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ABSTRACT

The use of mixed fermentations with *Starmerella bacillaris* and *Saccharomyces cerevisiae* is gaining attention in recent years due to their ability to modulate the metabolites production of enological interest. In the present study, four of the most popular planted red grape varieties (Cabernet sauvignon, Merlot, Pinot noir and Shiraz) were fermented using the aforementioned species and two different inoculation protocols (inoculation of *S. cerevisiae* after 24 and 48 h from the *Starm. bacillaris* inoculation), in order to evaluate their impact on the volatile composition and chromatic characteristics of wines. Analysis from chemical composition showed that titratable acidity and glycerol content exhibited marked differences among wines after fermentation. For volatile compounds, mixed fermented wines using an inoculation delay of 48 h led to reduction of volatile compounds (mainly esters). A shorter 24 h delay produced wines with higher values of color intensity than pure fermented wines. The differences observed between the inoculation protocols can be explained by the growth dynamics of both species during fermentation. These findings suggest that mixed fermentations posed a great potential in reducing metabolites which are considered negative for wine quality (mainly ethyl acetate and volatile fatty acids) and with an improvement of the chromatic profile of the wines.

Keywords: non-*Saccharomyces*, *Starmerella bacillaris*, mixed fermentations, chromatic profile, aroma profile

1. Introduction

Alcoholic fermentation is the transformation of grape sugars, mainly into ethanol and carbon dioxide. This process usually is carried out by successional evolution of indigenous or inoculated yeast species. It is recognized that yeast ecology during alcoholic fermentation is far more complex than what was believed until recently (Bokulich, Swadener, Sakamoto, Mills, & Bisson, 2015; Ciani, Comitini, Manazzu, & Domizio, 2010). Various physicochemical changes are occurring to turn grape juice into wine (Fleet, 2008). Besides ethanol, several metabolites are transformed or synthesized by yeasts, including glycerol, higher alcohols, and esters (Moreno-Arribas & Polo, 2009).

A large diversity of yeast species are involved in winemaking. Generally, spontaneous fermentation starts by the simultaneous growth of various non-*Saccharomyces* species, which are generally characterized by low fermentative power (Fleet, 2008). The growth of many of them is generally limited to the first days of fermentation, after which they die off. At this time, more strongly fermentative and more ethanol tolerant non-S*accharomyces* (mainly *Hanseniaspora* - anaform *Kloeckera* -, *Metschnikowia*, *Torulaspora*, *Candida* and *Kluyveromyces*) together with *Saccharomyces* spp. (predominantly *Saccharomyces cerevisiae*) take over the fermentation (Cravero et al., 2016; Varela & Borneman, 2016; Varela, 2016). This successional evolution of strains and species during fermentation is largely determined by their different sensibilities to the increasing levels of ethanol, temperature, dissolved oxygen content, and killer factors (Ciani & Comitini, 2015; Ciani, Capece, Comitini, Canonico, Siesto, & Romano, 2016; Albergaria & Arneborg, 2016). This, in turn, will have an impact on yeast biodiversity and thus on wine quality, as it can be possibly affected by pleasant or unpleasant attributes (Ciani et al., 2010; Jolly, Varela, & Pretorius, 2014). The adoption of fermentation practices, which limit the production of undesirable metabolites by favoring the growth of desirable yeasts, is fundamental in order to enhance wine quality.

Non-*Saccharomyces* yeasts possess higher intraspecific physiological diversity than *S. cerevisiae* strains, with potential negative and positive contribution to the chemical and sensorial profile of wines, through the production of metabolites of oenological interest (Ciani et al., 2010; Jolly et al., 2014). Among the latter the high production of glycerol, mannoproteins, organic acids that contribute to the total acidity, volatile compounds with pleasant notes and low production of acetic acid and ethanol, promoted their use in winemaking (Ciani et al., 2016; Mate & Maicas, 2017; Padilla, Gil, & Manzanares, 2016).

However, few non-*Saccharomyces* strains are able to consume high sugar levels from the must and therefore their use in combination with selected *S. cerevisiae* strains is necessary in order to complete the fermentation and take advantage of the unique characteristics of the first (Fleet, 2008). A successful mixed fermentation is considered when non-*Saccharomyces* yeasts could grow and achieve high levels of biomass before they die off. Therefore, the selection of suitable yeast strains in association with physicochemical parameters (temperature, sugar concentration, nitrogen availability and ethanol concentration) could be used to promote their growth and consequently their contribution to wine composition (Comitini, Capece, Ciani, & Romano, 2017; Fleet, 2003).

Among non-*Saccharomyces* yeasts, *Starmerella bacillaris* (synonym *Candida zemplinina*) has been described as a yeast with a positive effect on wine quality. Generally, strains of this species are known as high producers of glycerol, pyruvic acid and low producers of ethanol (Magyar, Nyitrai-Sárdy, Leskó, Pomázi, & Kállay, 2014; Mestre, Maturano, Combina, Mercado, Toro, & Vasquez, 2017; Rantsiou et al., 2017, Zara et al. 2014). However, contradictory results were observed for acetic acid production, indicating intraspecific variation (Englezos, Giacosa, Rantsiou, Rolle, & Cocolin, 2017). These phenotypic characteristics and its ability to tolerate relatively high concentrations of ethanol enable the use of this non-*Saccharomyces* yeast in mixed fermentations with selected *S. cerevisiae* strains. In the last years, several studies have made significant progresses in many aspects including the importance of inoculation density, timing, and combination of strains in the organoleptic properties of wines (Comitini et al., 2011, Englezos et al., 2016a, Sadoudi et al., 2012). However, several efforts must be undertaken in order to establish a link between an inoculation protocol and chemical composition of wines using the same couple of strains and fermentation conditions. Understanding the nature and origins of wine volatile metabolites may provide the potential to manipulate yeast ecology towards the production of wines with flavour, aroma, and chromatic characteristics desired by targeted consumer groups.

In the present study, we investigated the chemical composition, chromatic characteristics and volatile profiles of Cabernet sauvignon, Merlot, Pinot noir and Shiraz wines produced with mixed fermentations of *Starm. bacillaris* FC54 and *S. cerevisiae* Uvaferm BC® using an inoculation delay of 24 and 48 hours. Control fermentations with *S. cerevisiae* Uvaferm BC® were performed in parallel.

2. Materials and methods

2.1. Strains

Starm. bacillaris FC54 from the DISAFA collection (Department of Agricultural, Forest and Food Sciences, University of Turin, Italy) and *S. cerevisiae* Uvaferm BC® (Lallemand Inc., Montreal, Canada) were used. This couple of yeast strains were selected due to their ability to reduce the ethanol content of wines produced from musts with relatively high content of sugars (Englezos et al., 2016a). Both yeasts were routinely grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose, all from Biogenetics, Milan, Italy) and maintained on YPD plates (supplemented with 2% agar) at 4 °C.

2.2. Must preparation

Four red wine grape varieties (*Vitis vinifera* L.) cultivars namely Cabernet sauvignon, Merlot, Pinot noir and Shiraz were collected from the CNR-IPSP ampelographic collection of Grinzane Cavour (Cuneo province, north-west Italy, 44.651 N, 7.995 E). The harvest date of each grape variety was based on the degree of technological ripeness. Grapes of each variety were destemmed, crushed and the musts with the grape skins were heated at 60 °C for 1 h to promote the extraction of colour from the skins and deactivate indigenous yeast population (Boulton et al., 1996). After cooling down, the juice was separated from the skins using a stainless steel sieve and stored at 4 °C before fermentation. Pasteurization efficiency was checked by plating on Wallerstein laboratory nutrient (WLN) medium (Biogenetics). The composition of natural grape musts was adjusted to 250 ± 5 g/L of sugars and 180 ± 5 mg/L of yeast assimilable nitrogen (YAN) using the commercial product Fermaid O® (Lallemand Inc.) to provide a unified starting point for the yeasts. The chemical composition of the musts is reported in Table 1.

2.3. Fermentation trials

Three inoculation protocols were conducted for each grape variety: one pure fermentation with *S. cerevisiae* Uvaferm BC® and two mixed fermentations in which *S. cerevisiae* Uvaferm BC® was inoculated after 24 and 48 h after *Starm. bacillaris* FC54 inoculation. Thirty-six fermentations (4 grape varieties × 3 inoculation protocols × 3 replicates) were carried out each in a 1-L sterile glass bottle containing 800 mL of must under

semi-anaerobic conditions, using air-locks to maintain semi-anaerobic conditions during fermentation. Pure and mixed culture fermentations were inoculated with 5.0 x 10^6 cells/mL, which corresponds to a dose of 25 g/hL of active dry yeast (ADY) (Lallemand SAS, Toulouse, France), previously activated in a sterile glucose solution (5 %), incubated at 37 °C. After inoculation, the musts were incubated at 25 °C without agitation. The fermentation process was tracked by plate counting and chemical analysis described below. Fermentations were considered finished when the residual sugars were less than 2 g/L. Afterwards, the chemical composition, chromatic characteristics and volatile profiles of wines was analysed.

2.4. Microbiological analysis

Yeast growth dynamics during the fermentation process was determined by plate counts. Aliquots of one mililiter (1mL) were taken from each must during fermentation at days 0 (immediately after inoculation), 1, 2, 4, 7, and 10 (only for the mixed culture fermentation with 48 hours delay) and diluted appropriately in sterile Ringer's solution (Oxoid, Milan, Italy). One hundred microliter (100 µL) aliquots were plated onto WLN plates, which allows the visual differentiation of *Starm. bacillaris* and *S. cerevisiae* yeast species. Plates were incubated at 28 °C for 3-5 days before counting. In this medium, *Starm. bacillaris* forms flat, light to intense green colonies, while *S. cerevisiae* forms creamy white colonies, with light shades of green on the top facilitating the concurrent enumeration of both species during the fermentation process.

2.5. Must and wine analysis

Ethanol (% v/v), sugars, glycerol and organic acids (g/L) concentrations during and at the end of fermentation were determined by HPLC using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a HPX-87H column (Bio-Rad) following the chromatographic conditions proposed by Rolle et al. (2018). The official method OIV-MA-AS313-01:R2015 proposed by the International Organization of Vine and Wine (OIV, 2015) was applied to determine titratable acidity and the results are expressed in g/L as tartaric acid. pH was measured using an InoLab 730 pH meter (WTW, Weilheim, DE). Total YAN concentration was determined spectrophotometrically by using two enzymatic kits following the manufacturer's instructions (Megazyme International Ireland).

The production of fermentation-derived volatile compounds was assayed by Head

Space Solid Phase Micro-Extraction (HS-SPME) followed by Gas Chromatography-Mass Spectroscopy (GC-MS). Briefly, 5 mL of sample was placed in a 20 mL headspace glass vial, containing 2 g of NaCl and 200 μL of internal standard (prepared by adding 15.5 mg/L of 1-heptanol (analytical grade, 99.95%, Sigma, Milan, Italy) in a 10 % v/v ethanol solution). Afterwards, the vials were tightly sealed with 18-mm diameter screw caps with silicon septum (Supelco, Bellefonte, PA, USA) and shaken carefully to dissolve NaCl. Sample vials were place onto a Gerstel MPS2 XL (Gerstel, Baltimore, MD, USA) auto sampling device. The chromatographic conditions were as those reported by Englezos et al. (2018). Briefly, the program consisted of heating the vial at 40 °C for 10 min, inserting the fiber into the headspace of the sample vials for 20 min at 40 °C and desorbed in the GC inlet in splitless mode for 5 min at 250 °C, the ion source temperature was 150 ºC and interface was 280 ºC. Analyses were performed on an Agilent 7890C gas chromatograph (Little Falls, DE, USA) coupled to an Agilent 5975 mass selective detector and a DB-WAX capillary column (30 m x 0.25 mm inner diameter, 0.25 mm film thickness, J&W Scientific Inc., Folsom, CA, USA). The software used was Agilent G1702-90057 MSD ChemStation. The oven temperature was started at 40 °C, held for 5 min, increased to 200 °C at 2°C/min, held at that temperature for 10 min and increased to 220 °C at 5 °C/min. The carrier gas was Helium with a flow rate of 1 mL/min in constant flow mode. Mass spectra detection was carried out in total ion current mode (TIC mode) with a scan range of 33-330 m/z. The detection of the volatile compounds was carried by matching the retention time of each compound with either reported in the literature and in the online database (http://webbook.nist.gov/chemistry/) and pure standards, whenever available (2,3-butanediol isomers mixture, 2-methyl-1-propanol, 1-octanol, 2-phenylethanol, diethyl succinate, ethyl acetate, ethyl decanoate, ethyl dodecanoate, ethyl heptanoate, ethyl hexanoate, ethyl nonanoate, ethyl octanoate, ethyl phenylacetate, hexanol, hexanoic acid, hexyl acetate, linalool, methyl decanoate, octanoic acid and β-damascenone, all from Sigma). Concentration of each identified compound was calculated by a calibration with standard solutions analysed under the same conditions as the wine samples. Each replicate was analysed in duplicate.

The absorption spectrum of each sample was registered spectrophotometrically according by the OIV-MA-AS2-11:R2006 method (OIV, 2015), using an UV-1800 spectrophotometer (Shimazdu Corporation, Kyoto, Japan). The absorbance values were recorded at 5 nm intervals over the range of 380-780 nm wavelength using 2 mm path-length cuvettes, and the CIEL*a*b* coordinates were calculated. In the CIEL*a*b* color space, the chromatic coordinates are chroma or “saturation” (C*), clarity or lightness (L*), red/green

color (a*) (with +a* indicating red and –a* indicating green) and yellow/blue (b*) (with +b* indicating yellow and –b* indicating blue). The CIEL*a*b* color difference was calculated as: $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$ (Torchio, Rio Segade, Gerbi, Cagnasso, & Rolle, 2011).

2.7. Statistical analyses

Fermentation data were analysed using the IBM SPSS Statistics software package (version 19.0, IBM Corp., Armonk, NY, USA). The Tukey-b post hoc test for $p<0.05$ was used to establish significant differences by one-way Analysis of Variance (ANOVA). A Multifactorial ANOVA test was carried out to understand the effect of the two tested factors (variety and inoculation protocol) and to verify the existence of any interaction between them.

3. Results and discussion

3.1. Yeast growth during fermentation

The growth dynamics of viable cells in both pure and mixed culture fermentations are shown in Fig. 1. Both pure and mixed culture fermentations showed similar evolution patterns, independently of the grape variety used. For the pure culture fermentations *S. cerevisiae* Uvaferm BC® finished the alcoholic fermentation within 7 days and achieved a cell population of around 5.0 - 9.0 x 10^7 CFU/mL at the end of the exponential phase, which was maintained until the end of the process (Fig. 1, left panel).

In the mixed culture fermentations where *S. cerevisiae* Uvaferm BC® was sequentially inoculated after 24 h (Fig. 1, central panel) and 48 h (Fig.1, right panel) with respect to *Starm. bacillaris*, different evolution profiles were obtained, compared to pure culture fermentations. The inoculation of *Starm. bacillaris* affected *S. cerevisiae* (and vice versa) growth dynamics and cellular concentration in an inoculation delay dependent fashion. As shown in Fig. 1 (central panel), the early inoculation of *S. cerevisiae* (24 h delay) negatively affected the proliferation and dominance of *Starm. bacillaris* during fermentation, as the *S. cerevisiae* strain achieved a similar maximum cell concentration with respect to that obtained by the pure culture. On the other hand, in mixed fermentations with a 48 h delay, *Starm. bacillaris* growth showed a negative effect on *S. cerevisiae* growth. *Starm. bacillaris* dominated the fermentation process during the first 7 days and reached a maximum cellular

concentration of around 1.0 x 10^8 CFU/mL, which is almost 50 % higher than those of 24 h delay (around 5.0 x 10^7 CFU/mL). This increase in *Starm. bacillaris* viable cells led to a reduction in the number of viable cells of *S. cerevisiae* and almost 50% lower viable cell population was registered compared to pure culture fermentations with *S. cerevisiae*. This finding was in agreement with previous studies that demonstrated that the early growth of *Starm. bacillaris* negatively influenced the growth of *S. cerevisiae* (Englezos et al., 2016a, Sadoudi et al., 2012). Such negative effect may be ascribed to the enhanced competition of non-*Saccharomyces*, which was probably caused by completion of nutrients or cell-to-cell contact mechanisms as previously noted by Ciani & Comitini (2015), Albergaria & Arneborg (2016). Fermentation kinetics were in accordance with growth kinetics, in particular the sugar consumption of mixed fermentations with 24h delay was comparable to that of *S. cerevisiae* in pure culture. Conversely, the dominance of *Starm. bacillaris* on *S. cerevisiae* resulted in significantly lower sugar consumption rates.

3.2. Basic oenological parameters

The chemical composition of wines produced by pure and mixed culture fermentations are presented in Table 1. Regardless of the grape variety and inoculation protocol used, all fermentations resulted in complete sugar consumption (< 2.0 g/L), however the duration of fermentations differed between the inoculation protocols tested. In fact, results revealed that pure and mixed culture fermentations with 24 h delay completed the fermentation in 7 days, whereas 10 days were required for the mixed fermentations with 48 h delay (Fig. 2). The strong fructophilic character of *Starm. bacillaris* was confirmed during the first 1 to 2 days of fermentation. The two inoculation protocols resulted in different sugar consumption by *Starm. bacillaris* prior to *S. cerevisiae* inoculation. Compared to mixed fermentations with 24 h delay, inoculation of must with *S. cerevisiae* after 48 h of fermentation determined *Starm. bacillaris* to consume more sugars (almost twice), mainly fructose prior to *S. cerevisiae* inoculation (Fig. 2).

As a result, the inoculation protocol and in particular the inoculation time of *S. cerevisiae* influenced greatly the chemical composition of the wines. Compared to wines produced by *S. cerevisiae* in pure culture, the use of mixed cultures produced wines with more glycerol and less ethanol. Wines fermented using an inoculation delay of 24 and 48 h always contained higher levels of glycerol (1.1–5.9 g/L more glycerol) and lower ethanol (0.2–0.6 % v/v less ethanol). These differences were lower for the 24h inoculation delay.

Glycerol and ethanol yields were calculated using the data of sugar consumption and glycerol and ethanol production, respectively, at the end of fermentation. Mixed fermentations were distinguished by a relatively high glycerol and low ethanol yields. These differences were higher for the 48h inoculation delay. Therefore, the time of *S. cerevisiae* inoculation affected the production of metabolites, confirming earlier findings (Englezos et al., 2016a,b). In particular, glycerol could have a favourable impact on wine sensory perception. Due to its non-aromatic nature, it can significantly contribute to wine structure/body perception. In wines, levels between 7 and 15 g/L are frequently encountered and higher levels are thought to contribute also to the smoothness and viscosity of wine (Scanes, Hohmann, & Priori, 1998). Therefore, high glycerol levels contribute to wine traits and indicate that the overproduction of glycerol by *Starm. bacillaris* in mixed fermentations could improve the sensory qualities of the wine (Swiegers et al., 2005). Conversely, acetic acid significantly increased by increasing the delay of *S. cerevisiae*, although all the wines contained less than 0.43 g/L.

The titratable acidity parameter also showed great differences between inoculation protocols. Wines produced from mixed culture fermentations using a delay of 48 h generally had the highest values (5.95–6.60 g/L), while pure fermented wines contained the lowest values (5.34–5.76 g/L), and as a result, contributed accordingly to pH values. As for the other chemical parameters, these differences were higher in wines fermented using a delay of 48 h. These significant differences in the titratable acidity (increase in average of 0.82 g/L) and pH (average reduction of 0.30 units) compared to pure fermented wines, could not be explained by the principal organic acid concentrations measured in this study [citric, tartaric, malic and lactic acid (data not shown)]. To the contrary a decrease of succinic acid (average 0.18 g/L) was recorded in mixed fermented wines (48 h delay) with respect to those produced with Uvaferm BC® in pure culture. These findings suggest that *Starm. bacillaris* strain used in this study possess the capability to produce relative high concentrations of unmeasured organic acid compounds. Among these compounds, α-ketoglutaric and pyruvic acids were found in relative high concentrations in wines fermented by pure cultures of *Starm. bacillaris*, compared to pure fermented wines with *S. cerevisiae* (Magyar et al., 2014). The keto acids are produced either during the early stages of fermentation from sugar metabolism, or from the corresponding amino acids, alanine (pyruvic acid) and glutamate (α-keto glutaric acid), by the Ehrlich pathway (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). Additionally, pyruvic acid is a key product during the glycolysis and major source of redox balance during the ethanol production, hence a little is secreted from the cell. Thus, it can be

speculated that *Starm. bacillaris* strains swift carbon away from ethanol to this organic acid, in order to maintain intracellular $NADH/NAD^+$ redox balance. The acidogenic nature of *Starm. bacillaris* could have an impact on wine color stability, mainly due to the ability of the pyruvic acid to bind sulfur dioxide and swift the equilibrium of anthocyanins from the colorless to colored form (Mangani, Buscioni, Collina, Bocci, & Vincenzini, 2011; Morata et al., 2016). Additionally, pyruvic acid is an important key compound in carbon metabolism formed by yeasts and LAB (Morata, Calderón, González, Gómez-Cordovés, & Suárez, 2007). It is a precursor of many chemical compounds, which are involved in the formation of stable pigments such as vitisin A (malvidin-3-O-glucoside-pyruvate) (Asenstorfer, Markides, Iland, & Jones, 2003). Thus, this acidification property could be exploited in winemaking in order to make wines produced in warm climate regions more acid and increase microbiological stability at the end of the fermentation process.

Chromatic characteristics of wines produced by pure and mixed culture fermentations are presented in Table 2. CIEL*a*b* color measurements indicated that mixed fermented wines with 24 and 48 hours delay had a lower degree of lightness (L*) compared to pure fermented wines. Wines produced by mixed starter cultures also had the highest amount of redness (a*), yellowness (b*), and color intensity. These changes may be explained by the reduction of pH in wines due to the metabolic activity of *Starm. bacillaris* which is a good producer of organic acids (Magyar et al., 2014). Furthermore, in Merlot and Shiraz trials a significant decrease of the color hue parameter was observed, which is negatively influenced by the red color contribution in relation to the yellow component. Lower values were found in the mixed trials, with a significant effect also of the *S. cerevisiae* inoculation delay (24 or 48 h), thus meaning a higher red color contribution. This effect was not significantly observed in C. sauvignon and Pinot noir samples. Together with the evaluation of the color components for each produced wine, the ΔE* color difference was assessed between pure fermentations and each mixed fermentation sample (Table 2). When considering red wines, a ΔE* value of 3 was assessed as the general color tolerance perceptible by the human eye (Martínez, Melgosa, Pérez, Hita, & Negueruela, 2001). In our experiment, all the mixed fermentation wines evidenced a perceptible color difference (ΔE* higher than 3) when compared with pure fermentations, with the exception of the mixed 24h experiment in Pinot noir. For all the varieties considered the mixed 24h samples evidenced less overall color differences than mixed 48h samples (lower ΔE* parameter) both in relation to pure fermentations.

3.3 Volatile composition

Aroma compounds give the wine its typical odour. Yeast species represents one of the most important factors affecting wine fermentative volatile composition. Esters and alcohols mainly influence the general volatile composition of young red wines, while varietal components such as terpenes and norisoprenoids are present depending by the grape content (López, Ferreira, Hernández, & Cacho, 1999). Therefore, the fermentation process has an important role in defining the key aroma components of a young wine (Hirst & Richter, 2016). Table 3 lists the volatile compounds identified in wines fermented with different inoculation protocols. Thirty-five volatile compounds were listed and grouped in 4 aroma families, including alcohols, esters, fatty acids, terpenes and C_{13}-norisoprenoids and other compounds. Esters were the most abundant group in the wines, followed by alcohols, while fatty acids, terpenes and C_{13}-norisoprenoids were found to have smaller figures. In general, the content of the most volatile compounds varied significantly in function of the employment of *Starm. bacillaris* and of the inoculation delay of *S. cerevisiae*, while it was not influenced by the grape variety used. Wines fermented with mixed yeast cultures were distinct for their general lack of volatile compounds compared to pure fermented wines with *S. cerevisiae*. This reduction was particularly evident in wines in which *S. cerevisiae* was inoculated with a delay of 48 h with respect to the inoculation of *Starm. bacillaris*.

3.3.1 Higher alcohols

In addition to ethanol, yeasts also produce a large number of long-chain alcohols. These alcohols, called higher alcohols (also known as fusel alcohols) are secondary yeast metabolites produced from amino acid catabolism via the Ehrlich pathway (Hazelwood et al. 2008). Excessive concentrations of higher alcohols are strongly correlated with strong and pungent smell and taste, whereas optimal levels can impart fruity character in wines (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Both pure and mixed fermentations, independently of the grape variety used, produced the same levels of higher alcohols, at concentrations ranging from 17.8 mg/L to 21.8 mg/L, well below the threshold of 300 mg/L which have been found to contribute positively to wine complexity (Rapp & Mandery, 1986). This was true except Pinot noir wines, in which mixed fermented wine with a sequential delay of 48 h was distinguished from the other inoculation protocols by lower amounts of total higher alcohols (18.5 mg/L vs 22.6 mg/L).

Due to the strict correlation with yeast metabolism, the concentration of each higher alcohol in wine represents an important variable for yeast differentiation (Swiegers et al., 2005). A total of 7 alcohols were identified across the wines, with isoamylic alcohol, 2,3-butanediol (1), 2-methyl-1-propanol (isobutanol) and 2-phenyl ethanol as the major representatives. However, none of them surpassed their odour threshold (Cullere, Escudero, Cacho & Ferrerira, 2004; Ferreira, Lopez & Cacho, 2000; Guth, 1997; Li, 2006). Isobutanol and isoamylic alcohol are produced by yeasts during alcoholic fermentation through the conversion of leucine and isoleucine, respectively via Ehrlich pathway (Hazelwood et al., 2005). Mixed fermentations led to a lower production of isoamylic alcohol (herbaceous notes) and octanol (fruity notes) for all the grape varieties used. Similar results have been observed by Sadoudi et al. (2012) in Sauvignon blanc must fermented with *Starm. bacillaris* and *S. cerevisiae*, using 24 h delay. To the contrary, isobutanol production, which contributes to wine aroma with further herbaceous notes, tended to increase in sequential mixed fermented wines with increasing the inoculation delay of *S. cerevisiae*. The concentration of 2-phenyl ethanol, an aromatic compound associated with pleasant floral and rose notes, was not significantly different between pure and mixed fermented wines produced from Shiraz and Merlot grapes, in agreement with recent studies (Sadoudi et al., 2012l; Zara et al., 2014). C. sauvignon wines produced from mixed culture fermentation with 48 h delay contained significant higher level of this metabolite. Conversely, Pinot noir wines fermented with the above mentioned inoculation protocol contained significant lower level of this metabolite. Finally, (R,R; R,S-meso) 2,3-butanendiol was the only higher alcohol that didn't respond to yeast inoculation protocol, except for C. sauvignon wines in which the concentration of R,S-meso, 2,3-butanendiol increased in wines fermented with mixed cultures, using an inoculation delay of 48 h. These results let us hypothesize that both species have different preference on amino acid consumption and, as a result, the formation of individual higher alcohols is strictly correlated to the concentration of the respective amino acids in must.

3.3.2 Esters

Yeast derived esters are a class of volatile compounds with positive contribution to wine aroma, through the introduction of fruity and floral notes (Swiegers et al., 2005). Even small changes in the concentration of these secondary metabolites can have tangible effects on the sensory evaluation of the final product. Two classes of esters are synthesized by yeasts during fermentation, the acetate esters and the ethyl fatty acid esters. The first group is

414 produced through condensation of yeast-derived higher alcohols with acetyl-coA, catalysed
415 in the cell by a group of enzymes called alcohol acyl-transferases (AAT) genes, ATF1 and
416 ATF2 (Peddie, 1990). The other group of esters are formed by the reaction of ethanol with
417 volatile fatty acid (fatty acid degradation), formed during lipid biosynthesis (Saerens,
418 Delvaux, Verstrepen, & Thevelein, 2010). As seen in Table 3, a total of 16 esters were
419 identified in wines, including 12 ethyl esters and 4 acetate esters. Results demonstrated that
420 the use of *Starm. bacillaris* in combination with *S. cerevisiae* in mixed fermentations
421 remarkably reduced the production of ethyl and acetate esters, especially in mixed
422 fermentations with 48 h delay with respect to fermentations with *S. cerevisiae* alone for all
423 the grape varieties used in this study (9.0 mg/L vs 74.4 mg/L). Merlot and Shiraz wines
424 fermented with *S. cerevisiae* exhibited the highest content of total esters, while the C.
425 sauvignon and Pinot noir the lowest ones.
426 Among the identified esters, ethyl esters of straight-chain fatty acids such as ethyl hexanoate,
427 ethyl octanoate, ethyl dodecanoate and ethyl decanoate associated with pleasant floral and
428 fruity odors were the most abundant ethyl esters in the wines. The concentration of these
429 compounds tended to decrease in mixed fermentations apparently due to the involvement of
430 *Starm. bacillaris* in the fermentation process. This reduction was particularly evident in
431 mixed fermentations with 48 h delay, in accordance with the lower concentration of
432 corresponding fatty acids in these wines. The reduction in ethyl hexanoate by
433 *Starm. bacillaris*/*S. cerevisiae* mixed culture was also observed by Zara et al. (2014) who
434 found a decrease of this compound in pilot scale fermentations in which *S. cerevisiae* was
435 inoculated when *Starm. bacillaris* achieved 3 % (v/v) of ethanol than that produced by the
436 inoculation of *S. cerevisiae* alone. In general, ethyl esters significantly decreased in
437 concentration when *S. cerevisiae* was inoculated after 24 h of fermentation. This decrease
438 was more evident when *S. cerevisiae* was inoculated after 48 h from *Starm. bacillaris*
439 inoculation. However, Andorra et al. (2010, 2012) and Comitini et al. (2001), using a co-
440 inoculation protocol, observed no significant differences in ethyl esters. Additionally, a
441 decrease of ethyl hexanoate was observed in wines inoculated using an inoculum ratio
442 10.000:1 that favoured *Starm. bacillaris* growth. These findings highlight the importance of
443 the inoculation protocol and density on the chemical composition of the wines. Not all ethyl
444 esters influenced the wine aroma. According to the odour threshold, a small part of this
445 aroma family could contribute actively to wine aroma. In fact, ethyl butanoate, ethyl
446 decanoate, ethyl hexanoate, ethyl octanoate, which provides a pleasant fruity aroma, were

447 found at a concentration above their olfactory threshold in all wines obtained (20, 200, 14 and
448 5 μg/L; Francis et al., 2005).
449 Wines inoculated with mix starter cultures presented significant lower acetate esters
450 content, than those produced from pure cultures, suggesting that *Starm. bacillaris* possess
451 lower ability to synthetize volatile fatty acids than *S. cerevisiae*. This reduction was
452 particularly evident in mixed fermentations carried out with 48 h delay. Nevertheless, the
453 concentrations of 2-phenyl acetate (except in wines produced from Pinot noir grapes) and 3-
454 methyl-1-butanol acetate were above their odour threshold (250 and 30 μg/L; Francis &
455 Newton, 2005), and therefore only these compounds can contribute to wine aroma. In
456 function of *Starm. bacillaris*/*S. cerevisiae* interaction, the acetate esters that witnessed
457 significant reduction were ethyl acetate, hexyl acetate, 2-phenylethyl acetate and 3-methyl-1-
458 butanol acetate (isoamyl acetate). Among them hexyl acetate was the most notable,
459 displaying approximately a ten-fold decrease. This metabolite associated with fruitiness, is
460 not present in the grapes but its formed by yeast during fermentation. The reduction in hexyl
461 acetate, 2-phenylethyl acetate and isoamyl acetate was also observed by Sadoudi et al. (2012)
462 who found that concentrations of these compounds obtained by sequential mixed
463 fermentation of *Starm. bacillaris*/*S. cerevisiae* showed approximately five times lower values
464 that produced by *S. cerevisiae* alone. However, Andorra et al. (2010) using a co-inoculation
465 protocol to ferment Macabeo must which did not enable *Starm. bacillaris* growth, didn't
466 observed significant differences in total acetate esters concentration between wines fermented
467 with pure and mixed fermented wines.
468 Ethyl acetate, originating by yeasts during fermentation, contributes pleasant, fruity
469 notes to wines in concentrations lower than 150 mg/L. However, at concentrations above this
470 limit, this metabolite could negatively affect wine quality with negative descriptors such as
471 nail polish remover (Swiegers et al., 2005). All wines showed concentrations ranging from
472 3.2 mg/L to 5.3 mg/L of ethyl acetate, well below the level of 150 mg/L, contributing
473 positively to wine profile. As shown in Table 3 the concentration of this metabolite was
474 significantly different in response to inoculation protocol, decreasing in it's concentration in
475 mixed fermented wines. In our study, the concentration of acetate esters decreased in
476 response to *Starm. bacillaris* proliferation in mixed fermentations. The significant decrease of
477 ethyl acetate, isoamyl acetate and 2-phenylethyl acetate by *S. cerevisiae* has been associated
478 with over expression of IAH-encoded ester degrading enzyme (Lilly, Bauer, Lambrechts,
479 Swigers, Cozzolino, & Pretorius, 2006). Additionally, the increased levels of isoamyl acetate
480 in pure fermented wines could be explained by overexpression of a branched-chain amino

acid transferase gene BAT1, which is correlated with increased production of isoamylic alcohol the precursor of isoamyl acetate (Lilly et al., 2006).

3.3.4 Fatty acids

Three major volatile fatty acids were identified in wines produced from pure and mixed starter cultures, namely decanoic, dodecanoic and hexanoic acid (Table 3). Results revealed that their concentration ranged from 59 to 938 μg/L across the fermented wines, well below the level of 20 mg/L which enhance the butter-like and cheese notes (Ribéreau-Gayon, Dubourdieu, Doneche, & Lonvaud, 2006). As a result, they are less likely to affect negatively the aroma of wine, independently of the grape variety. Concentration of decanoic, dodecanoic and hexanoic acid associated with negative characters of fatty and rancid showed a reduction in response to *Starm. bacillaris* proliferation, showing the lowest concentration in sequential inoculated wines with 48 hours delay. In addition, the concentration of each fatty acid was below its odour threshold, and therefore are less likely to contribute to wine aroma (Francis & Newton, 2005).

Fatty acids concentration results are in agreement with Zara et al. (2014) observations and partly in disagreement with Sadoudi et al. (2012) findings. The former study demonstrated a significant increase of decanoic acid during mixed fermentations in Sauvignon blanc wines, compared to pure fermented wines with *S. cerevisiae*, while no significant differences were observed for octanoic acid production. To the contrary, the first study showed significant decrease of octanoic acids in Shiraz wines produced by the inoculation of *S. cerevisiae* when ethanol concentration was 2 % (v/v). Their observations, together with our results suggest that the changes in volatile fatty acids concentration during fermentation are strain and temperature dependent.

3.3.5 Terpenes and C_{13}-norisoprenoids

The last class of volatile compounds terpenes and C_{13}-norisoprenoids have a significant influence on the fruity and floral character of wines. Terpenes and C_{13}-norisoprenoids contribute to the varietal character of many wines, especially aromatic cultivars (Swiegers et al., 2005). During fermentation, this group of compounds also present in grapes in glycoside form can be released through acid-induced hydrolysis by grape endogenous and yeast hydrolytic enzymes (Moreno-Arribas & Polo, 2009). Yeast species have been shown to have different expression levels and activities of these enzymes (Strauss,

Jolly, Lambrechts, & van Resemburg, 2001; Charoenchai, Fleet, Henschke, & Todd, 1997). In the current study, five volatile compounds belonging to this class were identified including citronellol, geraniol, linalool, 4-terpineol and *β*-damascenone. Regarding their total concentration, no significant differences were registered between pure and mixed culture fermented wines. Wines from Shiraz grapes were an exception since the use of *Starm. bacillaris* in mixed culture fermentations significantly increased their concentration. For single compounds, both sequential mixed fermented wines showed higher amount of citronellol and linalool (citrus-like note) than pure fermented wines, indicating higher activity or higher expression *β*-glycosidase enzymes in *Starm. bacillaris* strain (Englezos, Rantsiou, Torchio, Rolle, Gerbi, & Cocolin, 2015). The concentration of *β*-damascenone was above its odour threshold (0.05 μg/L; Francis & Newton, 2005), and therefore contribute actively to the floral aroma of all the wines studies. In addition, pure fermented wines and mixed fermented wines with 24 h delay were distinguished by a higher amount of *β*-damascenone.

4. Conclusion

The results of this study demonstrated that inoculation protocol plays a decisive role in affecting wine volatile profile and colour characteristics, independently of the grape variety. Particularly, the early grow of *Starm. bacillaris* in mixed fermented wines markedly affected the growth of *S. cerevisiae* and consequently the final chemical composition of wines. This impact led to reduction total ester concentration and an increase in the concentrations of glycerol and total acids, compared to pure fermented wines with pure *S. cerevisiae*. For all the varieties mixed cultures affected positively the chromatic characteristics of the wines. Further work is required to confirm these results with different combinations of *Starm. bacillaris*/*S. cerevisiae* strains.

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Table 1

Final chemical parameters of musts and wines produced by pure and mixed culture fermentations

Grape variety	Inoculation protocol	Residual sugars (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	Ygly (g/s) (g/g)	Yeth (eth/s) (g/g)	pH	TA (g/L)
C. sauvignon	must	248.8 ± 1.6	3.14 ± 0.02	< 0.1	< 0.1	< 0.1	< 0.1	-	-	3.99 ± 0.01	4.33 ± 0.05
	pure	< 2.0	2.25 ± 0.04a	1.75 ± 0.01b	0.17 ± 0.01a	9.4 ± 0.1a	14.8 ± 0.1c	0.038 ± 0.001a	0.060 ± 0.001c	3.79 ± 0.14b	5.76 ± 0.14a
	mixed 24 h	< 2.0	2.44 ± 0.02b	1.76 ± 0.01b	0.14 ± 0.01a	11.0 ± 0.1b	14.6 ± 0.1b	0.044 ± 0.001b	0.059 ± 0.001b	3.64 ± 0.11ab	6.11 ± 0.22a
	mixed 48 h	< 2.0	2.63 ± 0.03c	1.61 ± 0.01a	0.27 ± 0.03b	15.3 ± 0.1c	14.2 ± 0.1a	0.061 ± 0.001c	0.057 ± 0.001a	3.49 ± 0.01a	6.60 ± 0.15b
Sign.			***	***	***	***	***	***	***	*	**
Merlot	must	248.1 ± 1.3	0.99 ± 0.02	< 0.1	< 0.1	< 0.1	< 0.1	-	-	3.81 ± 0.01	3.15 ± 0.07
	pure	< 2.0	0.89 ± 0.02a	1.46 ± 0.01c	0.21 ± 0.01b	9.2 ± 0.1a	14.9 ± 0.1c	0.037 ± 0.001a	0.060 ± 0.001c	3.63 ± 0.04b	5.34 ± 0.08a
	mixed 24 h	< 2.0	0.95 ± 0.01b	1.55 ± 0.01b	0.16 ± 0.01a	10.7 ± 0.1b	14.7 ± 0.1b	0.043 ± 0.001b	0.059 ± 0.001b	3.49 ± 0.1b	5.67 ± 0.18b
	mixed 48 h	< 2.0	1.29 ± 0.02c	1.31 ± 0.01a	0.33 ± 0.01c	14.9 ± 0.1c	14.3 ± 0.1a	0.060 ± 0.001c	0.058 ± 0.001a	3.3 ± 0a	6.30 ± 0.01c
Sign.			***	***	***	***	***	***	***	**	***
Pinot noir	must	254.0 ± 0.6	2.04 ± 0.01	< 0.1	< 0.1	< 0.1	< 0.1	-	-	4.06 ± 0.01	3.31 ± 0.05
	pure	< 2.0	1.59 ± 0.04a	1.65 ± 0.01c	0.12 ± 0.01a	9.7 ± 0.1a	15.2 ± 0.1c	0.036 ± 0.001a	0.060 ± 0.001c	3.82 ± 0.03b	5.35 ± 0.04a
	mixed 24 h	< 2.0	1.67 ± 0.02b	1.61 ± 0.01b	0.14 ± 0.01a	10.8 ± 0.1b	15.1 ± 0.1b	0.041 ± 0.001b	0.059 ± 0.001b	3.81 ± 0.05b	5.58 ± 0.08b
	mixed 48 h	< 2.0	1.72 ± 0.01c	1.39 ± 0.01a	0.43 ± 0.19b	14.6 ± 0.1c	14.7 ± 0.1a	0.056 ± 0.001c	0.058 ± 0.001a	3.53 ± 0.01a	5.95 ± 0.02c
Sign.			***	***	***	***	***	***	***	***	***
Shiraz	must	250.3 ± 1.0	2.23 ± 0.01	< 0.1	< 0.1	< 0.1	< 0.1	-	-	3.82 ± 0.01	4.35 ± 0.05
	pure	< 2.0	1.48 ± 0.01a	1.50 ± 0.01b	0.11 ± 0.01a	8.8 ± 0.1a	14.8 ± 0.1c	0.035 ± 0.001a	0.059 ± 0.001c	3.65 ± 0.02c	5.66 ± 0.19a
	mixed 24 h	< 2.0	1.68 ± 0.02b	1.52 ± 0.01c	0.13 ± 0.01b	10.4 ± 0.1b	14.6 ± 0.1b	0.042 ± 0.001b	0.058 ± 0.001b	3.43 ± 0.03b	6.47 ± 0.11b
	mixed 48 h	< 2.0	1.86 ± 0.01c	1.36 ± 0.01a	0.20 ± 0.01c	12.8 ± 0.1c	14.4 ± 0.1a	0.051 ± 0.001c	0.058 ± 0.001a	3.37 ± 0.01a	6.52 ± 0.01b
Sign.			***	***	***	***	***	***	***	***	***

The values are means ± standard deviation of three independent experiments. Sig: *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively between the three wines produced. TA: titratable acidity, Ygly (glycerol/sugar consumption): glycerol yield and Yeth (ethanol/sugar consumption): ethanol yield.

Table 2

Chromatic characteristics of wines produced by pure and mixed culture fermentations

Grape variety	Inoculation protocol	L*	a*	b*	Color hue	Color intensity (optical path 10 mm)	ΔE*
C. sauvignon	pure	56.22 ± 0.86c	47.76 ± 0.16a	14.79 ± 0.58	0.74 ± 0.02	2.03 ± 0.06a	
	mixed 24 h	53.97 ± 0.05b	53.57 ± 0.08b	15.46 ± 0.15	0.67 ± 0.01	2.22 ± 0.01b	6.33
	mixed 48 h	52.75 ± 0.16a	53.84 ± 0.08c	15.18 ± 0.05	0.64 ± 0.01	2.29 ± 0.01b	7.01
Sign.		***	***	ns	ns	***	
Merlot	pure	43.29 ± 0.12b	59.37 ± 0.49a	24.76 ± 0.3a	0.62 ± 0.01c	3.31 ± 0.02a	
	mixed 24 h	40.87 ± 0.46ab	61.3 ± 0.12b	26.1 ± 0.4a	0.59 ± 0.01b	3.65 ± 0.07b	3.37
	mixed 48 h	38.69 ± 1.82a	63.2 ± 0.84c	30.33 ± 1.45b	0.51 ± 0.01a	4.27 ± 0.25c	8.18
Sign.		**	***	***	***	***	
Pinot noir	pure	66.39 ± 0.82b	32.03 ± 0.6	16.64 ± 0.44a	1.01 ± 0.01	1.46 ± 0.05a	
	mixed 24 h	65.25 ± 1.13b	33.28 ± 1.05	17.06 ± 0.21a	1.03 ± 0.02	1.55 ± 0.05a	1.74
	mixed 48 h	62.08 ± 0.05a	31.01 ± 0.34	17.91 ± 0.07b	1.06 ± 0.01	1.71 ± 0.03b	4.61
Sign.		**	ns	**	ns	**	
Shiraz	pure	63.76 ± 0.52c	37.07 ± 0.18a	20.15 ± 0.15b	0.96 ± 0.01c	1.70 ± 0.03a	
	mixed 24 h	61.15 ± 0.21b	41.27 ± 0.13b	19.39 ± 0.31b	0.89 ± 0.01b	1.83 ± 0.02ab	5.00
	mixed 48 h	57.83 ± 1.11a	45.47 ± 0.31c	17.7 ± 0.89a	0.78 ± 0.01a	1.95 ± 0.17b	10.57
Sign.		***	***	**	***	*	

The values are means ± standard deviation of three independent experiments. Sig: *, **, *** and ns indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively. L*: luminosity; a*: red/green color component and b*: yellow/blue color component. ΔE* parameter was calculated considering average values of L*, a*, and b* color components, for each mixed fermentation sample with relation to the same variety pure fermentation sample.

711 **Table 3**

712 Volatile composition of the wines produced by pure and mixed culture fermentations

	Cabernet Sauvignon (Cs)			Merlot (M)			Pinot noir (Pn)			Shiraz (S)			Statistical differences						
Metabolites	Pure	Mixed FA 24h	Mixed FA 48h	Pure	Mixed FA 24h	Mixed FA 48h	Pure	Mixed FA 24h	Mixed FA 48h	Pure	Mixed FA 24h	Mixed FA 48h	Var. (a)	In.. (b)	a * b	Cs	M	Pn	S
Alcohols																			
Benzylic alcohol	4 ± 0^{b}	4 ± 0^{b}	3 ± 0^{a}	2 ± 1	2 ± 0	1 ± 0	11 ± 1^{b}	13 ± 1^{c}	7 ± 1^{a}	3 ± 1^{b}	3 ± 1^{b}	2 ± 0^{a}	***	***	***	***	ns	***	*
Hexanol	305 ± 7	310 ± 10	289 ± 33	286 ± 18^{a}	335 ± 7^{b}	283 ± 50^{a}	321 ± 11^{b}	339 ± 8^{b}	290 ± 30^{a}	222 ± 7^{a}	334 ± 49^{b}	302 ± 18^{b}	**	***	***	ns	**	**	***
Isoamylic alcohol	8152 ± 557^{b}	8596 ± 312^{b}	5895 ± 759^{a}	8206 ± 892^{b}	7804 ± 321^{b}	5017 ± 517^{a}	11286 ± 262^{c}	9864 ± 633^{b}	7294 ± 714^{a}	6216 ± 859	7542 ± 1708	6187 ± 519	***	***	***	***	***	***	ns
Octanol	13 ± 1^{c}	8 ± 0^{b}	2 ± 0^{a}	17 ± 2^{c}	13 ± 1^{b}	2 ± 0^{a}	7 ± 1^{c}	7 ± 1^{b}	4 ± 0^{a}	23 ± 2^{b}	23 ± 4^{b}	7 ± 1^{a}	***	***	***	***	***	***	***
(R,R)-2,3-Butanediol	191 ± 64^{ab}	162 ± 57^{a}	269 ± 75^{b}	230 ± 135	186 ± 23	454 ± 50	285 ± 125	318 ± 182	350 ± 78	269 ± 64	301 ± 47	220 ± 49	ns	ns	ns	ns	ns	ns	ns
(R,S-meso)-2-3-Butanediol	42 ± 16^{a}	44 ± 17^{b}	130 ± 35^{c}	66 ± 21	46 ± 6	210 ± 23	72 ± 34	94 ± 69	163 ± 52	65 ± 15	95 ± 24	93 ± 33	ns	***	ns	***	ns	ns	ns
2-Methyl-1-propanol	235 ± 23^{a}	429 ± 58^{ab}	747 ± 457^{b}	238 ± 33^{a}	349 ± 18^{b}	814 ± 47^{c}	441 ± 26^{a}	535 ± 59^{b}	957 ± 91^{c}	221 ± 37^{a}	405 ± 64^{b}	935 ± 98^{c}	***	***	ns	*	***	***	***
2-Phenylethanol	11950 ± 1415^{a}	11188 ± 1020^{a}	14442 ± 1563^{b}	11080 ± 1632	11933 ± 969	13679 ± 2658	10106 ± 1020^{ab}	11511 ± 646^{b}	9477 ± 1333^{a}	9039 ± 1448	10174 ± 1230	9999 ± 629	***	*	**	**	ns	*	ns
∑ Alcohols	20892 ± 1233	20741 ± 859	21776 ± 1609	20127 ± 1264	20668 ± 695	20460 ± 3385	22530 ± 1261^{b}	22681 ± 949^{b}	18540 ± 1736^{a}	16058 ± 729	18877 ± 2932	17746 ± 246	**	ns	ns	ns	ns	***	ns
Esters																			
Diethyl succinate	14 ± 1^{c}	8 ± 1^{b}	4 ± 1^{a}	15 ± 1	11 ± 1	12 ± 6	15 ± 1^{c}	10 ± 1^{b}	5 ± 1^{a}	20 ± 2^{c}	15 ± 3^{b}	8 ± 1^{a}	***	***	***	***	ns	***	***
Ethyl acetate	3256 ± 218^{a}	2855 ± 194^{b}	3299 ± 215^{a}	5520 ± 1033^{b}	3500 ± 135^{b}	3571 ± 114^{a}	4013 ± 122^{b}	3526 ± 203^{a}	3273 ± 346^{a}	5255 ± 389^{b}	4157 ± 617^{a}	3994 ± 401^{a}	***	***	***	**	***	***	**
Ethyl butanoate	101 ± 9^{b}	92 ± 6^{b}	70 ± 14^{a}	175 ± 17^{c}	119 ± 7^{b}	87 ± 9^{a}	113 ± 11^{c}	90 ± 4^{b}	74 ± 10^{a}	164 ± 18^{b}	152 ± 16^{ab}	130 ± 16^{a}	***	***	***	***	***	***	*
Ethyl decanoate	8053 ± 1935^{c}	5064 ± 250^{b}	1028 ± 208^{a}	15615 ± 2607^{c}	7114 ± 611^{b}	1543 ± 252^{a}	7990 ± 580^{c}	6452 ± 628^{b}	2641 ± 489^{a}	18238 ± 2760^{c}	10141 ± 2978^{b}	3115 ± 185^{a}	***	***	***	***	***	***	***
Ethyl dodecanoate	879 ± 134^{c}	532 ± 36^{b}	144 ± 24^{a}	1335 ± 194^{c}	750 ± 56^{b}	179 ± 10^{a}	850 ± 57^{c}	760 ± 52^{b}	255 ± 41^{a}	2347 ± 423^{c}	1138 ± 195^{b}	337 ± 11^{a}	***	***	***	***	***	***	***
Ethyl heptanoate	17 ± 2^{b}	21 ± 1^{c}	9 ± 3^{a}	12 ± 2^{b}	13 ± 1^{b}	7 ± 2^{a}	31 ± 1^{c}	27 ± 2^{b}	17 ± 3^{a}	20 ± 1^{a}	34 ± 5^{b}	23 ± 5^{a}	***	***	***	***	***	***	***
Ethyl hexanoate	2798 ± 404^{c}	2032 ± 142^{b}	484 ± 90^{a}	4658 ± 506^{c}	2939 ± 221^{b}	757 ± 194^{a}	2407 ± 144^{c}	1780 ± 157^{b}	720 ± 63^{a}	4118 ± 242^{b}	3495 ± 693^{b}	1317 ± 240^{a}	***	***	***	***	***	***	***
Ethyl (E)-2-hexenoate	32 ± 2^{a}	48 ± 2^{b}	30 ± 6^{a}	23 ± 2	27 ± 1	24 ± 5	24 ± 1^{b}	27 ± 2^{c}	18 ± 2^{a}	11 ± 2^{a}	24 ± 4^{b}	23 ± 4^{b}	***	***	***	***	ns	***	***
Ethyl octanoate	16015 ± 3546^{c}	10643 ± 656^{b}	2272 ± 549^{a}	27051 ± 4089^{c}	14598 ± 1348^{b}	3294 ± 490^{a}	18634 ± 1344^{c}	14836 ± 1344^{b}	6008 ± 1076^{a}	29032 ± 2994^{c}	18969 ± 5182^{b}	6273 ± 835^{a}	***	***	***	***	***	***	***
Ethyl nonanoate	26 ± 2^{b}	30 ± 1^{c}	16 ± 4^{a}	23 ± 1^{b}	24 ± 2^{b}	12 ± 3^{a}	53 ± 4	49 ± 5	53 ± 11	42 ± 6	45 ± 10	49 ± 23	***	ns	*	***	***	ns	ns
Ethyl 4-hydroxybutanoate	21 ± 6^{b}	14 ± 3^{a}	9 ± 1^{a}	24 ± 7^{b}	17 ± 2^{ab}	12 ± 8^{a}	31 ± 8^{b}	35 ± 12^{b}	16 ± 3^{a}	17 ± 1^{b}	19 ± 2^{b}	8 ± 1^{a}	***	***	ns	**	*	*	***
Ethyl 9-decenoate	7 ± 1^{c}	2 ± 1^{b}	0 ± 0^{a}	23 ± 4^{c}	7 ± 2^{b}	1 ± 0^{a}	6 ± 4^{b}	5 ± 1^{b}	1 ± 0^{a}	33 ± 7^{b}	31 ± 10^{b}	3 ± 1^{a}	***	***	***	***	***	**	***
Hexyl acetate	457 ± 42^{c}	127 ± 7^{b}	39 ± 9^{a}	960 ± 114^{c}	263 ± 21^{b}	66 ± 17^{a}	123 ± 6^{c}	76 ± 6^{b}	34 ± 3^{a}	1736 ± 102^{c}	555 ± 110^{b}	179 ± 28^{a}	***	***	***	***	***	***	***

Methyldecanoate	3 ± 1^{c}	2 ± 0^{a}	0 ± 0^{b}	7 ± 1^{c}	3 ± 0^{b}	1 ± 0^{a}	8 ± 1^{c}	6 ± 1^{b}	2 ± 0^{a}	7 ± 1^{c}	4 ± 2^{b}	1 ± 0^{a}	***	***	***	***	***	***	***
2-Phenylethyl acetate	631 ± 57^{c}	462 ± 28^{b}	326 ± 40^{a}	689 ± 55^{b}	618 ± 38^{b}	313 ± 14^{a}	217 ± 30^{b}	190 ± 10^{b}	148 ± 28^{a}	2179 ± 402^{c}	1416 ± 244^{b}	747 ± 59^{a}	***	***	***	***	***	**	***
3-Methyl-1-butanol acetate	5835 ± 510^{c}	2689 ± 254^{b}	1315 ± 299^{a}	8007 ± 838^{c}	3101 ± 221^{b}	1352 ± 275^{a}	4138 ± 184^{c}	2473 ± 173^{b}	1634 ± 229^{a}	11161 ± 1286^{c}	4660 ± 1127^{b}	2608 ± 434^{a}	***	***	***	***	***	***	***
∑ Esters	38145 ± 6716^{c}	24623 ± 1079^{b}	9046 ± 1078^{a}	64137 ± 8350^{c}	33105 ± 2518^{b}	11232 ± 1050^{a}	38654 ± 2141^{c}	30344 ± 2331^{b}	14897 ± 2131^{a}	74381 ± 5701^{c}	44855 ± 10625^{b}	18812 ± 1647^{a}	***	***	***	***	***	***	***
Fatty acids																			
Decanoic acid	83 ± 14^{c}	42 ± 5^{b}	13 ± 2^{a}	142 ± 14^{c}	80 ± 4^{b}	15 ± 2^{a}	97 ± 10^{b}	86 ± 7^{b}	5 ± 9^{a}	273 ± 56^{c}	124 ± 22^{b}	37 ± 10^{a}	***	***	***	***	***	***	***
Hexanoic acid	70 ± 9^{c}	45 ± 7^{b}	19 ± 1^{a}	101 ± 12^{c}	64 ± 3^{b}	22 ± 1^{a}	71 ± 9^{b}	58 ± 12^{b}	32 ± 4^{a}	124 ± 18^{c}	81 ± 8^{b}	40 ± 6^{a}	***	***	***	***	***	***	***
Octanoic acid	194 ± 25^{c}	98 ± 11^{b}	27 ± 3^{a}	285 ± 27^{c}	159 ± 9^{b}	31 ± 2^{a}	224 ± 20^{c}	188 ± 19^{b}	76 ± 20^{a}	541 ± 98^{c}	249 ± 45^{b}	92 ± 25^{a}	***	***	***	***	***	***	***
∑ Fatty acids	347 ± 46^{c}	185 ± 22^{b}	59 ± 2^{a}	528 ± 47^{c}	303 ± 14^{b}	68 ± 4^{a}	392 ± 37^{c}	332 ± 33^{b}	113 ± 24^{a}	938 ± 166^{c}	454 ± 75^{b}	169 ± 38^{a}	***	***	***	***	***	***	***
Terpenes and C13-norisoprenoids																			
Citronellol	12 ± 1^{a}	19 ± 1^{b}	16 ± 2^{b}	8 ± 3^{a}	14 ± 1^{b}	11 ± 1^{ab}	24 ± 1^{a}	29 ± 1^{b}	36 ± 6^{c}	9 ± 1^{a}	21 ± 4^{b}	24 ± 2^{b}	***	***	***	***	**	***	***
Geraniol	9 ± 1^{a}	13 ± 2^{b}	16 ± 2^{c}	13 ± 3	13 ± 1	12 ± 0	13 ± 1^{a}	16 ± 1^{b}	16 ± 3^{b}	12 ± 1^{a}	19 ± 3^{b}	24 ± 2^{c}	***	***	***	***	ns	*	***
Linalool	7 ± 1^{a}	9 ± 1^{a}	19 ± 3^{b}	8 ± 1^{a}	11 ± 0^{b}	15 ± 2^{c}	10 ± 1^{a}	12 ± 0^{b}	17 ± 2^{c}	15 ± 1^{a}	25 ± 3^{b}	42 ± 5^{c}	***	***	***	***	***	***	***
β-Damascenone	25 ± 4^{b}	23 ± 3^{b}	16 ± 3^{a}	9 ± 9	19 ± 1	9 ± 1	41 ± 4^{ab}	44 ± 3^{b}	37 ± 6^{a}	19 ± 2^{b}	20 ± 3^{b}	11 ± 7^{a}	***	***	ns	**	*	ns	*
4-Terpineol	1 ± 0	0 ± 0	0 ± 0	1 ± 1	1 ± 0	0 ± 0	10 ± 1^{b}	9 ± 0^{b}	6 ± 1^{a}	0 ± 0	0 ± 0	0 ± 0	***	***	***	ns	ns	***	ns
∑ Terpenes e C13-norisoprenoids	64 ± 5	65 ± 3	67 ± 8	39 ± 8	57 ± 2	48 ± 3	98 ± 6	108 ± 4	112 ± 12	55 ± 4^{a}	84 ± 9^{b}	101 ± 8^{c}	***	***	***	ns	ns	ns	***
Other metabolites																			
Benzaldehyde	0 ± 0^{a}	0 ± 0^{a}	10 ± 2^{b}	0 ± 0^{a}	0 ± 0^{a}	9 ± 1^{b}	3 ± 5^{a}	1 ± 3^{a}	9 ± 1^{b}	0 ± 0^{a}	0 ± 0^{a}	8 ± 1^{b}	ns	***	ns	***	***	*	***
γ-Butyrolactone	53 ± 10	43 ± 7	48 ± 5	105 ± 40	56 ± 5	52 ± 5	39 ± 4^{b}	40 ± 5^{b}	31 ± 4^{a}	36 ± 6	37 ± 4	34 ± 4	***	**	**	ns	*	*	ns
3-(Methylthio)-1-propanol	35 ± 6^{b}	34 ± 5^{b}	15 ± 1^{a}	35 ± 8^{b}	40 ± 4^{b}	18 ± 6^{a}	39 ± 19^{b}	44 ± 2^{b}	17 ± 2^{a}	26 ± 2^{b}	31 ± 3^{c}	15 ± 1^{a}	*	***	ns	***	**	*	***
∑ Other metabolites	88 ± 14	77 ± 10	73 ± 7	141 ± 48^{b}	96 ± 6^{ab}	79 ± 6^{a}	81 ± 23^{b}	85 ± 7^{b}	56 ± 7^{a}	62 ± 8	68 ± 7	57 ± 5	**	***	*	ns	*	*	ns

Aroma compounds in wines expressed in μg/L, as means ± standard deviation of three independent experiments (each replicate was analysed two times (total 6)). Sig: *, **, *** and ns indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively. a: variety and b: interaction

Figure captions

Fig.1

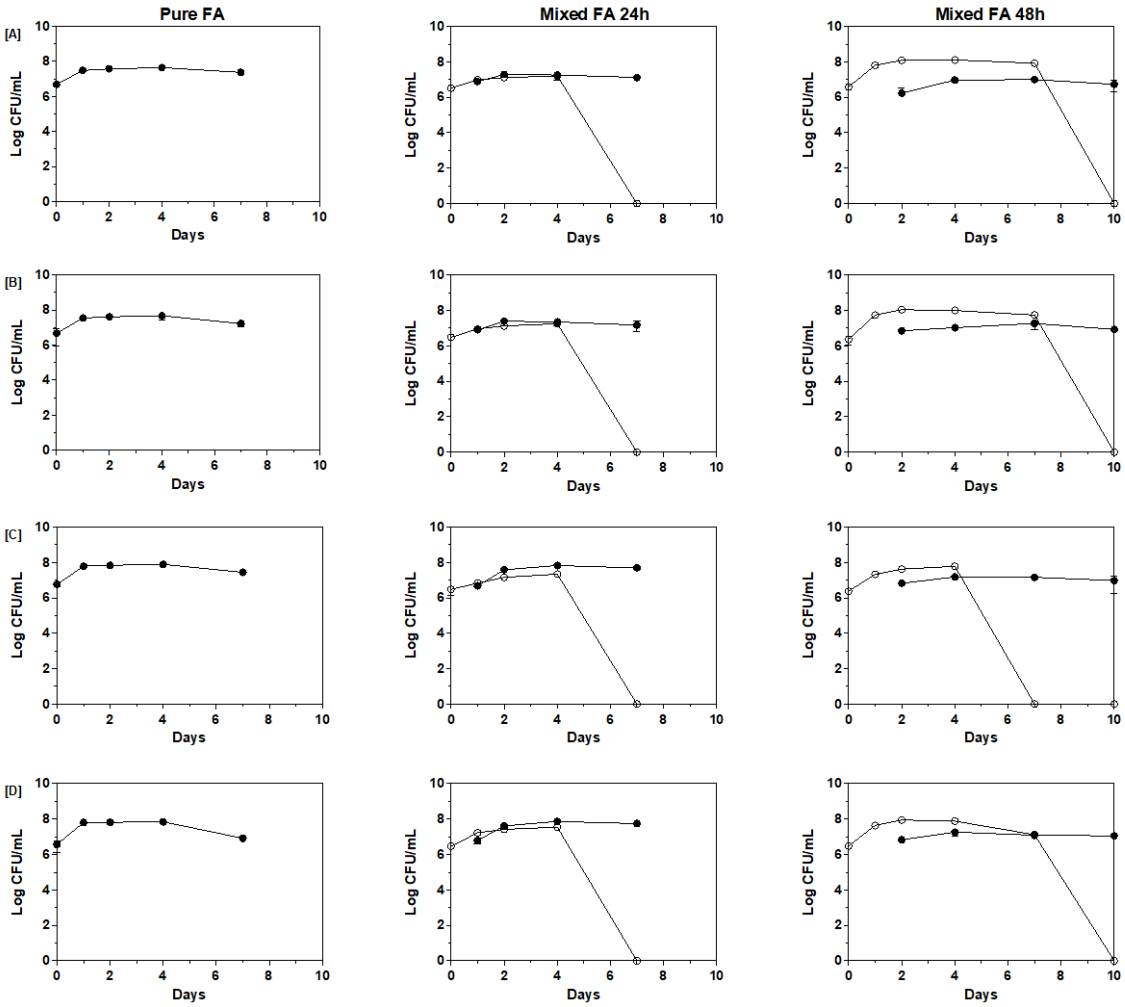
Growth dynamics of yeasts during pure (left panel) and mixed culture fermentations (inoculation of *S. cerevisiae* after 24 (central panel) and 48 h (left panel) from the *Starm. bacillaris* inoculation) using red grape musts: a) C. sauvignon, b) Merlot, c) Pinot noir and d) Shiraz. *Starm. bacillaris* strain FC54 (white circle) and *S. cerevisiae* Uvaferm BC® (black circle). Counts are the mean CFU/mL values ± standard deviations. Data are representative of three independent experiments.

Fig.2

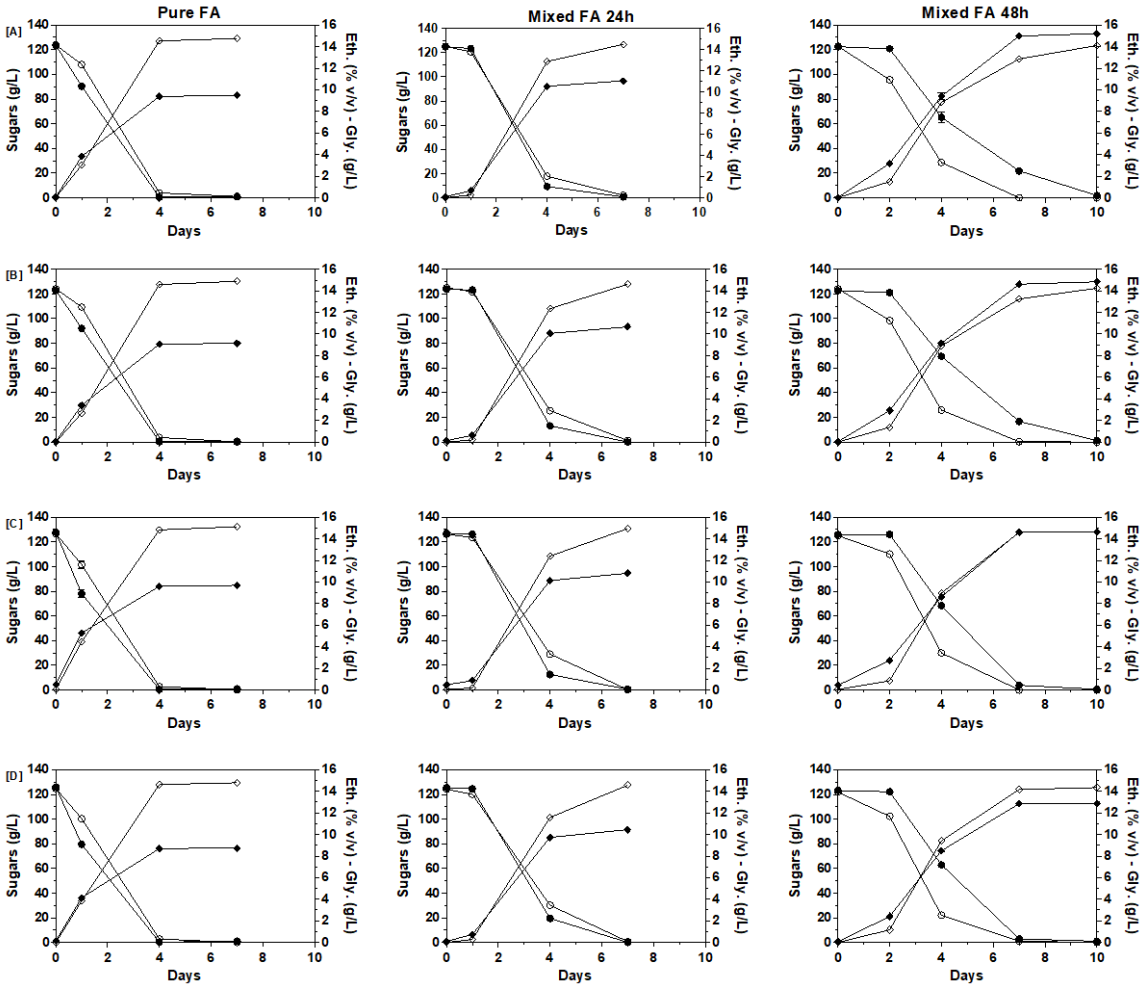
Evolution of metabolites during pure (left panel) and mixed culture fermentations (inoculation of *S. cerevisiae* after 24 (central panel) and 48 h (left panel) from the *Starm. bacillaris* inoculation) using red grape musts: a) C. sauvignon, b) Merlot, c) Pinot noir and d) Shiraz. Glucose (black circle), fructose (white circle), ethanol (white diamond) and glycerol (black diamond). Data are the mean ± standard deviations. Data are representative of three independent experiments.

Figures

Fig.1



771 **Fig. 2**



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