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Controlled mixed fermentation at winery scale using *Zygotorulaspora florentina* and *Saccharomyces cerevisiae*

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Abstract

Over the last few years the use of multi-starter inocula has become an attractive biotechnological practice in the search for wine with high flavour complexity or distinctive characters. This has been possible through exploiting the particular oenological features of some non-*Saccharomyces* yeast strains, and the effects that derive from their specific interactions with *Saccharomyces*. In the present study, we evaluated the selected strain *Zygotorulaspora florentina* (formerly *Zygosaccharomyces florentinus*) in mixed culture fermentations with *Saccharomyces cerevisiae*, from the laboratory scale to the winery scale. The scale-up fermentation and substrate composition (i.e., white or red musts) influenced the analytical composition of the mixed fermentation.

At the laboratory scale, mixed fermentation with *Z. florentina* exhibited an enhancement of polysaccharides and 2-phenylethanol content and a reduction of volatile acidity.

At the winery scale, different fermentation characteristics of *Z. florentina* were observed. Using Sangiovese red grape juice, sequential fermentation trials showed a significantly higher concentration of glycerol and esters while the sensorial analysis of the resulting wines showed higher floral notes and lower perception of astringency. To our knowledge, this is the first time that this yeasts association has been evaluated at the winery scale indicating the potential use of this mixed culture in red grape varieties.

Keywords: mixed fermentation, non-*Saccharomyces*, *Zygotorulaspora florentina* (*Zygosaccharomyces florentinus*), wine yeast, winery scale, organoleptic evaluation

1. Introduction

During spontaneous wine fermentation, the microbial succession of apiculate yeasts (*Hanseniaspora/Kloeckera*) followed by *Saccharomyces cerevisiae* is well documented, along with the occasional presence of other yeast species belonging mainly to the genera *Metschnikowia*, *Pichia*, *Candida*, *Lachancea* and *Torulaspora* (Fleet, 2003; Jolly *et al.*, 2014; Pretorius, 2000). As spontaneous fermentation is difficult to manage, and as the oenological profiles of the resulting wines are hard to predict and reproduce, inoculated fermentation with selected strains of *Saccharomyces* has become the common practice that is now used all over the world. Indeed, the use of starter cultures of *S. cerevisiae* coupled with the use of SO_2 suppresses the wild microflora, which allows better control of the fermentation process, while at the same time producing wines with the desired oenological profiles.

However, the positive influences of non-*Saccharomyces* yeast on the analytical and aromatic profile of wine have been documented in several studies (Bely *et al.*, 2008; Comitini *et al.*, 2011; Sabaudi *et al.*, 2012; Viana *et al.*, 2008). This contrasts with the introduction of selected *S. cerevisiae* starter cultures that simplify not only the wine microflora, but also the analytical composition of the wine. In this context, over the last two decades there has been a re-evaluation of the role of non-*Saccharomyces* yeast species in wine making, following various studies on the role, presence, persistence and oenological aptitude of non-*Saccharomyces* wine yeast (Domizio *et al.*, 2007; Egli *et al.*, 1998; Lema *et al.*, 1996; Martinez *et al.*, 1989; Mora *et al.*, 1990; Rojas *et al.*, 2001).

In mixed fermentations, *Saccharomyces* and non-*Saccharomyces* yeast do not passively co-exist; instead they show interesting interactions that can influence the analytical profile of the wine (Andorrà *et al.*, 2012, 2014; Anfang *et al.*, 2009; Ciani and Ferraro, 1998; Ciani and Comitini, 2015; Loira *et al.*, 2014; Sadoudi *et al.*, 2012). As non-*Saccharomyces* wine yeast have specific oenological traits that might have additive effects on wine flavour and aroma (Strauss *et al.*, 2001;

Viana *et al.,* 2008), counteract undesirable microflora (Oro *et al.,* 2014; Ramírez *et al.,* 2015) or reduce the ethanol content in wine (Canonico *et al.,* 2016; Contreras *et al.* 2014; Quirós *et al.,* 2014) the use of controlled mixed fermentations of *Saccharomyces* and non-*Saccharomyces* wine yeast has been particularly encouraged more recently (Ciani *et al.,* 2006, 2010; Gobbi *et al.,* 2013; Jolly *et al.,* 2006; Mendoza *et al.,* 2007; Moreira *et al.,* 2008; Navarrete-Bolaños, 2012) even when these yeast are generally recognized as spoilage yeasts (Domizio *et al.,* 2011a, 2011b).

In a previous study, a selected strain of *Zygotorulaspora florentina* (formerly *Zygosaccharomyces florentinus*), as used in mixed fermentations at different inoculum ratio with *S. cerevisiae,* caused an increase of the production of polysaccharides and a modulation of the final concentrations of the various volatile compounds (Domizio *et al.,* 2011a). In the present study, the impact of inoculation modalities (e.g., co-inoculation, sequential fermentation) on the interactions between the two selected strains and on the aromatic profiles of the final wines were first evaluated at laboratory scale, and then, at industrial (winery) scale using the Sangiovese red grape variety.

2. Materials and methods

2.1. Yeast strains and media

The yeast strains used in this study were the commercial *S. cerevisiae* strain Lalvin EC1118 (Lallemand Inc.) and *Z. florentina* strain 42 from the Yeast Culture Collection of the Department of Agricultural Biotechnologies (GESAAF, University of Florence, Italy), which were maintained at -80 °C. The *Z. florentina* strain 42 was identified by D1-D2 domain analysis.

After revitalisation, these two yeast strains were routinely cultured on YPD agar (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 20 g/L agar) at 27 °C for 48 h, and maintained at 4 °C for the duration of study. The media used to evaluate viable cell counts during mixed fermentations were WL nutrient agar (Oxoid, Hampshire, UK) and lysine agar (Oxoid, Hampshire, UK). WL nutrient agar plates were used to differentiate the fermenting yeast from must samples (Pallman et

al. 2001), and lysine agar for the viable counts of the non-*Saccharomyces* yeast population. The viable yeast cells in the single culture fermentations of *S. cerevisiae* and *Z. florentina* (controls) were determined using YPD agar plates.

2.2. Fermentation trials

2.2.1. Laboratory-scale fermentation

The laboratory-scale fermentation trials were performed in triplicate under static conditions at 25°C in 1-L sterile Erlenmeyer flasks filled with 750 mL pasteurised commercial white grape must (Folicello s.s., Modena, Italy). The analytical characteristics of this grape juice were: pH 3.61; initial reducing sugars 224 g/L; titratable acidity (as tartaric acid) 5.55 g/L; ammonia nitrogen 21.7 mg/L; primary amino nitrogen 154.4 mg/L; and polysaccharides 171.1 mg/L.

Based on the result obtained in our previous study (Domizio *et al.*, 2011a), the mixed culture fermentations were inoculated with 10^7 cell/mL *Z. florentina* and 10^6 cell/mL *S. cerevisiae* (i.e., at the ratio 10:1) as three inoculation modalities: (i) simultaneous inoculation; (ii) sequential inoculation of *S. cerevisiae* after 24 h; and (iii) sequential inoculation of *S. cerevisiae* after 48 h. Pure culture fermentations of *Z. florentina* and *S. cerevisiae* were also carried out as controls and inoculated with 10^7 cell/mL and 10^6 cell/mL, respectively.

The flasks were inoculated with pre-cultures grown in the same grape must at 25°C for 72 h. The volumes to be inoculated were determined on the basis of cell counting under a light microscope with a Thoma Zeiss chamber. The flasks were equipped with Müller valves that contained sulphuric acid and were weighed every day until the end of the fermentation (constant weight for two consecutive days). During the fermentation, samples were taken for the determination of viable cell counts.

2.2.2. Winery-scale fermentation

Industrial (winery-scale) fermentations were carried out in 10 hL steel tanks at the 'Consorzio Tuscania' experimental winery, located in the Chianti area (Tuscany), using the Sangiovese grape variety. After harvest, the grapes were kept refrigerated during transport to the cellar and then processed the following day. The must composition was: fermentable sugars 23% (w/v); total acidity (as tartaric acid) 7.4 g/L; pH 3.25; total sulphur dioxide 40 mg/L; ammonia nitrogen 28.2 mg/L; primary amino nitrogen 172.5 mg/L; and polysaccharides 189.3 mg/L.

Mixed fermentations (co-inoculum and sequential) were inoculated with the same ratio used at the laboratory scale, 10^7 cell/mL *Z. florentina* and 10^6 cell/mL *S. cerevisiae*. In the sequential fermentations, *S. cerevisiae* was added after 48 h of fermentation. Control fermentations were carried out by inoculation of 10^6 cell/mL *S. cerevisiae*. The *Z. florentina* and *S. cerevisiae* strains were inoculated as fresh cultures and active dried yeast (ADY), respectively. The fermentations were performed in triplicate at 25 ±2 °C. Must aeration and cap management were carried out by pumping-over, according to the winery protocol. Alcoholic fermentation evolution was followed by periodically measuring the density by a double scale hydrometer (density and Baumé). During the fermentation, cell concentrations were evaluated as viable cell counts.

2.3. Analytical determinations

The measurements for ethanol, total sulphur dioxide, total acidity, volatile acidity, and pH were carried out using current analytical methods and according to the Official European Union Methods (EC, 2000). Ammonia nitrogen was determined using an enzymatic method (kit n. 112732; Roche Diagnostics, Germany), while the available amino nitrogen was evaluated following the procedures described by Dukes and Butzke (1998), applying the o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay.

Volatile compounds were quantified using gas-liquid chromatographic analyses using the procedure reported in Domizio *et al.* (2011a). Acetaldehyde, ethyl acetate, ethyl lactate and higher alcohols were assayed using 3-Methyl-2-butanol (IUPAC: 3-Methylbutan-2-ol) as internal standard

and a gas chromatograph (GC-2014;Shimadzu, Kyoto, Japan) equipped with a flame ionisation detector and a polyethylene glycol column (Zebron ZB-WAX Plus;30 m × 0.32 mm ID×0.25 mm film thickness; Phenomenex, Torrance, CA, USA). The other volatile compounds were quantified using 3-Octanol (IUPAC: Octan-3-ol) as internal standard and a fused silica capillary column (Supelcowax 10; 30 m × 0.25 mm ID × 0.25 mm film thickness; Supelco, Bellefonte, PA, USA), by the same GC apparatus described above.

The total polysaccharides, sugars, glycerol, L-malic and DL-lactic acids in the samples were evaluated using an HPLC apparatus (Varian Inc., CA, USA) equipped with a 410 series autosampler (20 µl loop), a 210 series pump, and a 356-LC refractive index detector, according to the procedures described in Domizio *et al*. (2011a, 2011b).

In particular, isocratic separation of total polysaccharides was carried out using a Supelco TSK G-OLIGO-PW (808031) column (30 cm × 7.8 mm ID) and a Supelco TSK-GEL OLIGO (808034) guard column (4 cm × 6 mm ID) (Bellefonte, PA, USA). Instead, the isocratic separation of sugars, glycerol, L-malic and DL-lactic acids were carried out using a Rezex-ROA-Organic Acids (30 + 15) cm × 7.8 mm ID column (Phenomenex, Torrance, CA, USA).

The areas of the peaks of interest were integrated using the Galaxie Chromatography Data System version 1.9.302.530 (Varian Inc., CA, USA).

2.4. Interdelta polymorphisms of S. cerevisiae

Molecular monitoring of the inoculated starter cultures was carried out to determine the level of dominance over the indigenous *S. cerevisiae* strains during the industrial fermentation (Li *et al.*, 2012; Mercado *et al.*, 2011), using interdelta PCR amplification.

S. cerevisiae colonies were isolated randomly from YPD agar plates, and used for cell counts at the end of the industrial fermentations. The molecular characterisation by PCR-amplification of interdelta sequences was performed on the isolate clones (20 for each tank). DNA was extracted from the *S. cerevisiae* clones, cultured at 25 °C for 24 h on YPD agar, and a cells

heat-treatment was used. In particular, a loop from each colony was re-suspended in 5 µL sterile distilled water in 0.2 mL PCR tubes and heated to 95 °C for 5 min, with a single step at 4 °C.

The amplification PCR reactions were performed on a Thermal Cycler (Bio-Rad, Hercules, CA, USA), with the PCR mixture added directly to these unpurified DNA suspensions. Interdelta sequences were amplified according to the procedure described by Gobbi *et al*. (2013) using δ-primers proposed by Ness *et al.*, (1993) and optimized by Legras and Karst, (2003). The amplification products were separated by electrophoresis on 1.5% (w/v) agarose gels at 75 V for 1 h in 0.5× TBE buffer. The gels were stained with ethidium bromide and visualised under UV light (Gel Doc 1000 transilluminator; Bio-Rad, Hercules, CA, USA), equipped with a photographic system.

2.5. Wine sensory evaluation

After about 6 months of aging, the wines produced by the industrial-scale fermentations were bottled into 750 mL cork-capped bottles. A formal evaluation was performed by eight trained panellists (i.e., oenologists, viticulturists, professional wine-makers), over two training tasting sessions, to determine the aroma profiles of the wines. The evaluation of these samples was carried out in randomised complete blocks, with each treatment replicate sampled twice by each panellist. The sensory characteristics were quantified on a 1-9 scale using a series of descriptors that are normally used for evaluation of Sangiovese wines. The score of every sensory descriptor was measured using a 1-9 point scale, and normalised before the statistical analyses.

2.6. Statistical analysis

Tests of statistical significance for the chemical and sensory data were carried out by one-way analysis of variance using the SuperANOVA software (version 1.1, for Macintosh OS 9.1). The significant differences among these data were determined using Duncan testing, and they were considered significant for $P<0.05$.

3. Results and discussion

3.1. Laboratory-scale fermentation

Figure 1 shows the fermentation kinetics of the pure and mixed fermentations that were carried out at the laboratory scale, to evaluate the inoculation modality of mixed fermentations according to the yeast interactions. Differences in the fermentation kinetics among the various mixed fermentations were evident. In particular, the fermentation kinetics appeared to be driven by the presence of *S. cerevisiae*. Indeed, the longer the time when *Z. florentina* acted in the absence of *Saccharomyces*, the lower the fermentation kinetics. This was supported by the following observations: (i) the lower fermentation kinetics of the trials inoculated with *S. cerevisiae* after 48 h, compared to *S. cerevisiae* inoculation at 24 h; (ii) the lowest fermentation kinetics when *Z. florentina* was in pure culture; and, in contrast, (iii) the highest fermentation kinetics when *S. cerevisiae* was in pure culture.

For the growth kinetics, the *S. cerevisiae* strain showed similar patterns in the pure *S. cerevisiae* fermentation (Fig. 2a) as in the co-inoculum fermentation (Fig. 2c). Both of the fermentations reached a concentration of about 3.2×10^7 cell/mL at 4 days after inoculation. On the contrary, in the sequential fermentations, the presence of *Z. florentina* resulted in slower growth of *S. cerevisiae*, (Fig. 2d, e). The lower growth of *S. cerevisiae* did not appear to be related to nitrogen competition. Indeed, during the first days of fermentation, the residual nitrogen (as ammonium and available amino nitrogen) in the sequential mixed fermentations was higher than in both the pure *S. cerevisiae* culture and the co-inoculum fermentations. In agreement with this, fermentation of the pure *Z. florentina* culture (Fig. 2b) compared to that of the pure *S. cerevisiae* culture (Fig. 2a) showed lower nitrogen consumption during the first days of fermentation, probably also due to the higher initial *Z. florentina* cell concentration with respect to that of *S. cerevisiae*. Moreover, in the pure *Z. florentina* culture, there was a significant increase in the available amino nitrogen that started after the first week of fermentation. This increase might be the consequence of yeast

autolysis (Andorrà *et al*., 2010) or be related to the proteolytic activity of *Z. florentina*, even if this strain did not show protease activity in agar plate tests during the preliminary laboratory screening (Domizio *et al.*, 2011a). The lower growth of *S. cerevisiae* in sequential fermentation might be due to the competition for other nutritional factors or to the production of some inhibitory compounds by the *Z. florentina* strain.

The growth of *Z. florentina* appeared to be clearly influenced by the presence of *S. cerevisiae*. Indeed, the *Z. florentina* cell concentrations declined according to the *S. cerevisiae* inoculation time. In particular, when *S. cerevisiae* was co-inoculated, the *Z. florentina* cell concentration started to decline after the second day of fermentation and was not detectable after 10 days. In contrast, in both of the sequential fermentations *Z. florentina* cell concentration started to decline after seven days of fermentation, but at day 10 it was still present at 10^6 cell/mL and 10^7 cell/mL (sequential fermentation at 24 h and 48 h, respectively).

The analytical profiles of the wines obtained from these pure and mixed fermentations at 18 days are reported in Table 1. Similar final ethanol concentrations were present in the pure *S. cerevisiae* culture and the mixed fermentations with *Z. florentina*. In contrast, the residual sugar was significantly different in the mixed 48 h fermentations (19.50 g/L) and in the fermentation of the pure *Z. florentina* culture (87.5 g/L). This behaviour highlights the low fermentation capacity of *Z. florentina*. There were also slightly lower ethanol concentrations in the co-inoculation fermentations with respect to the pure *S. cerevisiae* culture, and although these differences did not reach statistical significance, they might be associated with the lower growth of *S. cerevisiae*, as already seen (Fig 2c). This is also in agreement with the lower final conversion ratio for the co-inoculum and the pure *S. cerevisiae* culture, evaluated as the ethanol (% volume)/fermented sugars (% weight) ratio at the end of the alcoholic fermentation (0.5745 *vs* 0.5962, respectively). This behaviour might represent a natural way to decrease the ethanol concentration in wine, which would meet the new market requests for wine with lower ethanol concentrations. Indeed, this approach has already been

positively evaluated using other non-*Saccharomyces* yeast in mixed fermentations (Contreras *et al.*, 2014, 2015; Fleet, 2008; Gobbi *et al.*, 2014; Pickering, 2000; Quirós *et al.*, 2014).

For the volatile acidity, the pure *Z. florentina* fermentation and the co-inoculation fermentation showed the lowest values compared with all of the other fermentations.

The glycerol concentration was similar in all of the trials, with the exception of the pure *Z. florentina* fermentation, which was significantly lower (5.39 g/L); this is in agreement with our previous findings (Domizio *et al.*, 2011a). For the polysaccharides concentrations, the pure *Z. florentina* culture was characterised by high polysaccharides release, which was about double that of the control pure *S. cerevisiae* fermentation, as also in agreement with our previous findings (Domizio *et al*. 2011a; 2014). In the mixed fermentations, the concentration of polysaccharides appears to be correlated to the abundance of *Z. florentina*, which suggests an additive effect on the production of this metabolites. In this context. Domizio *et al.*, (2014) found that this strain *of Z. florentina* release high quantity of polysaccharides starting from the onset of the alcoholic fermentation. These compounds originate from the cell wall and therefore essentially mannoproteins. These last one have been recognized to have many positive oenological properties such as improving mouth-feel and fullness (Vidal *et al.*, 2004), decreasing astringency (Quijada-Morín *et al.*, 2014), adding complexity and aromatic persistence (Chalier *et al.*, 2007), increasing sweetness and roundness (Guadalupe *et al.*, 2007), and reducing protein and tartrate instability (Gerbaud *et al.*, 1997; Gonzalez-Ramos *et al.*, 2008).

Among the volatile compounds (Table 1), the acetaldehyde concentrations were low in all of the trials. For the other volatile compounds, the amylic alcohols (i.e., 2-methyl-1-butanol,3-methyl-1-butanol) had low concentrations in all of the trials, and considering the sum of these two alcohols, this was lower in the pure *Z. florentina* culture (94 mg/L) than the pure *S. cerevisiae* culture (195 mg/L). In particular, there were significant differences among the trials for 3-methyl-1-butanol, but not for 2-methyl-1-butanol. These different production levels influenced the relevant concentrations in the mixed fermentations (which when combined ranged from 134-160 mg/L), which suggests an

additive effect due to the low concentrations produced by the pure *Z. florentina* culture fermentations. Moreover, in all of the mixed fermentations, there were significantly increased concentrations of 2-phenylethanol (32-40 mg/L) (which gives a rose-like flavour to wine) compared to both of the pure fermentations (22-24 mg/L). This would suggest a clear synergistic effect that was derived from the interactions between these two yeast, confirming the findings of our previous study (Domizio *et al.*, 2011a).

Among the acetate esters, ethyl acetate was the main ester produced. This compound contributes to the fruity aroma, although depending on the sensory threshold of the wine, at high concentrations (>100-150 mg/L) it is responsible for a solvent-like aroma. There was a significant reduction in the ethyl acetate concentrations in all of the trials compared with the control pure *S. cerevisiae* fermentation. As acetate ester production during alcoholic fermentation is strain dependent and has been shown to also be dependent on the balance of ester synthesis by alcohol acetyl-transferases and ester hydrolysis by ester-hydrolases (Fukuda *et al.,* 1998; Inoue *et al.,* 1997), these data suggest a metabolic interactions between these two yeast that promotes an interesting reduction in ethyl acetate in these wines.

The other two acetates analysed, isoamyl acetate (with a banana-like aroma) and phenyl ethyl acetate (with a fruity and flowery flavour) were present in the wines at concentrations that reflected the additive contributions from the two inoculated yeast, *S. cerevisiae* and *Z. florentina*. In particular, the higher production of isoamyl acetate by *S. cerevisiae* influenced the isoamyl acetate concentration in the wine that was dependent on the presence of *S. cerevisiae* in the mixed fermentations. The same behaviour was seen for the production of phenylethyl acetate by *Z. florentina*.

For the other ethyl esters, a significant reduction in ethyl exanoate was observed in all of the mixed fermentations, as compared with the pure *S. cerevisiae* fermentation, which reflected the lower production of ethyl exanoate by *Z. florentina*, as shown in particular by the pure *Z. florentina* fermentation. On the contrary, although ethyl decanoate was highly expressed by *Z florentina* in its

pure fermentation, there were significantly lower, and comparable, concentrations of ethyl decanoate in all of the other fermentations.

3.2. Winery-scale fermentation

After the preliminary laboratory trials carried out to evaluate the influence of the inoculation modalities on the interactions between the two inoculated strains and on the aromatic profiles of the final wines, winery scale trials were performed with *Z. florentina*/*S. cerevisiae* mixed culture using Sangiovese red grape variety (fermentation with grape skin). Before processing at the winery scale, the grapes were kept at low temperature (see Materials and methods), and as a consequence, the temperature of the must was about 20 °C at inoculation, where it then remained for the following 2 days. This will be why at the beginning of the fermentation the sugar consumption (evaluated by density evolution) was low despite of the high cell concentrations (>10^6 cell/mL) (Fig. 3). However, after 4 days of fermentation, the *S. cerevisiae* strain reached higher concentrations (about half a logarithmic order) in the control (Fig. 3a) with respect to the co-inoculation fermentations (Fig. 3b), which confirmed what is reported for the previous fermentations at laboratory scale, despite of the different inoculation modalities (fresh and ADY form). The sugar consumption in the 48 h sequential fermentations (Fig. 3c) was lower than the control and co-culture fermentations. The delayed inoculation after 48 h of *Saccharomyces* will have allowed the *Z. florentina* yeast, as well as any other non-*Saccharomyces* yeast present, to survive longer with respect to the co-inoculations. As seen above, the lower growth of *S. cerevisiae* in the sequential fermentation, and in turn, the lower sugar consumption seen in the relevant fermentations, might have been due to competition for nutritional factors or to production of some inhibitory compounds by the *Z. florentina* strain.

In terms of dominance over the indigenous *S. cerevisiae* strains during the industrial fermentation, six different interdelta PCR patterns have been observed (data not shown). However, the *S. cerevisiae* EC1118 starter strain showed the same pattern as biotype I, which was the

dominant biotype in the control fermentation (78.4%), in the co-inoculation (84.4%).) and in the sequential 48-h delayed inoculation (74.3%). This indicated the high dominance of the *S. cerevisiae* EC1118 starter strain over the indigenous *S. cerevisiae* strains, excluding their significant contributions and, at the same time, the correlation between the chemical and sensorial profiles of the inoculated yeast strain.

The sequential cultures showed more heterogeneous *S. cerevisiae* populations, with five different biotypes, while the pure cultures and the co-cultures showed only two and three biotypes, respectively.

Unlike the laboratory scale fermentations, all winery scale trials showed comparable ethanol concentration (about 12.5%). Moreover, a significantly higher glycerol concentration was observed in the sequential fermentations (around 25%) with respect to the control and co-culture fermentations (Table 2). Instead, despite the higher concentration of polysaccharides in the winery fermentation trials if compared with laboratory fermentation trials, no differences were observed among them. Indeed, in the laboratory-scale fermentations, there were significantly higher polysaccharide concentrations in the mixed fermentations if compared with *S. cerevisiae* control fermentation (Table 1), reflecting the additive contributions from *Z. florentina*. On the contrary, the presence of substances deriving from the red grape skin, such as fatty acids and sterols, had probably influenced differently the polysaccharide release by the *Z. florentina* strains in the winery fermentation trials. Moreover, the higher amount of polysaccharides observed in *S. cerevisiae* fermentation control trials performed at winery scale, in comparison with that observed at laboratory scale, might be related to the polysaccharide release by other non-*Saccharomyces* yeasts present at high concentrations since the beginning of alcoholic fermentation. In agreement, Domizio *et al.* (2014) reported that most of the non-*Saccharomyces* yeasts are characterized by the capacity to release into a synthetic grape juice higher quantity of polysaccharide than *Saccharomyces* yeasts.

The data relevant to the volatile compounds (Table 2) showed a significant decrease in the acetaldehyde concentration in the sequential 48-h fermentation with respect to the relevant *S.*

cerevisiae control (-22.3%) and the co-culture as well (-18.6%). Of note, the acetaldehyde concentrations were low in all of these winery fermentations. With respect to their relevant *S. cerevisiae* control, the hexanol (green/grass aroma) concentrations were significantly lower in the co-culture (-23.9%) and sequential (-19.6%) fermentations.

A different profile of the ester compounds present in the wines obtained at laboratory and industrial scale fermentation was also observed. For instance, an increase of the ethyl acetate concentration in all the winery fermentation compared with the laboratory scale fermentation was detected. However, it ranged from 79 mg/L and 108 mg/L, below the threshold (approximately 150 mg/L) above which it causes off aroma in red wine. On the other hand, the presence at high concentrations of wild non-*Saccharomyces* yeasts at the beginning of the fermentation could have determined an increase of this spoilage metabolite, and their persistence for a longer time on the sequential fermentation trial can explain the reduction of the ethyl acetate only in the co-inoculated trials compared with the *S. cerevisiae* control trial. Also some ester compounds, such as isoamyl acetate, ethyl hexanoate and ethyl butyrate present in lower concentration in the mixed fermentation *versus* the *S. cerevisiae* control fermentation at the laboratory scale fermentation, were instead increased in all of the wines produced by the mixed culture fermentations at the winery level. On the contrary, the production of high levels of 2-phenylethanol by *Z. florentina* observed in mixed fermentations at laboratory scale, including those performed previously (Domizio *et al.*, 2011a), was not exhibited during the industrial fermentations, with the red grape juice. As 2-phenylethanol contributes greatly to the floral note of wine, the use of mixed fermentation with *Z. florentina* might permit this desirable character to be better expressed in white wine, rather than in red wine.

The organoleptic evaluation of the wines 6 months after bottling (Fig. 4) showed some significant differences according to Duncan's test ($p \leq 0.05$). In particular, the floral character was higher perceived in both of the wines obtained from the mixed fermentations (i.e., co-inoculation, sequential inoculation), with respect to the relevant controls. Moreover, the wine obtained from the sequential fermentations was less astringent and at the same time showed a lower fruity intensity,

with respect to both of the other fermentations (i.e., control, co-inoculation). The minor astringency might be linked to the higher glycerol concentration in the sequential fermentation, while the lower fruity intensity might be related to some extent to the slightly higher volatile acidity and ethyl acetate content. The differences in the organoleptic evaluation observed at the end of fermentation are not always fully justified by the analytical profile of wines. On the other hand, it is well known that aromatic compounds during wine maturation can modify and deeply interact and, depending on their different concentrations, might result in different sensorial perception (Escudero *et al*., 2007).

4. Conclusions

The data from the present study indicate that various parameters influenced the presence of the two inoculated strains *S. cerevisiae* and *Z. florentina* during the alcoholic fermentation and consequently the final quality of the wines obtained. An increase of polysaccharides and 2-phenylethanol content and a reduction of volatile acidity was observed in the trials carried out at laboratory scale to evaluate the influence of the inoculation modalities (e.g., co-inoculation, sequential fermentation) on the analytical and aromatic profiles of the final wines. On the contrary, at the industrial scale using red grape juice the higher content of polysaccharides and 2-phenylethanol was no longer evident, and these trials were characterized by a significant higher glycerol production (just in the sequential culture), a significant increase of isoamyl acetate, ethyl hexanoate and ethyl butyrate, all responsible compounds of the fruit aroma, and a significant decrease on the hexanol concentration. However, the differences in the hexanol content were probably too low to determine variation on green/grass aroma perception among the relevant wines. The sensorial analysis of the wine obtained by mixed fermentations with *S. cerevisiae* and *Z. florentina* at the winery scale suggests the possibility of obtaining higher floral notes compared with the wines obtained by inoculation with only *S. cerevisiae*. However, fruity notes were perceived only in the co-inoculated wine and at the same level as in the control (*S. cerevisiae* inoculated). On

the other hand, the lower astringency perception, as observed in the sequential fermentation, represents an interesting character that would be exploitable for commercial red wine production. Further studies are required to confirm the influence of white or red winemaking on this specific yeast-yeast interactions and consequently on the relevant final analytical wine composition. Indeed, compounds such mannoproteins and 2-phenylethanol, specifically present through all the fermentation trials so far performed with the association *Z. florentina*-*S. cerevisiae* using white grape juice, let us speculate that the use of mixed fermentation with *Z. florentina* might permit these desirable characters to be better expressed in white wine, rather than in red wine.

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FIGURE CAPTIONS

Figure 1. Fermentation kinetics (as CO_2 production) of the micro-fermentations. Pure cultures of *S. cerevisiae* (-□-) and *Z. florentina* (-x-), their co-culture (-■-), and their sequential cultures with *S. cerevisiae* at 24 h (-▲-) and 48 h (-●-).

Figure 2. Viable cells evolution of *S. cerevisiae* (—●—) and *Z. florentina* (-•-●-•-) and kinetics of ammonia (black columns) and free amino nitrogen consumption (white columns) during fermentations with different inoculation treatments. These include pure culture fermentations of *S. cerevisiae* (a) and of *Z. florentina* (b), mixed culture fermentations at different inoculum times of *S. cerevisiae*, as co-culture with *Z. florentina* (c) and mixed culture fermentation with *S. cerevisiae* addition delayed 24 h (d) and 48 h (e).

Figure 3. Density evolution (columns) and viable cells evolution (lines) during industrial fermentation. These include control culture fermentation (a), co-culture fermentation (b), and sequential fermentations with *S. cerevisiae* addition after 48h (c). *S. cerevisiae* (—□—), *Z. florentina* (-•-▲-•-), and indigenous non-*Saccharomyces* yeast (--■--).

Figure 4. Organoleptic evaluation of the wines produced on an industrial scale using the different inoculation treatments, after 6 months aging. The data represent the means of two complete replicates based on nine-point scales. Within each organoleptic descriptor: a, mean significantly different from the control wine, according to Duncan's test ($p \leq 0.05$); *, emphasizes the descriptors under which at least one wine was perceived differently from the others.

Control, pure *S. cerevisiae* fermentations (••▲••), and co-culture fermentations (- - ■- -) and sequential-culture fermentations (—♦—) of *S. cerevisiae* and *Z. florentina* respectively.

Figure 1

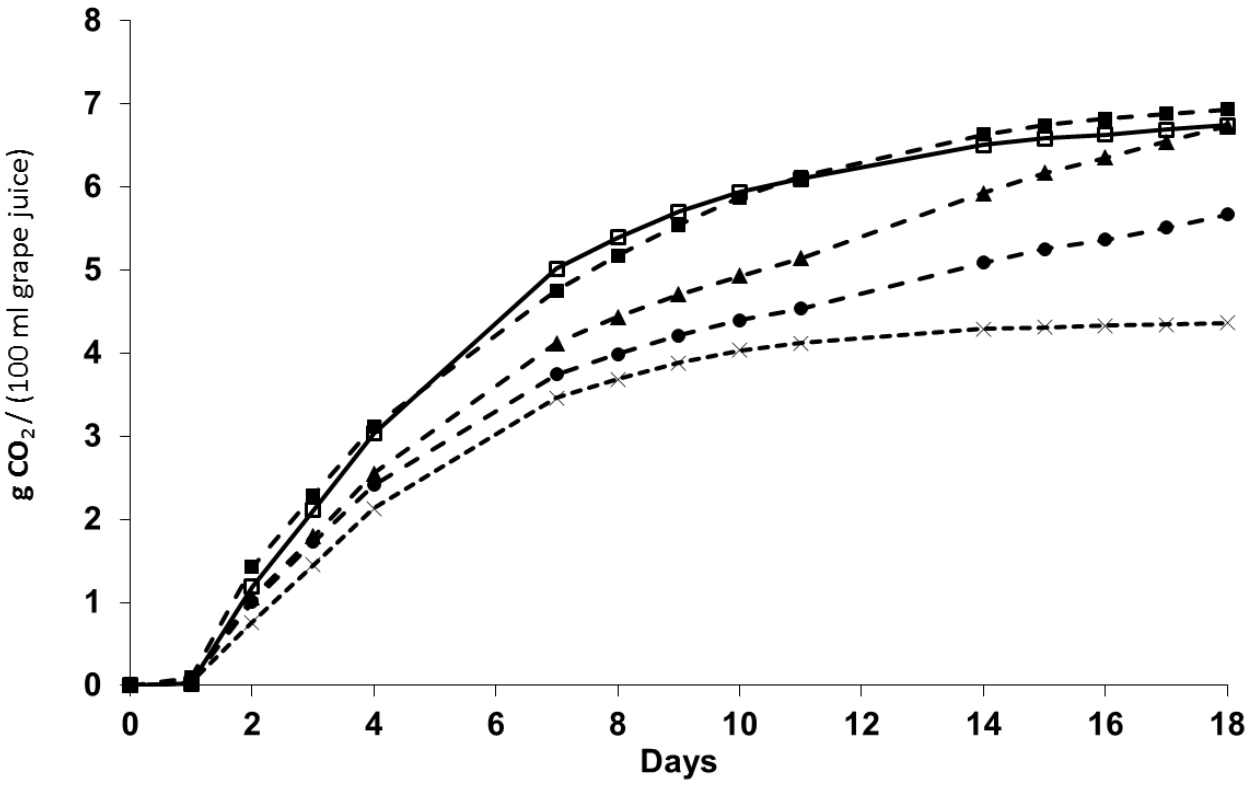

Figure 2

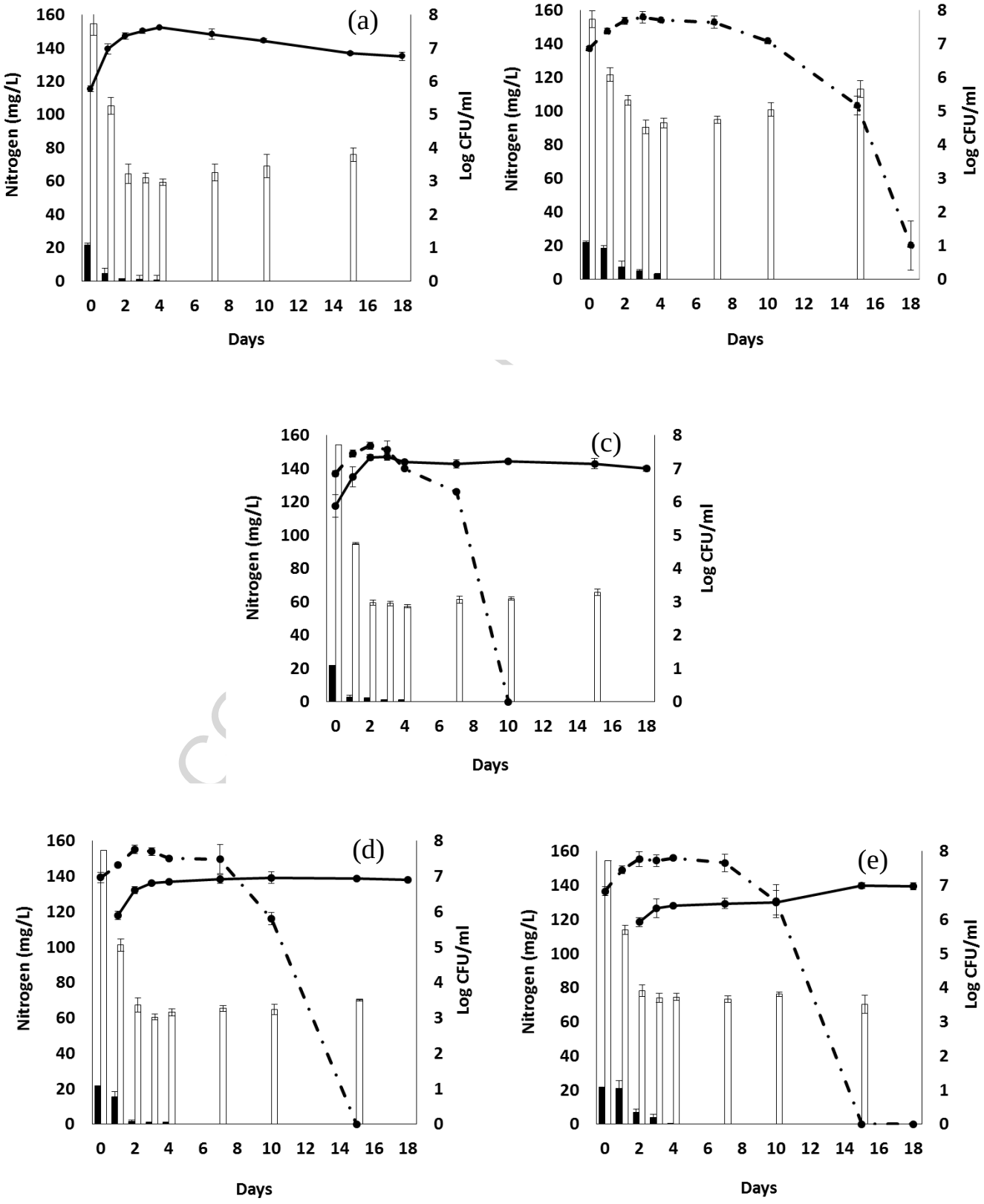

Figure 3

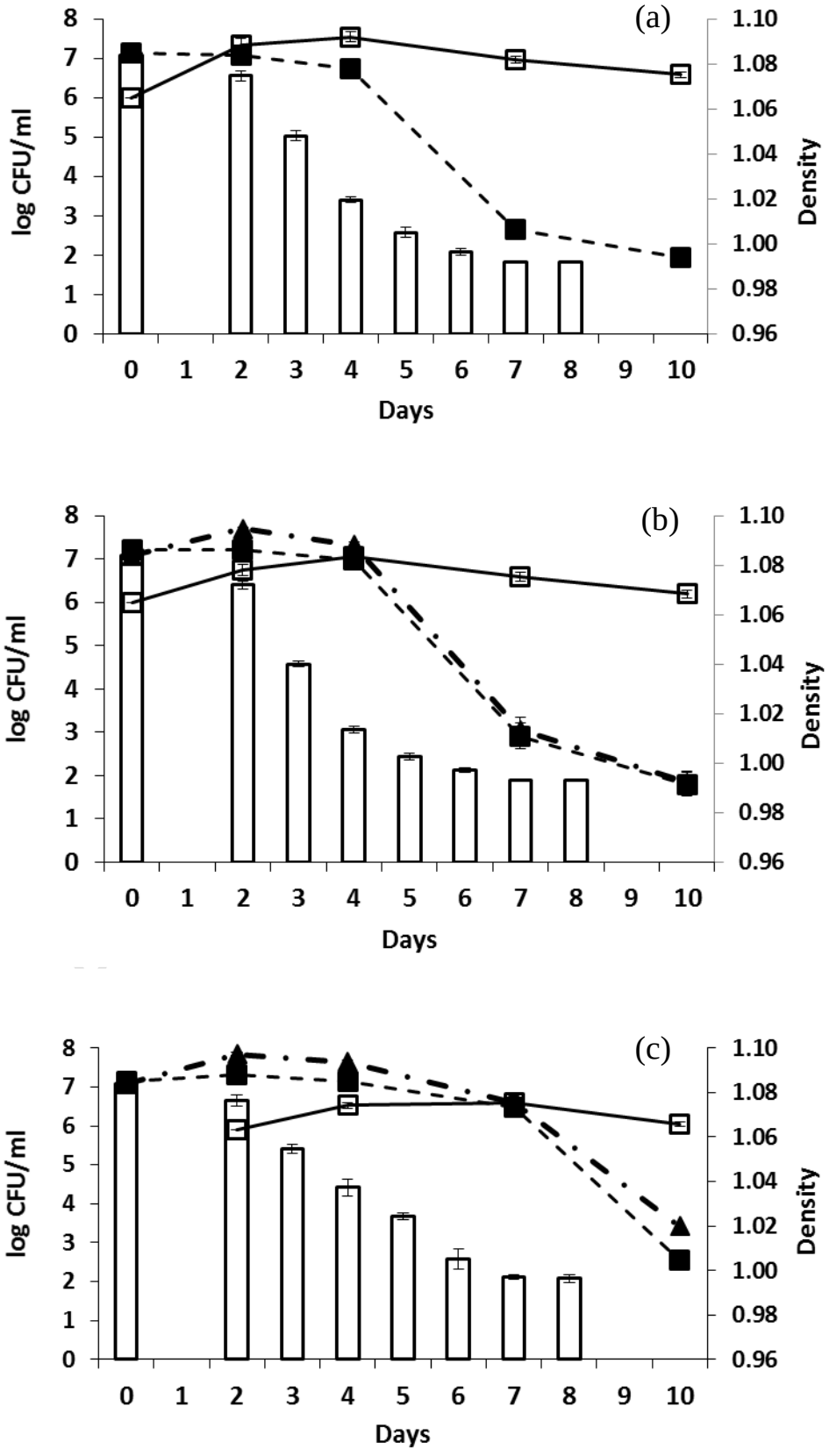

Figure 4

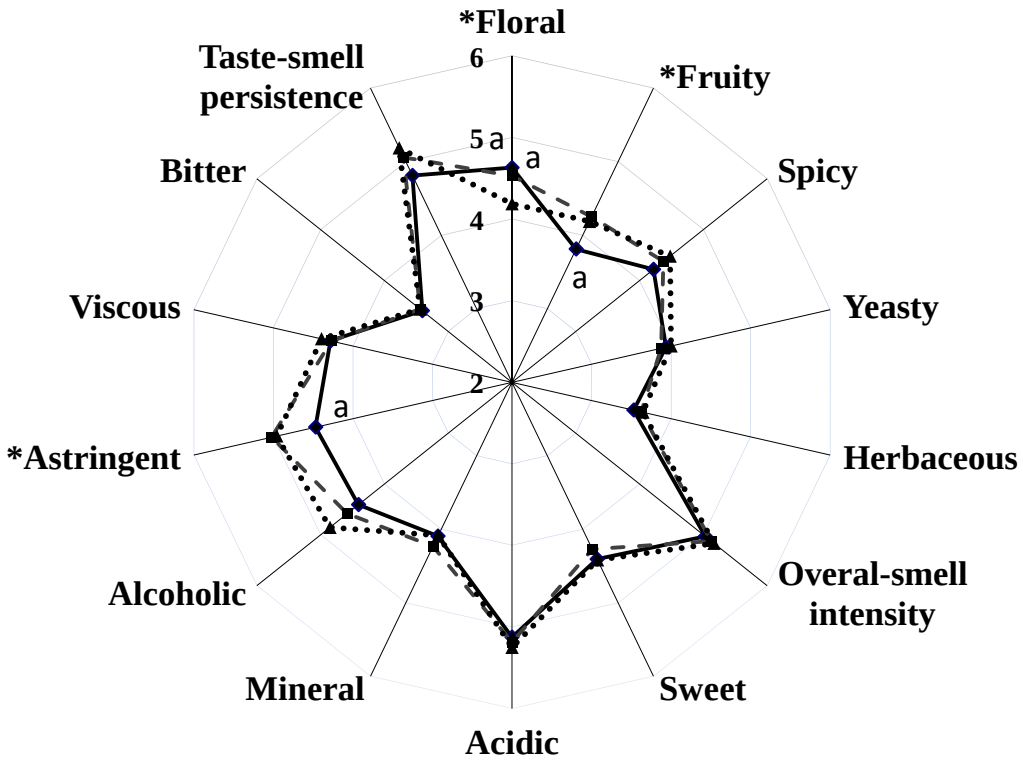

Table 1. Influence of mode of inoculation on the analytical profiles of the microfermentations carried out at 25 °C

Analysis	**Inoculation mode**				
	Pure cultures		**Co-culture**	**Sequential cultures**	
	S. cerevisiae	***Z. florentina***	***Z. florentina* + *S. cerevisiae***	***Z. florentina* + *S. cerevisiae* (24 h)**	***Z. florentina* + *S. cerevisiae* (48 h)**
Ethanol (%; v/v)	13.04±0.29^{b}	8.40±0.16^{a}	12.68±0.08^{b}	12.53±0.20^{b}	12.47±0.15^{b}
Residual sugars (g/L)	5.93±0.18^{a}	87.50±1.02^{c}	3.30±0.05^{a}	12.30±0.12^{ab}	19.50±0.11^{b}
pH	3.53±0.07^{ab}	3.43±0.03^{a}	3.57±0.02^{b}	3.42±0.04^{a}	3.40±0.03^{a}
Total acidity (g/L)	7.26±0.35^{ab}	6.64±0.14^{a}	6.73±0.16^{a}	7.88±0.63^{b}	7.65±0.15^{b}
Volatile acidity (g/L)	0.44±0.05^{b}	0.29±0.03^{a}	0.32±0.04^{a}	0.42±0.08^{b}	0.43±0.04^{b}
D,L-lactic acid (g/L)	0.16±0.05^{a}	0.27±0.11^{a}	0.21±0.05^{a}	0.24±0.10^{a}	0.18±0.06^{a}
Glycerol (g/L)	7.04±0.48^{b}	5.39±0.09^{a}	6.95±0.12^{b}	6.88±0.23^{b}	6.32±0.25^{b}
Δ-Polysaccharides (mg/L)	100.3±4.3^{a}	218.6±5.7^{c}	135.2±3.5^{b}	156.1±2.6^{b}	157.3±2.9^{b}
Volatile compounds (mg/L)					
Acetaldehyde	18.42±3.37^{a}	16.18±5.01^{a}	14.68±5.06^{a}	15.69±4.71^{a}	15.88±5.31^{a}
1-Propanol	27.44±5.09^{ab}	28.66±1.96^{ab}	20.40±2.11^{a}	35.00 ±3.26^{b}	34.09±4.15^{b}
2-Methyl-1-propanol	108.66±8.33^{c}	17.50±3.26^{a}	44.68±9.81^{b}	31.40±5.85^{ab}	22.95±1.85^{ab}
2-Methyl-1-butanol	24.02±3.01^{a}	36.85±3.36^{a}	33.18±2.75^{a}	35.32±7.23^{a}	36.97±1.69^{a}
3-Methyl-1-butanol	171.11±4.32^{c}	57.82±2.34a	126.74±6.77^{bc}	98.96±9.84^{b}	105.95±6.47^{b}
Hexanol	0.90±0.05^{a}	1.55±0.04^{b}	1.15±0.10^{ab}	1.07±0.08^{ab}	1.19±0.11^{ab}

2-Phenylethanol	22.23±1.70[a]	23.53±1.80[a]	39.97±0.92[b]	32.30±0.84[b]	32.07±0.83[b]
Ethyl acetate	19.58±3.13[b]	12.09±2.61[a]	10.67±4.81[a]	8.85±4.32[a]	7.15±5.12[a]
Isoamyl acetate	0.77±0.19[c]	0.01±0.01[a]	0.46±0.01[b]	0.27±0.10[ab]	0.19±0.03[ab]
Phenylethyl acetate	0.11±0.02[a]	0.78±0.10[b]	0.54±0.10[b]	0.98±0.43[b]	0.77±0.23[b]
Ethyl lactate	<0,01	<0,01	<0,01	<0,01	<0,01
Ethyl hexanoate	0.44±0.09[c]	0.03±0.00[a]	0.36±0.04[b]	0.22±0.03[ab]	0.18±0.02[ab]
Ethyl octanoate	0.44±0.05[b]	0.00±0.00[a]	0.16±0.05[ab]	0.03±0.01[a]	0.03±0.01[a]
Ethyl decanoate	0.10±0.05[a]	0.72±0.07[b]	0.15±0.02[a]	0.14±0.07[a]	0.11±0.02[a]

Data are means ±standard deviations of three separate replicates.

Values with different superscript letters ([a, b, c]) within each row are significantly different, according to Duncan test (P≤0.05).

Table 2. Influence of mode of inoculation on the analytical profiles of the wines at the end of the industrial fermentations

Analysis	**Control culture** ***S. cerevisiae***	**Co-culture** ***Z. florentina*** **+ *S. cerevisiae***	**Sequential culture** ***Z. florentina*** **+ *S. cerevisiae* (48 h)**
Ethanol (%; v/v)	12.57±0.15^{a}	12.49±0.18^{a}	12.36±0.20^{a}
pH	3.35±0.01^{a}	3.35±0.02^{a}	3.37±0.02^{a}
Total acidity (g/L)	7.09±0.24^{a}	7.51±0.11^{b}	7.72±0.18^{b}
Volatile acidity (g/L)	0.31±0.02^{a}	0.31±0.02^{a}	0.38±0.02^{b}
Malic acid (g/L)	2.15±0.11^{a}	2.10±0.15^{a}	2.05±0.07^{a}
D.L-lactic acid (g/L)	0.22±0.01^{a}	0.19±0.02^{a}	0.20±0.02^{a}
Glycerol (g/L)	9.08±0.15^{a}	9.42±0.27^{a}	11.59±0.36^{b}
Δ-Polysaccharides (mg/L)	165.6±8.0^{a}	179.7±18.4^{a}	172.9±16.6^{a}
Volatile compounds (mg/L)			
Acetaldehyde	47.18±5.14^{b}	45.05±8.83^{b}	36.67±4.55^{a}
Methanol	76.41±3.40^{b}	71.73±1.73^{ab}	67.62±1.44^{a}
1-Propanol	75.87±6.75^{b}	61.14±6.35^{ab}	52.51±3.53^{a}
2-Methyl-1-propanol	54.03±3.26^{a}	60.26±1.01^{ab}	73.76±1.57^{b}
2-Methyl-1-butanol	33.18±1.30^{a}	32.95±2.11^{a}	33.65±2.90^{a}
3-Methyl-1-butanol	134.90±3.98^{a}	136.19±4.82^{a}	136.93±3.86^{a}
Hexanol	0.46±0.01^{b}	0.35±0.02^{a}	0.37±0.01^{a}
2-Phenylethanol	21.53±0.08^{a}	23.69±1.50^{a}	22.74±2.55^{a}
Ethyl acetate	95.10±10.84^{ab}	79.80±8.06^{a}	108.20±2.94^{b}
Isoamyl acetate	0.17±0.01^{a}	0.28±0.02^{ab}	0.55±0.17^{c}
Phenylethyl acetate	0.05±0.01^{a}	0.04±0.01^{a}	0.04±0.00^{a}
Ethyl lactate	1.16±0.14^{ab}	1.13±0.05^{a}	1.20±0.22^{b}
Ethyl butirrate	0.09±0.02^{a}	0.34±0.02^{bc}	0.44±0.02^{c}
Ethyl hexanoate	0.07±0.01^{a}	0.38±0.02^{c}	0.26±0.03^{b}
Ethyl octanoate	0.25±0.05^{b}	0.18±0.02^{a}	0.18±0.08^{a}
Decanoic acid	0.18±0.01^{a}	0.18±0.02^{a}	0.19±0.01^{a}

Residual sugars in all the fermentation were <1 g/L

Data are means ±standard deviations of three separate replicates.

Values with different superscript letters ($^{a.\ b.\ c}$) within each row are significantly different, according to Duncan test (P≤0.05)

HIGHLIGHTS

- By mixed fermentation *Zygotorulaspora florentina* positively affect wine quality.
- Increasing of glycerol and polysaccharides in mixed fermentation was observed
- Decreasing of acetaldehyde in mixed fermentation *Z.florentina/S.cerevisiae* was observed
- Wines with higher floral notes and lower astringency were obtained