Zygotorulaspora florentina and *Starmerella bacillaris* in multistarter fermentation with *Saccharomyces cerevisiae* to reduce volatile acidity of high sugar musts

L. LENCIONI[1], M. TACCARI[1,2], M. CIANI[2] and P. DOMIZIO[1]

[1] Dipartimento di Gestione dei Sistemi Agrari, Alimentari e Forestali, Università degli Studi di Firenze, 50144 Florence, Italy
[2] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, 60131 Ancona, Italy
Corresponding author: Dr Paola Domizio, email paola.domizio@unifi.it

Abstract

Background and Aims: The possibility to decrease wine volatile acidity (VA) is an important aspect in wine production. This applies in particular to wines that are produced from musts with high sugar concentration, where the osmotic pressure promotes an increase in acetic acid production by *Saccharomyces cerevisiae*. This study aimed to identify suitable yeast strains and fermentation temperature to undertake the alcoholic fermentation of high sugar musts.

Methods and Results: To lower VA during fermentation of high sugar musts, two non-*Saccharomyces* yeast strains, *Zygotorulaspora florentina* and *Starmerella bacillaris*, were used in multistarter fermentations with *S. cerevisiae* at a fermentation temperature of 14 and 20°C. The fermentation temperature influenced the yeast behaviour and the composition of the two mixed fermentations.

Conclusions: Independent of fermentation temperature, the mixed fermentations with *Z. florentina* performed best to reduce VA.

Significance of the Study: Mixed fermentations with the non-*Saccharomyces* yeast strains *Z. florentina* and *S. bacillaris* may represent a valuable approach for the fermentation of high sugar musts.

Keywords: *high sugar must, mixed fermentation, non*-Saccharomyces *yeast,* Starmerella bacillaris, Zygotorulaspora florentina

Introduction

Alcoholic fermentation of high sugar must by *Saccharomyces cerevisiae* is often associated with the production of a high concentration of acetic acid (Erasmus et al. 2003, 2004). Indeed, up-regulation of the genes encoding aldehyde dehydrogenases and the subsequent increase in acetic acid production have been reported for *Saccharomyces* yeast in response to osmotic stress conditions (Hohmann 2002, Erasmus et al. 2004). High acetic acid concentration can have a considerable impact on the quality of a wine, as this imparts a vinegar-like character. Thus, depending on the type of wine, the concentration of volatile acidity (VA) might exceed the European Economic Community legal limit that is specific for each type of wine. In this context, mixed fermentation with *Saccharomyces* and *Starmerella bacillaris* (synonym *Candida zemplinina*) (Rantsiou et al. 2012) or *Torulaspora delbrueckii* (Bely et al. 2008) has been proposed for the fermentation of high sugar musts, to reduce the VA of the resulting wines. Such reduction in VA has been explained mainly as a consequence of the osmophilic characters of these non-*Saccharomyces* yeasts. Therefore, they might be suitable for the initial stages of multistarter fermentation, to lower the sugar concentration of the must, to make it more suitable for subsequent fermentation by *S. cerevisiae*.

Several authors have highlighted that non-*Saccharomyces* yeasts in mixed fermentation can strongly impact the sensory features of the wines. Most of the non-*Saccharomyces* yeasts are able to produce a wide range of hydrolytic enzymes, involved in the release of aromatic compounds from grape precursors, to produce a high concentration of interesting esters, such as isoamyl acetate (banana-like aroma) and 2-phenyl-ethyl acetate (rose-like aroma), and to release a high quantity of mannoproteins, which have many positive oenological properties (Viana et al. 2008, Ciani et al. 2010, Andorrà et al. 2012, Sadoudi et al. 2012, Domizio et al. 2014, 2017).

Strains belonging to *S. bacillaris* have been shown to have a strong fructophilic character, and to be characterised by the production of a low concentration of ethanol and acetic acid and a high concentration of glycerol (Sipiczki 2003, Magyar and Tóth 2011, Tofalo et al. 2012, Englezos et al. 2015). In contrast, there have been few studies on *Zygotorulaspora florentina* yeast. In recent studies, Domizio et al. (2011) and Lencioni et al. (2016) used the *Z. florentina* #42 strain (formerly known as *Zygosaccharomyces florentinus*) in pure and mixed fermentations with *S. cerevisiae* of must with a standard level of sugar; they reported not only a reduction in VA but also an enhancement of the concentration of polysaccharides, phenyl-ethyl acetate and 2-phenyl ethanol in wine.

In the present study, the effect of temperature on the oenological performance of *Z. florentina* and *S. bacillaris* was evaluated during mixed fermentations with *S. cerevisiae* in high sugar musts, to assess their potential use for the production of some types of dessert wines, such as Vinsanto wine and similar wine styles that are not fortified.

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Materials and methods

Yeast strains

The yeast strains *Z. florentina* #42, *S. bacillaris* #22, and *S. cerevisiae* #SDB1 were obtained from the Yeast Culture Collections of the Dipartimento di Gestione dei Sistemi Agrari, Alimentari e Forestali (GESAAF), Università degli Studi di Firenze and the Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche. Both non-*Saccharomyces* strains were isolated from grape and must of different origin. In particular, the strain *Z. florentina* #42 was isolated during alcoholic fermentation of Vinsanto and the strain *S. bacillaris* #22 from dried grapes. They were cryopreserved, and when necessary subcultured at 6-month intervals on yeast extract–peptone dextrose agar (Oxoid, Basingstoke, England), and maintained at 4°C.

Microfermentation trials

The fermentation performance of these yeast strains was evaluated in pure and multistarter microfermentation trials, which were carried out in 250 mL Erlenmeyer flasks containing 200 mL commercial white grape must (pH 3.2; TA 7.68 g/L, as tartaric acid). The grape must was supplemented with diammonium phosphate to a final concentration of 230 mg/L yeast assimilable nitrogen, and equimolar glucose and fructose to a final concentration of 35% (m/v). The enriched musts were then pasteurised for 15 min at 104°C.

The flasks were inoculated with 48 h pre-cultures grown in the same medium at 25°C, with the cell concentration determined by counting under light microscopy. At both 14 and 20°C, 1×10^5 cells/mL were used for the *S. cerevisiae* pure fermentation (Control), while the multistarter, or mixed fermentations were inoculated with 1×10^4 cells/mL for *S. cerevisiae* plus 1×10^6 cells/mL for *Z. florentina* or *S. bacillaris*.

Such inoculation levels were based on the results of previous investigation (Comitini et al. 2011, Domizio et al. 2011) of the growth competition between these non-*Saccharomyces* strains and *S. cerevisiae*. The flasks were stoppered with Müller valves that contained sulfuric acid to allow the CO_2 to escape, and mass loss was evaluated until the end of fermentation (i.e. constant mass for 2 consecutive days). The grape must was fermented in duplicate at 14 and 20°C under static conditions. Samples were taken at regular intervals from each flask to evaluate the viable cell count. An aliquot (100 μL) of serial dilutions of each sample was plated onto Wallerstein Laboratory nutrient agar medium (WL) (Oxoid Unipath, Basingstoke, England), which is a differential medium that allows the putative identification of wine yeasts on the basis of colour and morphology of the colonies. It was used for the count of viable *S. cerevisiae* and non-*Saccharomyces* yeasts (Pallmann et al. 2001).

Wine composition

Ethanol, residual sugar, VA and pH were determined on the wines produced, according to the Official European Union Methods (European Commission 2000). The ethanol yield (Y_{EtOH}) was calculated as the ratio (m/m) between the ethanol produced and the sugar consumed. Fermentation purity was calculated as the ratio between the VA [expressed as acetic acid equivalents (g/L)] and the ethanol concentration (% v/v).

The concentration of polysaccharides and glycerol was evaluated according to Domizio et al. (2017). In particular, polysaccharides were determined by isocratic separation on a Supelco TSK G-OLIGO-PW (808031) column (30 cm × 7.8 mm i.d.) and a Supelco TSK-GEL OLIGO (808034) guard column (4 cm × 6 mm i.d.) (Supelco, Bellefonte, PA, USA), and glycerol by isocratic separation on a Rezex-ROA-Organic Acids (30 + 15) cm × 7.8 mm i.d. column (Phenomenex, Torrance, CA, USA). The area of the peaks of interest was integrated using the Galaxie Chromatography Data System version 1.9.302.530 (Varian, Palo Alto, CA, USA). The concentration of acetaldehyde, ethyl acetate, and higher alcohols was determined by gas chromatography (Lencioni et al. 2016).

Statistical analysis

The data were analysed using the Statgraphics Plus v.2.1 software (Statpoint Technologies, Warrenton, VA, USA). To evaluate the statistical significance of the chemical data of the wines, data were subjected to one-way ANOVA (general linear model). Significant differences between the data were determined using the Duncan test, with $P < 0.05$ considered as significant.

Results and discussion

Cell growth and fermentation kinetics

Figure 1 shows the cell growth and fermentation kinetics of *S. cerevisiae* in the pure fermentations and in the mixed fermentations with *Z. florentina* #42 and *S. bacillaris* #22 in grape must containing 35% (m/m) sugars, at 14 and at 20°C. Independent of the fermentation temperature, both non-*Saccharomyces* yeast strains reached a cell concentration ranging from 3 to 4×10^7 CFU/mL during the first 7 days of the alcoholic fermentation (Figure 1a,e). After 4 weeks of fermentation, however, the higher temperature of 20°C resulted in a dramatic decrease in the viable count of both *Z. florentina* and *S. bacillaris* yeast strains, and after 40 days of alcoholic fermentation they showed a cell concentration of around 6×10^5 CFU/mL (Figure 1f,g). In contrast at 14°C, *Z. florentina* and *S. bacillaris* had a higher cell concentration after 40 days of fermentation (3.5×10^7, 6.4×10^7 CFU/mL, respectively) (Figure 1b,c). Both non-*Saccharomyces* yeast strains then showed a significant decrease over the following days, showing a cell concentration of around $0.5–1 \times 10^6$ CFU/mL by day 70 of the alcholic fermentation. The *S. cerevisiae* pure fermentation, independent of the fermentation temperature, after 7 days of alcoholic fermentation had reached a cell concentration of about 10^7 CFU/mL, and persisted at this cell concentration to the end of the fermentation (day 70). The same cell growth was seen for *S. cerevisiae* during the mixed fermentations at each temperature tested. Despite the lower initial cell concentration of *S. cerevisiae* compared to that of *S. bacillaris* in the mixed fermentations (1×10^4 vs 1×10^6 cells/mL), a similar cell number (~10^7 CFU/mL) was reached after only 7 days of fermentation, and at each temperature (Figure 1c,g). *Zygotorulaspora florentina*, however, appeared to affect the growth of *S. cerevisiae*, which reached only 1×10^6 and 2×10^6 CFU/mL at 14 and 20°C, respectively, after 7 days of fermentation (Figure 1b,f). After 40 days of fermentation, however, and to the end of the fermentation, there was a similar cell number of *S. cerevisiae* in both of these mixed fermentations, at each temperature tested.

The fermentation kinetics were in agreement with the growth kinetics. In particular, similar fermentation kinetics were seen in the mixed fermentations with *S. bacillaris* and in the *S. cerevisiae* pure fermentations. In contrast, the fermentation kinetics of the mixed fermentations with *Z. florentina* were slower, which was particularly evident at

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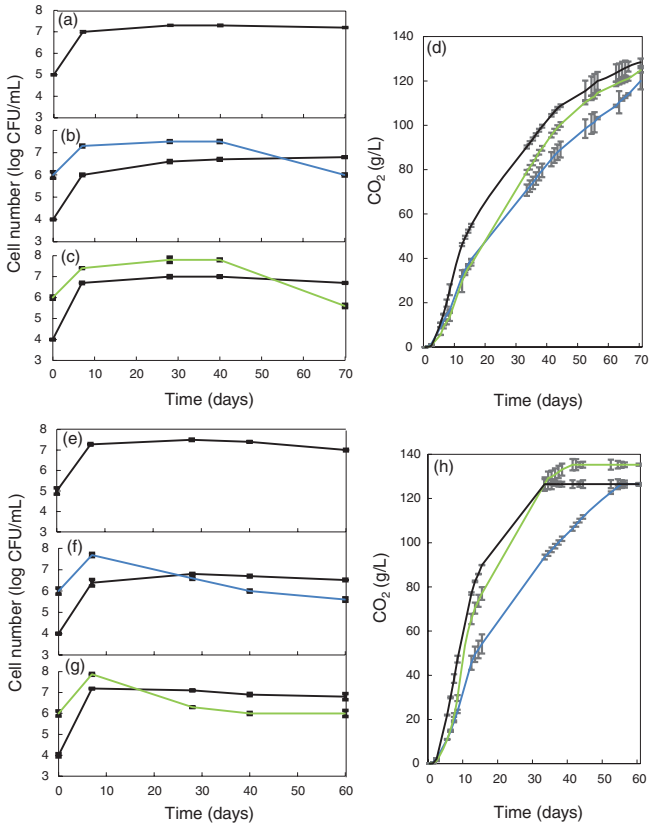

Figure 1. Effect of mixed fermentation on (a–c, e–g) yeast growth and (d, h) fermentation kinetics in 35% sugar must at (a–d) 14°C and at (e–h) 20°C. Growth of *Saccharomyces cerevisiae* (#SDB1) (——) in (a, e) pure culture, (b, f) in mixed culture with *Zygotorulaspora florentina* #42, and (c, g) in mixed culture with *Starmerella bacillaris* #22; growth of *Z. florentina* #42 (b, f) in mixed culture (——); growth of *S. bacillaris* #22 (c, g) in mixed culture (——). Fermentation kinetics (d) at 14°C and (h) at 20°C of *S. cerevisiae* (#SDB1) in pure culture (——), in mixed culture with *Z. florentina* #42 (——) and with *S. bacillaris* #22 (——). Data are means ±SD (n = 2).

the higher temperature (20°C), compared to the Control *S. cerevisiae* pure fermentations (Figure 1d,h).

Wine composition

The data for the main fermentation parameters and compounds analysed for these wines are given in Table 1. The ethanol production by *S. cerevisiae* in the pure fermentations was not influenced by temperature. In contrast, there was a significant increase in the ethanol concentration with the increase in temperature for both of the mixed fermentations. At 14°C, however, there was a significantly lower ethanol concentration in the mixed fermentations of *S. cerevisiae* with *Z. florentina*. This is in agreement with the higher residual sugars at the end of fermentation and the lower fermentation kinetics (Figure 1) that were seen in this mixed fermentation, compared to that of the Control. Increased ethanol yield was generally obtained in the mixed fermentations. Although there was lower ethanol production at 14°C, the mixed fermentation of *S. cerevisiae* with *Z. florentina* had the highest ethanol yield. In contrast, at 20°C, the yield of both of the mixed fermentations was similar and higher compared to that of the Control.

The fermentation temperature greatly influenced the production of VA. In particular, comparing the fermentation of the Control with that of the *S. cerevisiae* with *S. bacillaris* mixed fermentations, the change from 14°C to the higher temperature of 20°C resulted in an increase in the VA of 187 and 59%, respectively. In contrast, there was no change in VA production in the *S. cerevisiae* with *Z. florentina* mixed fermentations at each fermentation temperature. Moreover, independent of the fermentation temperature, all of the mixed fermentations with *S. cerevisiae* and *Z. florentina* showed the lowest VA, which ranged from 0.18 to 0.20 g/L. The lowest value of fermentation purity evaluated as the amount of VA formed in relationship to ethanol produced [(VA g/L)/(ethanol % v/v)], in the mixed fermentations of *S. cerevisiae* with *Z. florentina* (Table 1), account for this behaviour. Therefore, the typical feature of *S. bacillaris* yeast, such as growth at high sugar concentration and low production of acetic acid was confirmed in the present study for the *S. bacillaris* #22 strain during these mixed fermentations of high sugar must. Similar features were also shown for the *Z. florentina* #42 strain. Compared to *S. bacillaris*, however, mixed fermentations of *S. cerevisiae* with *Z. florentina* #42 produced an even lower concentration of acetic acid. This is consistent with previous studies, where for fermentation of grape juice containing 22% sugar, *Z. florentina* #42 produced less acetic acid in the mixed fermentation, compared to *S. cerevisiae* as the pure fermentation Control (Domizio et al. 2011, Lencioni et al. 2016). The low VA during the alcoholic fermentation might be a typical feature of *Z. florentina*. Indeed, Domizio et al. (2011) reported low VA production during pure fermentations with four *Z. florentina* strains compared to three *S. cerevisiae* strains, when tested under the same fermentation conditions. It is worth emphasising that the *Z. florentina* #42 strain was isolated during alcoholic fermentation of Vinsanto, a dessert wine that is produced in Tuscany and other areas of central and northern Italy. Vinsanto is produced mainly from non-aromatic dried grapes that are characterised by high sugar concentration (Domizio and Lencioni 2011), and thus *Z. florentina* #42 is probably well adapted to such specific extreme conditions.

A significant increase in glycerol concentration was seen at the higher temperature for all fermentations. In particular, independent of the temperature, the mixed fermentation of *S. cerevisiae* with *S. bacillaris* showed significantly higher glycerol concentration, compared to that of the pure *S. cerevisiae* fermentations and to the mixed fermentation of *S. cerevisiae* with *Z. florentina*. The high production of glycerol has been reported to be a phenotypic trait among different strains of *S. bacillaris* (Sipiczki 2003, Magyar and Tóth 2011, Englezos et al. 2015). This explains the higher glycerol concentration found in the mixed fermentation trials of *S. bacillaris*/*S. cerevisiae*. In contrast, the higher glycerol production by the strain *Z. florentina* appears to be related to a specific response of the yeast to the high temperature.

Fermentation temperature also significantly influenced the concentration of polysaccharides released into the fermentation for both the *S. cerevisiae* pure fermentations and the mixed fermentations of *S. cerevisiae* with *S. bacillaris*. Instead, there was similar release of polysaccharides into the mixed fermentations of *S. cerevisiae* with *Z. florentina* at each fermentation temperature (245 and 274 mg/L, respectively). In particular, the release of polysaccharides by the *S. cerevisiae* with *Z. florentina* inoculations was significantly increased over that of the relevant *S. cerevisiae* pure fermentations. This is in agreement with previous studies that have shown that these two yeast strains can enhance the release of polysaccharides in pure and mixed fermentations with *S. cerevisiae* (Domizio et al. 2011, Lencioni et al. 2016). The polysaccharides released during the alcoholic fermentation by these two non-*Saccharomyces* yeasts were identified as mannoproteins (Domizio et al. 2014). Mannoproteins have been reported to have many

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Table 1. Composition of and volatile byproducts of wines obtained from microfermentation trials with mixed inoculation of *Saccharomyces cerevisiae* and *Zygotorulaspora florentina* and *Starmerella bacillaris* in high sugar grape must at 14 and 20°C.

	14°C			20°C		
Wine composition	***S. cerevisiae***	***Z. florentina* + *S. cerevisiae***	***S. bacillaris* + *S. cerevisiae***	***S. cerevisiae***	***Z. florentina* + *S. cerevisiae***	***S. bacillaris* + *S. cerevisiae***
Major oenological parameters						
Ethanol (% v/v)	16.6 ± 0.0c	15.9 ± 0.1a	16.4 ± 0.1bc	16.1 ± 0.2ab	17.4 ± 0.0d	17.6 ± 0.2d
Residual sugar (g/L)	33.2 ± 1.9a	69.4 ± 11.7c	40.3 ± 8.6b	41.5 ± 5.8b	32.8 ± 3.8a	30.0 ± 4.2a
Y_{EtOH}†	0.41a	0.44c	0.42b	0.41a	0.43c	0.44c
Volatile acidity‡ (g/L)	0.30 ± 0.01c	0.18 ± 0.01a	0.37 ± 0.01d	0.86 ± 0.05f	0.20 ± 0.02b	0.59 ± 0.08e
Fermentation purity§	0.018b	0.011a	0.022b	0.053d	0.011a	0.034c
Glycerol (g/L)	7.0 ± 0.2a	7.3 ± 0.1a	9.4 ± 0.1b	8.5 ± 0.0b	9.2 ± 0.14b	10.3 ± 0.0c
Polysaccharides (mg/L)	162.1 ± 7.6a	245.5 ± 12.1d	198.3 ± 11.8ab	216.2 ± 4.3bc	274.8 ± 8.6d	259.1 ± 6.6d
Major volatile byproducts (mg/L)						
Acetaldehyde	94.0 ± 8.7c	120.5 ± 1.5d	66.7 ± 0.4a	235.9 ± 18.5e	105.3 ± 0.6d	74.7 ± 1.9b
Ethyl acetate	131.2 ± 13.0[c]	54.7 ± 5.3a	116.7 ± 0.3b	148.7 ± 17.4c	110.2 ± 3.5b	132.6 ± 3.3c
Propanol	40.0 ± 0.5a	77.8 ± 0.1d	58.8 ± 1.4b	57.3 ± 2.8b	103. ± 0.8e	64.2 ± 1.3c
Isobutanol (2-methylpropan-1-ol)	27.3 ± 5.2a	27.6 ± 0.2a	26.5 ± 0.6a	86.2 ± 7.8c	73.4 ± 4.7bc	64.6 ± 1.5b
Amyl alcohol (2-m-1-ol)	22.9 ± 0.8c	17.3 ± 0.0b	12.9 ± 0.7a	45.8 ± 3.6e	49.5 ± 2.5e	32.1 ± 0.7d
Isoamyl alcohol (3-methylbutan-1-ol)	169.0 ± 3.6b	112.1 ± 3.6a	109.9 ± 0.7a	294.2 ± 16.7c	227.3 ± 2.6d	189.1 ± 4.6c

Data are means ±SD (n = 2); within each row, data with different letters are significantly different (Duncan's LSD, $P \leq 0.05$). *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. bacillaris*, *Starmerella bacillaris*; *Z. florentina*, *Zygotorulaspora florentina*. †Ethanol yield expressed as ethanol/sugar consumed (m/m) ratio. ‡Expressed as acetic acid equivalents. §Expressed as volatile acidity/ethanol (g/L/%) ratio.

positive oenological properties, such as reduction of protein and tartrate instability, addition of complexity and aromatic persistence, decrease in astringency, increase in sweetness and roundness, and improvement of mouthfeel (Caridi 2006). All these features are important also in dessert wine production (Domizio and Lencioni 2011).

Main volatile by-products

Table 1 reports the concentration of the main volatile compounds produced during the fermentations, which included acetaldehyde, ethyl acetate, and higher alcohols. Acetaldehyde and ethyl acetate can have different roles in winemaking, which depend on the type of wine. For instance, in dessert wines with 'oxidative aging' such as Vinsanto wine, acetaldehyde is related to the sensory perception of the typical 'oxidised character' of the wine (Domizio and Lencioni 2011). In this context, compared to 14°C, the higher temperature of 20°C with the *S. cerevisiae* pure fermentation resulted in a large increase in acetaldehyde production, from 94 to 236 mg/L, respectively. For both of the mixed fermentations at 20°C, however, acetaldehyde concentration was significantly lower compared to that of the Controls, and it was similar at each fermentation temperature. In particular, the mixed fermentations of *S. cerevisiae* with *Z. florentina* produced wines with an acetaldehyde concentration of 105–120 mg/L, which is consistent with the typical range found in some dessert wines such as Vinsanto (Domizio and Lencioni 2011). In contrast, the mixed fermentations of *S. cerevisiae* with *S. bacillaris* always showed a lower range of acetaldehyde (66–74 mg/L), which remained below the threshold of 100 mg/L over which the defect of 'rotten apples' may result. The production of ethyl acetate in the mixed fermentations of *S. cerevisiae* with *Z. florentina* #42 was particularly influenced by temperature, and ranged from 54 at 14°C to 110 mg/L at 20°C, and the mixed fermentations of *S. cerevisiae* with *S. bacillaris* #22, had a similar ethyl acetate concentration of 116 and 132 mg/L, respectively, at each temperature. All mixed fermentations, however, had a concentration of ethyl acetate below the reported detection level in wine of 150 mg/L (Lambrechts and Pretorius 2000).

The concentration of the individual amyl alcohols, 3-methyl-1-butanol and 2-methyl-1-butanol in the mixed fermentation trials was generally lower, than that of the Controls (Table 1). In particular, the concentration of the combined amyl and isoamyl alcohols at 14 and 20°C in the mixed fermentations of *S. cerevisiae* with *Z. florentina* and *S. bacillaris* (129.4 and 122.9 mg/L; 276.9 and 221.2 mg/L, respectively) was significantly lower than that of the Controls (191.9 and 339.9 mg/L, respectively).

Conclusions

The present study demonstrates that mixed fermentations of *S. cerevisiae* with either *Z. florentina* #42 or *S. bacillaris* #22 represent promising controlled multistarter fermentations for the production of some types of dessert wine, as both of these non-*Saccharomyces* yeasts can provide a valuable profile of the main analytical compounds for the wine produced. To the best of our knowledge, this is the first time that a *Z. florentina* yeast strain has been used in the fermentation of high sugar must. Of particular significance is the low VA produced under all conditions tested by mixed fermentations of *S. cerevisiae* with *Z. florentina*. On the basis of the main volatile compounds monitored in the present study, the different responses obtained by the two non-*Saccharomyces* yeast strains during these mixed fermentations with *S. cerevisiae* can be exploited for the production of different types of

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dessert wines. In particular, these mixed fermentations at the higher temperature of 20°C appear to produce wines that are characterised by the alcohol concentration and the main volatile compounds produced during the fermentation. In contrast, the mixed fermentations at the lower temperature of 14°C appear to produce wines that are mainly characterised by higher residual sugar content, which would therefore be sweeter. These data thus support the concept that such controlled multistarter fermentations of *S. cerevisiae* with selected non-*Saccharomyces* yeast can be used to control the formation of VA, improving at the same time the complexity of wines.

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