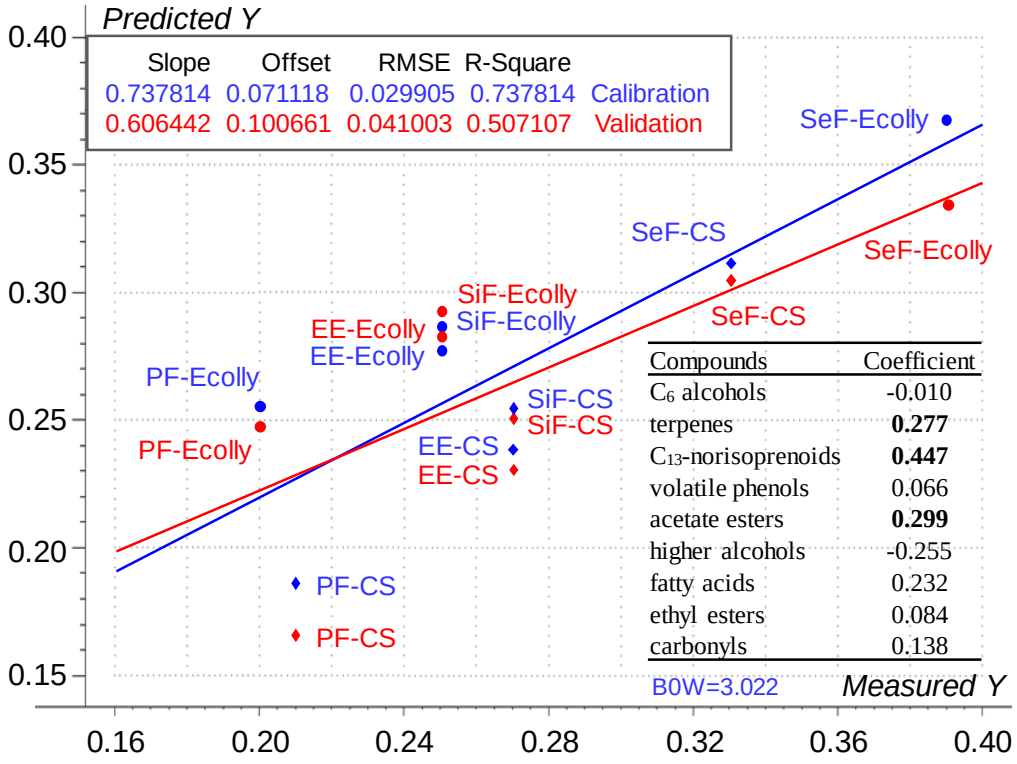
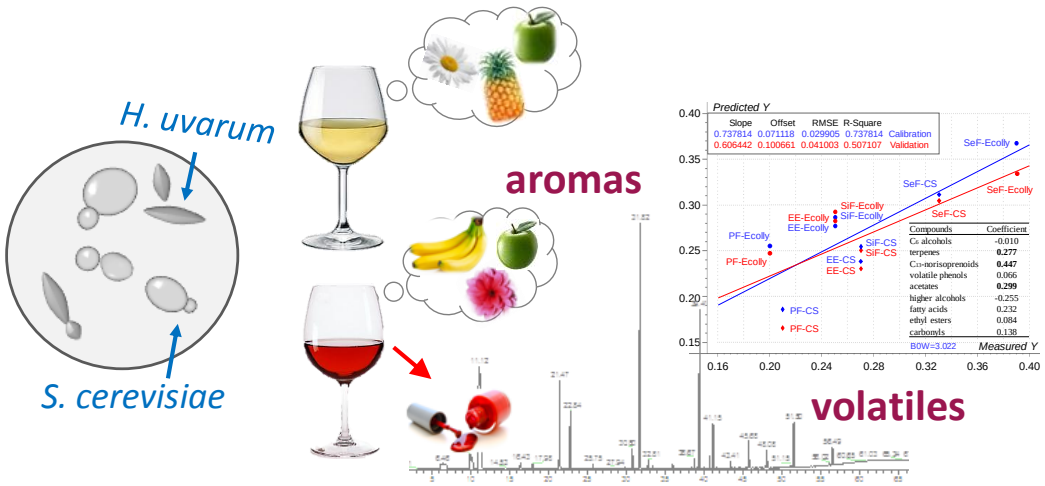


Fig. 7. PLS regression of floral trait from volatiles in Ecolly and CS wines.

Graphical abstract



Different mixed fermentation strategies resulted into distinctive chemical and sensory profiles of wines.

Highlights

- Wine aromas were distinctively modulated by different mixed fermentations.
- *H. uvarum* Yun268 contributed to wine volatiles via β-glucosidase and fatty acids.
- Extracellular extract enhanced varietal compounds rather than fermentative ones.
- Correlation between aroma traits and impact compounds were built using PLSR.