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Oxygen availability and strain combination modulate yeast growth dynamics in mixed culture fermentations of grape must with *Starmerella bacillaris* and *Saccharomyces cerevisiae*

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ABSTRACT

Starmerella bacillaris (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast that has been proposed as a co-inoculant of selected *Saccharomyces cerevisiae* strains in mixed culture fermentations to enhance the analytical composition of the wines. In order to acquire further knowledge on the metabolic interactions between these two species, in this study we investigated the impact of oxygen addition and combination of *Starm. bacillaris* with *S. cerevisiae* strains on the microbial growth and metabolite production. Fermentations were carried out under two different conditions of oxygen availability. Oxygen availability and strain combination clearly influenced the population dynamics throughout the fermentation. Oxygen concentration increased the survival time of *Starm. bacillaris* and decreased the growth rate of *S. cerevisiae* strains in mixed culture fermentations, whereas it did not affect the growth of the latter in pure culture fermentations. This study reveals new knowledge about the influence of oxygen availability on the successional evolution of yeast species during wine fermentation.

Keywords: *Starmerella bacillaris*; Mixed culture fermentations, Oxygen; Yeast interactions; Volatile metabolites

1. Introduction

Ethanol levels in wines have been rising over the last decade in many wine-producing countries, as a consequence of the high sugar content of the grapes currently used in wine production. This trend has often been attributed to global warming and the consumer preferences for well structured and full bodied wines produced from fully matured grapes (Mira de Orduña, 2010). The excessive sugar in the musts affects the fermentation process. High ethanol levels produced during the fermentation process may be toxic for the yeast cell by altering its membrane fluidity and this in turn may lead to arrested or sluggish sugar-to-ethanol conversion (Henderson and Block, 2014). Similarly, malolactic fermentation (MLF) a secondary bacterial fermentation occurring in red wines, during which *Oenococcus oeni* and other lactic acid bacteria (LAB) deacidify wine by conversion of malic to lactic acid, may be negatively affected (Zapparoli et al., 2009). Furthermore, ethanol can create sensory imbalance in the wine by increasing the perception of bitterness and hotness, as well as decreasing the perception of some wine aromas and flavour attributes (Goldner et al., 2009). From a commercial point of view, it can lead to an increase of the consumer's costs in countries where taxes are levied according to alcohol concentration (Sharma et al., 2014). Lastly, wine consumers are increasingly concerned with high ethanol content because of its harmful effect on human health (both physical and mental). Therefore, there is growing interest in reducing ethanol concentration in wine.

To this end, several techniques are being developed, targeting various steps of the winemaking process, starting from the vineyard to the winery, including grapevine and clonal selection, pre-fermentation, fermentation and post-fermentation strategies (Longo et al., 2016, Pickering, 2000; Varela et al. 2015). Among the available strategies, the choice should be economically relevant and at the same time, should not compromise organoleptic balance and other sensory characteristics of wine (Varela et al. 2015). The selection of yeasts able to convert glucose and fructose towards multiple secondary metabolites rather than ethanol, seems to be best suited for this purpose, since they do not require specific equipment (Tilloy et al., 2015). Indigenously isolated *Saccharomyces cerevisiae* strains exhibit similar ethanol yield values and as a consequence the research is focusing on developing *S. cerevisiae* and isolating non-*Saccharomyce*s strains with improved phenotypes, able to divert carbon away from ethanol production (Ciani et al., 2016, Tilloy et al., 2015). Non-*Saccharomyces* yeasts are an integral part of the indigenous mycobiota present on grapes and at least at the initial stages of most spontaneous or inoculated grape must fermentations (Cravero et al., 2016,

106 Varela et al., 2016a, 2016b). In pure culture fermentations, these species are generally
107 characterized by low fermentation efficiency (inability of completing alcoholic fermentation)
108 and as a result the inoculation of the same must with selected *S. cerevisiae* strains, results
109 fundamental in order to ensure complete fermentation of sugars (Andorrà et al., 2012; Tofalo
110 et al., 2016). This can be achieved simultaneously or sequentially (Ciani et al., 2010).
111 Conducting mixed culture wine fermentations, by controlled inoculation of selected non-
112 *Saccharomyces* and *S. cerevisiae* strains is a strategy that takes advantage of the unique
113 features of the former yeast group (Varela et al., 2016b).

114 Mixed fermentations and the employment of non-*Saccharomyces* species have
115 received growing attention over the recent years from the winemaking community. They
116 reflect yeast biodiversity of indigenous wine microbiota and modulate the production of
117 specific chemical compounds, as a consequence of the early growth of non-*Saccharomyces*
118 species (Ciani et al., 2010; Fleet, 2008; Jolly et al., 2014). Their efficiency is associated with
119 the promotion of the growth and metabolic activity of the selected non-*Saccharomyces* yeasts
120 by outcompeting or reducing the activity of the *S. cerevisiae* strain (Varela, 2016b). To this
121 end, numerous winemaking variables could be manipulated to encourage non-*Saccharomyces*
122 growth rate and contribution to the chemical composition and sensory quality of the wine.
123 These variables, include sugar concentration, fermentation temperature, inoculum density,
124 nitrogen and oxygen availability, inhibitory or stimulatory substances produced by the
125 growth of yeasts or bacteria, fungicide residues from the grapes and sulphur dioxide (SO_2)
126 addition (Fleet and Heard, 1993).

127 The application of non-*Saccharomyces* yeasts, in co-inoculation or sequential
128 inoculation with *S. cerevisiae* has been investigated in recent years for reducing the ethanol
129 yield (Bely et al., 2013; Canonico et al., 2016; Contreras et al. 2015a, 2015b; Giaramida et
130 al., 2013; Quirós et al. 2014, Varela et al., 2016c). Among them, *Starmerella bacillaris*
131 (synonym *Candida zemplinina*) is known as a high glycerol and low ethanol producer
132 (Englezos et al., 2015; Masneuf-Pomarede et al., 2015; Tofalo et al., 2012). We recently
133 reported a microbiological approach for reducing the ethanol content in wines based on
134 mixed culture fermentations of *Starm. bacillaris* and *S. cerevisiae* (Englezos et al. 2016a). In
135 this approach, *S. cerevisiae* was sequentially inoculated 48 hours after *Starm. bacillaris*,
136 leading to a marked decrease in the ethanol content up to 0.5 – 0.7 % (v/v), compared to *S.*
137 *cerevisiae* in pure culture fermentation. An important question still open after this study was
138 if strain compatibility and environmental factors could affect microbial growth and as a
139 consequence metabolites production. In this context, oxygen availability and strain

compatibility were considered to have great influence on fermentation speed as they impact on yeast metabolism and growth during fermentation (Hansen et al., 2011, Jolly et al., 2014). As a proof of concept, the objective of the present study was to acquire further knowledge about the impact of these parameters on mixed fermentation performance, carried out using conventional and evolutionary engineered (optimized for glycerol production/ethanol reduction) *S. cerevisiae* strains as partners of *Starm. bacillaris* stains.

2. Materials and methods

2.1. Strains

In the present study two *Starm. bacillaris* and two *S. cerevisiae* strains were used as starters. The *S. cerevisiae* strains were the commercial strains Uvaferm BC® and IONYS WF®, both from Lallemand Inc. (Montreal, Canada). The *Starm. bacillaris* strains used in this study were FC54 (yeast culture collection of DISAFA, Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Torino, Italy) and MUT 5705 (Mycotheca Universitatis Taurinensis-MUT, DBIOS, University of Torino, Italy), called CBE4 in previous studies (Englezos et al., 2015). All strains were selected for their enological traits in laboratory scale fermentations (Englezos et al., 2015, 2016a, Tilloy et al., 2014).

2.2. Fermentation trials

Fermentations were carried out in red must, without skins and seeds from Barbera grapes, which is the most planted red grape variety in Piedmont region (Northwest Italy). Barbera must contained 246.4 g/L sugars, pH 3.0, total acidity 10.0 g/L (expressed as g/L of tartaric acid) and 130 mg/L of yeast assimilable nitrogen (YAN) composed by 60 mg/L of inorganic nitrogen and 70 mg/L of organic nitrogen. The must was supplemented with 50 mg/L of organic nitrogen using the commercial product Fermaid O® (Lallemand Inc., Montreal, Canada) to achieve an initial YAN concentration of 180 mg/L. Before fermentation the must was pasteurized at 60 °C for 1 hour, as previously described by Englezos et al (2016b) and the absence of viable yeast populations was checked by plate counting on wallerstein laboratory nutrient (WLN) medium (Biogenetics, Milan, Italy).

Two sets of inoculation protocols were performed: a pure culture fermentation with *S. cerevisiae* strains and a mixed culture fermentation where *S. cerevisiae* strains were

inoculated 48 h after *Starm. bacillaris* inoculation. Mixed fermentations were carried out using the 4 different combinations of *Starm. bacillaris* and *S. cerevisiae* strains (FC54 and Uvaferm BC®, MUT 5705 and Uvaferm BC®, FC54 and IONYS WF®, MUT 5705 and IONYS WF®). All strains were inoculated as active dry yeast (ADY) and rehydrated according to manufacturer's instructions, except for strain MUT 5705 which was preadapted in the same must for 48 h at 25 °C. Prior to inoculation, yeast cells were counted by a Thoma hemocytometer chamber using methylene blue dye as a marker of cell viability. Then, appropriate amounts of inoculum were used to reach an initial cell population of about 5.0 x 10^6 cells/mL, that corresponds to a dose of 25 g/hL of ADY.

Triplicate fermentations were performed without and with the addition of oxygen (condition I and II respectively) in 1000 mL sterile glass bottles containing 800 mL Barbera grape must at 25 °C without agitation. After inoculation the bottles were closed with air locks containing sterile paraffin oil, to allow only the CO_2 to escape from the fermenting medium and prevent external contamination. For oxygen addition, the fermenting musts were saturated (about 7 mg/L of O_2) with pure oxygen (Rivoira, Milan, Italy) 24 and 48 hours after yeast inoculation. To estimate the dissolution of oxygen during fermentation, another grape must sample (inoculated with Uvaferm BC®) was micro-oxygenated and the oxygen content was controlled using a Nomasense oxygen analyzer (Nomacorc, SA). In order to improve O_2 solubility, the must was maintained in medium/high agitation (about 150 rev min^{-1}) on a rotary shaker (Velp Scientifica, Monza and Brianza, Italy) during oxygen addition. Samples were micro-oxygenated with Ox-evolution and ceramic diffuser (Intec, Pramaggiore, VE, Italy) with 10 mg/min oxygen flow rate for 10 minutes.

Fermentations were considered to be finished when the level of residual sugars was below 2 g/L. At this time, wines produced under the two conditions were kept at 4 °C to allow sedimentation of the solid parts. Wines were poured in 33cl glass bottles, supplemented with SO_2 in order to achieve a final concentration of 50 mg/L of total SO_2 and kept at 4 °C and analysed for chemical and volatile composition.

2.3. Microbiological analysis

The growth dynamics of the inoculated strains during the fermentation were determined by counting the viable cell population on WLN medium. Aliquots of 1 mL were periodically collected from each fermentation and serially diluted in sterile Ringer's solution

(Oxoid, Milan, Italy). Colony counting was performed after 3-5 days of incubation at 28 °C. The bromocresol green present in WLN medium acts as a dye, which *Starm. bacillaris* strains metabolize and therefore form flat, light to intense green colonies due to the acidogenic nature of this species (Sipiczki, 2004). On the other hand, *S. cerevisiae* strains do not take up this dye in the same way (strain dependent) and as a consequence generally form creamy white colonies, with different light shades of green on the top, facilitating the concurrent enumeration of the two species throughout the fermentation process.

2.4. Calculation of yeast growth performance parameters

The maximum specific growth rate (μ_{max}), defined as the rate of increase in cell number per time unit was calculated as follows: μ_{max}= (lnNf –lnN0)/(tf-t0), where Nf the yeast concentration (cfu/mL) at the final time point considered (tf) and N0 the initial yeast concentration, at the beginning of fermentation (t0). The generation number (g) defined as the number of cell divisions was calculated as follows: g = (logNf – logN0)/Log2. Generation time or doubling time (G) is called the time required for a cell to duplicate and divide itself and was calculated using the following formula: G=ln(2)/μ_{max}. All equations were calculated with the data from the exponential phase of growth for each strain. Strains were compared on the basis of their maximum population production and the time employed to reach this value.

2.5. Chemical analysis

Extracellular glucose, fructose, glycerol, primary organic acids (g/L) and ethanol (% v/v) concentrations were quantified after 2 days and at the end of fermentation, using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) instrument, equipped with an Aminex HPX-87H cation exchange column. The column was eluted with 0.0065 mol/L sulfuric acid (H_2SO_4) at a flow rate of 0.8 mL/min and a column temperature of 65 °C, using the protocols described by Rolle et al. (2012). The pH of the wines was determined by using the InoLab 730 pH meter (WTW, Weilheim, DE), while total acidity (TA) was determined and expressed in g/L of tartaric acid according to the official method proposed by the International Organization of Vine and Wine (OIV, 2008). The initial YAN concentration in the must, in terms of inorganic and organic nitrogen was determined

spectrophotometrically by using two enzymatic kits according to the manufacturer's instructions (Megazyme International, Bray, Ireland).

2.6. Volatile profile

Volatile metabolites were identified and subsequently quantified by HSPME-GC-MS immediately after the end of fermentation, using the protocols reported by Englezos et al. (2016b). Identification was carried out by matching the retention time of each compound with either those registered in the NIST Spectra database (http://webbook.nist.gov/chemistry/) or those of pure standards (Sigma-Aldrich, Milan, Italy) analysed in the same conditions, whenever available. The identified compounds were further verified, by calculating the Kováts retention index (KRI), using an alkane standard mixture C10-C40 (Sigma, Milan, Italy) as a reference for the retention times. An internal standard (1-heptanol) was added to each sample to semi-quantify the volatile compounds. Determinations were obtained by measuring the relative peak area of the identified compounds with those of the internal standard. Each replicate was analysed in duplicate.

2.6. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics software package (version 19.0, IBM Corp., Armonk, NY, USA). Significant differences between samples were determined using one-way Analysis of Variance (ANOVA). When significance was reached, a Tukey-b post-hoc test comparison at $p < 0.05$ was performed. The effect and interaction of oxygen addition, as well as the *S. cerevisiae* and *Starm. bacillaris* strain combination were analyzed by factorial ANOVA.

3. Results

3.1. Enumeration of yeast cell population

The yeast growth dynamics in pure and mixed culture fermentations were estimated using the plate count data and are illustrated in Figs. 1 and 2, respectively. *S. cerevisiae* strains, grown under semi-anaerobic conditions (condition I) in pure culture fermentations

showed similar growth dynamics during the first two days of fermentation, reaching population of about 1.0 x 10^8 cfu/mL (Fig. 1). Oxygen addition (condition II) influenced the exponential growth rate of the cells, in a strain dependent manner, since only cell populations of the laboratory-evolved strain IONYS WF® was positively affected (Table 1). The four growth parameters (generation number, time, maximum specific growth rate and cell viability) values registered for the strain IONYS WF® in the fermentations in which oxygen was added were two fold higher in comparison with the fermentation performed under semi-anaerobic conditions (respectively, 4.3 generations, 11.1, 0.063 h^{-1}, 11.1 and 1.0 x 10^8 cfu/mL for condition II and 2.1 generations, 23.8, 0.031 h^{-1} and 4.9 x 10^7 cfu/mL, for condition I). The stationary phase was observed from the 2nd to the 7th day of fermentation. *S. cerevisiae* strains showed different patterns of cell death after sugar exhaustion: strain IONYS WF® maintained the same cell viability (1.0 x 10^7 cfu/mL in both conditions) at the late stages of the fermentation, whereas Uvaferm BC® decreased to 1.0 x 10^5 cfu/mL.

The growth dynamics of the mixed culture fermentations using 4 different combinations of *Starm. bacillaris* and *S. cerevisiae* strains, under the two conditions of oxygen availability, are shown in Fig. 2. Conversely to that observed for *S. cerevisiae* strains in pure culture fermentations, both *Starm. bacillaris* strains, showed an oxygen-addition-dependent response, with significant differences between the two conditions (Fig. 2, Table 2). As can be seen in Fig. 2, oxygen addition supported both *Starm. bacillaris* strains to grow faster and reach a higher density at the beginning of the stationary phase with viable counts well above 1.0 x 10^8 cfu/mL. Oxygen addition showed a clear positive effect on both growth and fermentations parameters (Table 2) leading to a significant increase of 1.6 and 1.3 times of the generation number and 3.2 and 1.3 times of the μ_{max} for the strains FC54 and MUT 5705, respectively. Accordingly, the doubling time was reduced 3.2 and 1.4 times, respectively.

Concerning the coexistence of each of the two *Starm. bacillaris* strains in mixed fermentations with Uvaferm BC®, independently of the fermentation conditions applied, both strains dominated the fermentation process, with values of 10^8 cfu/mL, in the first 7 days and they became undetectable after 14 days. On the other hand, the survival time and dominance of both *Starm. bacillaris* strains over *S. cerevisiae* in mixed fermentations with IONYS WF®, was extended up to day 14 only in the presence of higher levels of oxygen in the musts (with cell viability above 1.0 x 10^7 cfu/mL).

The initial inoculation of the must with *Starm. bacillaris* strains in the mixed fermentations had a negative effect on growth and the performance of the two *S. cerevisiae*

306 strains, regardless of the oxygen addition. In both fermentation conditions, *S. cerevisiae*
307 strains reached the maximum cell density of about 5.0 x 10^7 cfu/mL, that was almost 50%
308 lower than the one registered in pure culture fermentations. In addition to this, the
309 supplementation of the must with oxygen, imposed the hardest condition for *S. cerevisiae*
310 growth. The most evident changes were the threefold and sevenfold decrease of the
311 generation number (from 1.6 - 2.4 to 0.2 - 0.7) with consequent decrease of the maximum
312 specific growth rate (from 0.022 – 0.034 to 0.003 to 0.010) and increased doubling time
313 (from 28.1 to 229.9) (Table 1). When the cells achieved the stationary phase, the viable cell
314 population remained stable for 7 days and decreased to 10^5 cfu/mL at the end of
315 fermentation.
316
317 *3.2. Conventional enological parameters*
318
319 The chemical composition of the wines produced by pure and mixed culture
320 fermentations is presented in Table 3. All fermentations, except the pairs FC54 with IONYS
321 WF® (condition II) and MUT 5705 with IONYS WF® (condition I and II) ended up with
322 residual sugar content of less than 4 g/L, although the durations of the fermentation differed.
323 Regarding the duration of fermentation, marked differences between the inoculation
324 protocols applied were registered. In fact, pure and mixed culture fermentations with *S.*
325 *cerevisiae* Uvaferm BC® completed the fermentation after 1 and 2 weeks respectively,
326 whereas 3 weeks were required for the corresponding fermentations with the evolved strain
327 IONYS WF® (data not shown). *Starm. bacillaris* strains exhibited a faster sugar uptake
328 (almost doubled) during the first 48 hours of fermentation in the presence of higher levels of
329 dissolved oxygen in the must, which is consistent with the growth dynamics data observed
330 before (Supplementary Table S1).
331 Wines produced with *S. cerevisiae* IONYS WF® (either by pure or mixed culture
332 fermentations), contained significantly more glycerol (increase up to 9.8 g/L), while the
333 ethanol content was reduced by 1.0% (v/v) than pure fermented wines with Uvaferm BC®.
334 On the other hand, mixed fermented wines using Uvaferm BC® as a partner of *Starm.*
335 *bacillaris* strains lead to an increase of the glycerol content by 4.7 - 5.8 g/L, while the ethanol
336 content was reduced by 0.5 % (v/v).
337 A significant decrease in pH with a parallel increase in titratable acidity of 1.0 to 3.4
338 g/L, was seen for the wines produced using only IONYS WF® and mixed culture

fermentations independently of the *S. cerevisiae* used. The differences in these parameters were higher in the wines produced from the evolved strain IONYS WF® in pure culture fermentations. The aeration conditions altered the chemical composition of the wines, especially the acetic acid content. In the presence of higher levels of dissolved oxygen in the fermentation medium, *S. cerevisiae* strains showed a slight to moderate increase of acetic acid (0.02-0.07 g/L), while in mixed fermentations the final content of this acid was almost two-fold higher, except for the pairs with MUT5705.

Glycerol and ethanol yields were calculated using the data obtained at the end of the fermentation. Pure and mixed culture fermentations with IONYS WF® strain, were clearly differentiated from the fermentations performed with Uvaferm BC®, on the basis of high glycerol and low ethanol yields. Glycerol yield in pure culture fermentations with IONYS WF® and mixed culture fermentations with FC54 was almost two times higher (about 0.0075 – 0.0078 g/g), than that registered for the Uvaferm BC® in pure culture fermentation (about 0.0059 – 0.0061 g/g). On the contrary, pure fermentations with IONYS WF® and mixed fermentations independently of the *S. cerevisiae* strain used showed the lowest levels of ethanol yield. Compared to Uvaferm BC®, the ethanol yields were reduced by 0.002 and 0.004 in the mixed and pure culture fermentations with IONYS WF®, respectively.

3.3 Volatile composition

A total of thirty-eight (38) volatile compounds were identified, semi-quantified using an internal standard and subsequently subdivided into five chemical classes, namely alcohols, fatty acids, esters, terpenes and other compounds. In order to uncover the influence of the fermentation conditions and strain combination on the chemical and volatile composition a univariate analysis was performed and the output is presented in Supplementary Table 2. Esters was the most abundant group in the samples, followed by alcohols, fatty acids and terpenes. Significant differences between pure and mixed culture fermentations were registered for each aroma family and for the majority of the individual compounds, independently of the oxygen addition. Pure fermented wines with IONYS WF®, contained higher concentrations of alcohols and esters compared to the strain Uvaferm BC®. Mixed fermented wines contained significantly lower levels of volatile compounds relative to wines produced with *S. cerevisiae* alone.

The total amount of alcohols in the wines was strongly associated with the concentrations of 2-phenyl-ethanol and isoamyl alcohol, which in combination constituted up

373 to 95 % of total alcohols. Wines produced from pure culture fermentations, independently of
374 the *S. cerevisiae* strain, contained significantly higher levels of individual alcohols, except for
375 the 2-methyl-1-propanol and hexanol. As observed for alcohols, wines inoculated first with
376 the two *Starm. bacillaris* strains showed significant decreased concentration of esters (for all
377 the individual compounds), independently from the addition of oxygen and strain used, while
378 the majority of the compounds were not affected by the fermentation conditions applied (16
379 out of 21). Conversely, to the abovementioned aroma categories, significantly higher levels
380 of monoterpenes were found in mixed fermentations, and the couple FC54 and IONYS WF®
381 was found to have the highest levels.
382 A principal component analysis (PCA) was conducted using the data presented in the
383 Supplementary Table S2, in order to uncover a possible correlation among the different
384 enological parameters and identify compounds able to explain the interaction of the strains as
385 well as to check reproducibility of the experiment (Fig. 3, panels A and B). Replicates were
386 clustered very close to each other indicating a good fermentation reproducibility of the pure
387 and mixed culture fermentations. The resulting PCA plot explained 67 % of the total variance
388 for the first two principal components (Fig. 3, panel A). The first principal component (PC1,
389 45 % of the variance) was mostly correlated to alcohols, esters and fatty acids and negatively
390 correlated to residual sugar concentration. The second principal component (PC2, 22 % of the
391 variance) was positively correlated to glycerol yield, total acidity and terpenes and negatively
392 correlated to ethanol yield.
393 Fig. 3 (Panel B) shows the distribution of the pure and mixed fermented wines with and
394 without the addition of oxygen, in the plane defined by the first two principal components.
395 Regardless of the oxygen addition, wines produced by pure culture fermentations were
396 located on the right part of the plot and can be separated from those fermented by mixed
397 cultures (left part) on the basis of the higher levels of alcohols, esters and fatty acids. On the
398 other hand, PCA was not able to differentiate wines produced by mixed culture
399 fermentations, except the wines produced by a combination of the strains FC54 and IONYS
400 WF® under semi-anaerobic conditions (condition I), while the others were grouped together
401 or separated as a function of the chemical composition. Wines produced with FC54 and
402 IONYS WF® under semi-anaerobic conditions were characterized by high levels of linalool
403 and glycerol yield. Interestingly, mixed fermented wines, independently of the couple of
404 strains and fermentation conditions applied were separated from the other wines due to the
405 higher levels of 3-methylbenzaldehyde, benzaldehyde, γ-butyrolactone, hexanol, 2-methyl-1-
406 propanol and linalool. Pure fermented wines were separated according to the strain used, with

wines from IONYS WF® on the upper part of the plot, while wines from Uvaferm BC® on the bottom. Wines with Uvaferm BC® were characterized by high pH values and high ethanol yields, on the other hand wines with IONYS WF® contained higher levels of alcohols and esters, like 2-phenylethanol and 2-phenyl acetate. Mixed fermented wines were clearly differentiated from those fermented by pure cultures due to the lower levels of aroma compounds.

4. Discussion

In recent years the use of non-*Saccharomyces* yeasts in association with *S. cerevisiae* strains is gaining positive attention from the wine making industry across the world (Ciani et al., 2010). The first commercially available non-*Saccharomyces* yeast was a "yeast blend" released in Denmark from Chr. Hansen in 2003. It was called Vinoflora® " Melody.nsac and Vinoflora" Harmony.nsac and contained a blend of *Torulaspora delbrueckii* with *S. cerevisiae* and *Kluveromyces thermotolerans* (now classified as *Lachancea thermotolerans*) (Jolly et al., 2014). Since that time, the number of non-*Saccharomyce*s yeasts available for commercial use from other yeast manufactures has increased, providing a wide variety of species.

Among these yeasts, many studies have proposed the use of *Starm. bacillaris* in mixed culture fermentations with *S. cerevisiae* strains, mainly due to the ability of the former to consume large quantities of fructose and to increase the glycerol and total acidity, while reducing the ethanol content in wines (Giaramida et al., 2012, Englezos et al., 2016a, Rantsiou et al., 2012, Sadoudi et al., 2012). We have previously shown that inoculation with *Starm. bacillaris* followed by inoculation of *S. cerevisiae* after 2 days of fermentation, leads to the production of Barbera wines with significant higher glycerol and lower ethanol levels, compared to the wines produced by the same *S. cerevisiae* strain in pure fermentation (Englezos et al., 2016a). However, for any practical applications, better knowledge about the impact of some winemaking practices that promote oxygen addition as well as the physiological and metabolic interactions between conventional and evolutionary engineered (optimized for glycerol production/alcohol reduction) *S. cerevisiae* and *Starm. bacillaris* strains must be known.

In the present study, we experimentally tested the impact of oxygen addition and combination of *Starm. bacillaris* with *S. cerevisiae* strains on yeast growth dynamics and wine profile in terms of technological performance and volatile composition. The results

showed that oxygen addition promoted the growth of the two *Starm. bacillaris* strains by increasing their generation number and, as a consequence, the sugar consumption in the first two days of fermentation. Thus, oxygen increased their survival and the coexistence for longer period with *S. cerevisiae* strains in mixed culture fermentations. This result agrees well with a previous study that demonstrated a decreased death rate of non-*Saccharomyces* yeasts like *T. delbrueckii* and *L. thermotolerans*, in the presence of *S. cerevisiae*, at higher levels of oxygen concentration (Hansen et al., 2001). It is generally acknowledged that the death of non-*Saccharomyces* yeasts in wine fermentations is attributed to their sensitivity to the increasing ethanol concentration in the must (Fleet, 2003). As a consequence, the non-*Saccharomyces* species that are present until the middle-end stages of the fermentation, may have also a higher tolerance to ethanol (Ciani and Comitini, 2015). Recent studies have demonstrated that *Starm. bacillaris* is able to withstand and grow at relative medium-high concentration of ethanol (Englezos et al, 2015; Tofalo et al., 2012). This fact led us to speculate, that the earlier death of *Starm. bacillaris* in mixed culture fermentations without oxygen addition, may be the result of the low oxygen levels in the medium. Further to the importance of this parameter on growth and performance of non-Sa*ccharomyces* yeasts (Hansen et al., 2001), several authors demonstrated that *S. cerevisiae* produced unknown metabolites that can negatively affect the performance of non-*Saccharomyces* in mixed fermentations (Albergaria et al., 2016; Ciani and Comitini, 2015). Among these metabolites, which are considered toxic for non-*Saccharomyces* yeasts, medium-chain fatty acids (hexanoic, octanoic and decanoic acids), were found in higher levels in pure fermented wines and probably influenced negatively the growth of *Starm. bacillaris* strains in the mixed culture fermentations (Viegas et al., 1989).

The association of *Starm. bacillaris* and *S. cerevisiae* strains also influenced significantly the fermentation kinetics resulting in wines with different compositions, in agreement with previous reports (Englezos et al., 2016a). However, the concentration of the conventional enological parameters in the sequentially inoculated wines were quite similar to that of IONYS WF® in pure culture. As expected, pure fermented wines with IONYS WF® had a marked increased glycerol production and decreased ethanol production than the conventional *S. cerevisiae* strain, due to the ability of the former to divert carbon towards glycerol and away from the production of ethanol (Tilloy et al., 2015). Mixed fermentations led to the production of wine with significantly higher levels of glycerol, total acidity and with reduced ethanol and pH, compared to the control wine fermented with Uvaferm BC® in pure culture. Additionally, glycerol production was significantly higher in the wines

produced by FC54 and IONYS WF®, compared to wines produced by IONYS WF® in pure fermentations without the addition of oxygen (condition I). These changes in mixed culture compared to pure culture fermentations are in agreement with previous studies using a conventional *S. cerevisiae* strain (Andorrà et al., 2010; Englezos et al., 2016a, Giaramida et al., 2013). However, it should be underlined that mixed culture fermentations with IONYS WF®, except the pair FC54 with IONYS WF® (condition I) ended up with residual sugar more than 4 g/L. Such negative effect may be ascribed to nutrient limitation, presence of growth-inhibitory compounds and cell-to-cell contact mechanism dependent on the presence of viable *Starm. bacillaris* cells at high concentration (Ciani and Comitini, 2015). The results, suggest that *S. cerevisiae* strain selection has a fundamental role on the fermentation of the mixed fermentations with *Starm. bacillaris* and *S. cerevisiae*, as previously described by Englezos et al. (2016a).

Additionally, in mixed fermentations using the conventional *S. cerevisiae* strain, pH reduction and concomitant increase of the total acidity respectively was observed at a level which could not be explained by the principal organic acid concentrations and/or any secondary compound analyzed in this study (citric, tartaric, succinic, malic, and lactic acid) (Supplementary Table S3). This character is probably related to the metabolic activity of *Starm. bacillaris* strains, which are good producers of α-ketoglutaric and pyruvic acids (Magyar et al., 2014). 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.

For any yeast strain and inoculation protocol, the impact that it has on flavour and aroma profile of the wines is of critical importance (Swiegers et al., 2005). The wines produced from sequential inoculations contained significantly lower volatile compounds compared to the respective controls, except for few individual compounds. For example, mixed fermented wines, independently of the couple used significantly increased the concentration of six aromatic compounds, namely 3-methylbenzaldehyde, benzaldehyde, γ-butyrolactone, hexanol, 2-methyl-1-propanol and linalool, compared to pure *S. cerevisiae* fermentation, indicating the presence of different metabolic pathways and interactions between the two species that probably are involved in the formation of individual volatile compounds.

Higher alcohols, are the most important group of volatile compounds produced by yeast and are divided in two subgroups, the aromatic and branched-chain alcohols (Moreno-Arribas et al., 2009). Among these alcohols, branched-chain higher alcohol, 2-methyl-1-

propanol is synthesized in the yeast cell through the Enrich-pathway, which involves the transamination of the amino acid precursor valine to form the α-ketoisovaleric acid, necessary for the formation of the corresponding alcohol (Swiegers et al., 2005). 2-methyl-1-propanol production was significantly higher in the wines produced by mixed cultures, compared to wines produced by pure Uvaferm BC® fermentation. This result agrees with previous findings, indicating the ability of mixed fermentations to produce high levels of this compound. However, in contrast to previous studies, low levels of the aromatic alcohol 2-phenylethanol, were found in this study (Andorrà et al., 2012, Englezos et al., 2016b). The use of different strains and/or fermentation conditions (such as, grape variety, temperature, pH, YAN, degree of turbidity etc.) may explain the differences.

Fermentation derived esters is a group of volatile compounds that are largely responsible for wine fruitiness and play a key role in the sensory composition of young red wines (Moreno-Arribas et al., 2009). Fermentative esters are mainly produced by the yeast metabolism through a reaction between alcohols with lipids and acetyl-CoA by acetyltransferase enzymes. The fermentation esters associated with wine fruitiness are divided in two groups: a. acetate esters (mainly: ethyl acetate, 2-phenyl ethyl acetate, 3-methyl-1-butanol acetate (isoamyl acetate), hexyl acetate) and b. ethyl fatty acid esters (mainly: ethyl butanoate, ethyl C_3 – ethyl C_{14}). Ester production was greatly influenced by the inoculation strategy rather than the strain combination, since mixed fermented wines tended to produce almost 3 times less esters compared the pure fermented wines. Ethyl decanoate, ethyl dodecanoate, ethyl hexanoate, ethyl octanoate, 2-phenylethyl acetate, hexyl acetate and 3-methyl-1-butanol acetate were the most abundant esters in all fermentations, however, their levels were significantly lower in sequentially inoculated wines compared to the control wines. These results verify previous findings by Sadoudi et al. (2012) on lower levels of acetate esters, however contradicting the levels of the major ethyl esters previously detected (Andorra et al., 2010, 2012). Additionally, Andorra et al. (2010) reported that co-inoculation of Macabeo must produced wines with increased concentration of ethyl esters, indicating that factors such as grape variety and inoculation delay of *S. cerevisiae* are involved in the esters formation in the mixed fermentations.

Terpenes concentration is a good parameter to reflect the fruity characteristics of the wines, even those produced from non varietal attribute grapes, like Barbera wines. Their levels were significantly higher in pure fermented wines with IONYS WF® and mixed fermented wines independently of the strains used compared to pure fermented wines with Uvaferm BC®. Mixed fermented wines with FC54 and IONYS WF® without the addition of

oxygen (condition I) presented the highest levels of terpenes, indicating a synergic effect of the two strains. Terpenes is a group of volatile compounds, which are not present in the must, and their content in the wines depends on the action of *β*-glycosidase enzymes which are produced by the yeast metabolism. Citronellol, linalool and nerolidol, which were investigated in this study, are the major representative compounds of this group and contribute to floral and fruity attributes. Their increase in the sequential inoculated wines, probably depend on the secretion of extracellular enzymes, like *β*-glycosidase by *Starm. bacillaris* strains, as previously reported by Englezos et al. (2015). Similarly, an indigenous *Starm. bacillaris* strain has been reported to increase terpene concentration in Sauvignon blanc wines produced by pure fermentation (Sadoudi et al., 2012). The same authors, reported significant lower concentrations of these metabolites in the wine co-inoculated with *S. cerevisiae*, probably due to negative interactions between the two species. The inoculation delay used by these authors was 24 hours while it was 48 hours in the present study, therefore it seems that length of inoculation delay and strain selection may impact the results.

Finally, PCA analysis including the main conventional enological parameters and volatile compounds revealed that the aroma profile of wines produced from co-fermentation of non-*Saccharomyces* with *S. cerevisiae* yeasts were different. This finding implies that the inoculation protocol (pure or mix fermentation) is more effective to modulate the chemical composition of the wines than the combination of *Starm. bacillaris* with *S. cerevisiae* strains in mixed culture fermentations. *S. cerevisiae* strain had a fundamental impact on aroma profile of pure fermented wines, in particular IONYS WF® strain increased significantly the concentrations of 2-phenyl ethanol, 2-phenylethyl acetate, 3-methyl-1-butanol acetate and other compounds associated with positive attributes. Lastly, the formation of off-odours linked to volatile compounds was measured by the concentration of ethyl acetate (nail polish remover) and volatile fatty acid formation (fatty) due to their negative sensory perception. Both compounds were found in levels lower than their odor detection threshold, impacting positively the overall aroma of the produced wines (Ribéreau Gayon et al. 2006).

5. Conclusion

In conclusion, the results obtained in this study demonstrated that oxygen addition, promoted *Starm. bacillaris* growth parameters and in particular their persistence in mixed fermentations. Nevertheless, this persistence did not influence greatly the chemical and volatile composition of the wines (or the majority of them), except the acetic content of the

577 wines. Mixed fermented wines showed a relative low concentration of volatile compounds,
578 compared to the respective control wines. Additionally, they did not contain high
579 concentrations of metabolites, which are considered harmful for wine quality and acceptance
580 from consumers.
581
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584
585 **References**
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702

Table 1

Growth parameters of *S. cerevisiae* strains in pure and mixed culture fermentations.

Strains and inoculation strategy	Condition	Generation number (g)	Doubling time (G)	Maximum specific growth rate (μmax, h^{-1})
Pure culture fermentations				
Uvaferm BC®	I	2.6 ± 0.2c,C	18.8 ± 1.3a,A	0.037 ± 0.003cd,C
	II	2.7 ± 0.2c,C	17.0 ± 0.1a,A	0.041 ± 0.000d,C
IONYS WF®	I	2.1 ± 0.6bc,β	23.8 ± 7.0a,α	0.031 ± 0.009bcd,β
	II	4.3 ± 0.3d,γ	11.1 ± 0.8a,α	0.063 ± 0.004e,γ
Mixed culture fermentations				
FC54 & Uvaferm BC®	I	1.7 ± 0.2b,B	28.1 ± 4.6a,A	0.025 ± 0.004bc,B
	II	0.6 ± 0.1a,A	83.8 ± 10.3b,B	0.008 ± 0.001a,A
MUT 5705 & Uvaferm BC®	I	1.6 ± 0.3b,B	31.5 ± 5.3a,A	0.022 ± 0.004b,B
	II	0.4 ± 0.0a,A	222.9 ± 14.9c,C	0.003 ± 0.000a,A
FC54 & IONYS WF®	I	2.4 ± 0.5bc,β	21.1 ± 5.0a,α	0.034 ± 0.007bcd,β
	II	0.7 ± 0.1a,α	72.8 ± 12.3b,β	0.010 ± 0.001a,α
MUT 5705 & IONYS WF®	I	1.7 ± 0.5b,β	29.7 ± 10.4a,α	0.025 ± 0.007bc,β
	II	0.2 ± 0.0a,α	212.0 ± 28.3c,γ	0.003 ± 0.000a,α
$Sign^1$		***	***	***
$Sign^2$		***	***	***
$Sign^3$		***	***	***

The values are means ± standard deviation of three independent experiments. Different superscript Latin letters within the same column indicate significant differences (Sig^1) between *S. cerevisiae* strains independent the inoculation strategy applied (Tukey-b test, $P < 0.05$). Different Upper Latin letters indicate significant differences (Sig^2) between *S. cerevisiae* strains in mixed fermentations performed with *S. cerevisiae* Uvaferm BC® (Tukey-b test, $p < 0.05$). Different Greek letters within the same column indicate significant differences (Sig^3) between *S. cerevisiae* strains in mixed fermentations performed with *S. cerevisiae* IONYS WF® (Tukey-b test, $p < 0.05$). $Sign^{1,2,3}$: *** indicate significance at $p < 0.001$. Condition I, II: without and with addition of oxygen, respectively.

Table 2

Growth parameters of *Starm. bacillaris* strains mixed culture fermentations.

Strains	Condition	Generation number (g)	Doubling time (G)	Maximum specific growth rate (μmax. h^{-1})
FC54	I	4.8 ± 0.1ab	20.4 ± 0.9e	0.034 ± 0.001a
	II	8.0 ± 0.5d	6.3 ± 0.4ab	0.111 ± 0.008e
MUT5705	I	4.2 ± 0.3ab	11.5 ± 0.8d	0.061 ± 0.004b
	II	5.6 ± 0.8b	8.3 ± 0.6c	0.084 ± 0.006c
Sign		***	***	***

The values are means ± standard deviation of six independent experiments. Different Latin letters within the same column indicate significant differences (Sig^{1}) between *Starm. bacillaris* strains independent the fermentation condition strategy applied (Tukey-b test, $p < 0.05$). $Sign^{-}$: *** indicate significance at $p < 0.001$. Condition I, II: without and with addition of oxygen, respectively.

Table 3

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

Strains and inoculation strategy	Condition	Residual sugars (g/L)	Acetic acid (g/L)	Succinic acid (g/L)	Glycerol (g/L)	Ethanol (% v/v)	$Y_{(g/s)}$ (g/g)	$Y_{(eth/s)}$ (g/g)	pH	TA (g/L)
Uvaferm BC®	I	0.5 ± 0.1a,AB	0.36 ± 0.01b,A	1.54 ± 0.01bc,C	9.3 ± 0.1a,A	14.7 ± 0.1d,B	0.038 ± 0.001a,A	0.060 ± 0.001c,B	3.35 ± 0.02c,A	6.70 ± 0.02a,A
	II	0.7 ± 0.1a,B	0.38 ± 0.02b,A	1.59 ± 0.01c,C	9.3 ± 0.1a,A	14.8 ± 0.1d,B	0.038 ± 0.001a,A	0.060 ± 0.001c,B	3.24 ± 0.06abc,A	6.72 ± 0.03a,A
IONYS WF®	I	3.1 ± 0.2b	0.12 ± 0.01a,α	2.71 ± 0.03f,β	18.4 ± 0.1d,α	13.6 ± 0.1a	0.076 ± 0.001d,α	0.056 ± 0.001a	3.19 ± 0.08ab	10.13 ± 0.02f,β
	II	1.1 ± 0.2a	0.19 ± 0.02a,β	2.63 ± 0.04e,β	18.5 ± 0.2d,α	13.8 ± 0.1b	0.075 ± 0.001d,α	0.056 ± 0.001a	3.11 ± 0.01a	9.63 ± 0.12e,α
FC54 & Uvaferm BC®	I	0.7 ± 0.1a,B	0.34 ± 0.02b,A	1.48 ± 0.01b,B	14.0 ± 0.1b,B	14.2 ± 0.1c,A	0.057 ± 001b,B	0.058 ± 0.001b,A	3.28 ± 0.02bc,A	7.69 ± 0.02b,B
	II	0.3 ± 0.3a,A	0.62 ± 0.01c,B	1.38 ± 0.01a,A	14.9 ± 0.2c,C	14.2 ± 0.1c,A	0.061 ± 0.001c,C	0.058 ± 0.001b,A	3.34 ± 0.05bc,A	7.97 ± 0.05c,C
MUT5705 & Uvaferm BC®	I	0.7 ± 0.1a,B	0.60 ± 0.01c,B	1.32 ± 0.01a,A	15.1 ± 0.1c,C	14.2 ± 0.1c,A	0.062 ± 0.001c,C	0.058 ± 0.001b,A	3.19 ± 0.08abc,A	7.94 ± 0.06c,C
	II	0.4 ± 0.1a,AB	0.55 ± 0.15c,B	1.37 ± 0.06a,A	15.1 ± 0.3c,C	14.3 ± 0.1c,A	0.061 ± 0.001c,C	0.058 ± 0.001b,A	3.22 ± 0.11abc,A	8.25 ± 0.20d,D
FC54 & IONYS WF®	I	2.6 ± 1.5b	0.36 ± 0.03b,γ	1.83 ± 0.04d,α	19.1 ± 0.2e,β	13.7 ± 0.1ab	0.078 ± 0.001e,β	0.056 ± 0.001a	3.2 ± 0.03abc	9.41 ± 0.15e,α
	II	32.3 ± 2.3	0.77 ± 0.03	0.98 ± 0.01	15.6 ± 0.2	12.4 ± 0.1	0.073 ± 0.001	0.058 ± 0.001	3.22 ± 0.01	7.04 ± 0.03
MUT 5705 & IONYS WF®	I	60.1 ± 2.5	0.79 ± 0.01	0.93 ± 0.01	15.6 ± 0.1	10.5 ± 0.2	0.084 ± 0.001	0.056 ± 0.001	3.14 ± 0.01	7.55 ± 0.08
	II	57.8 ± 10.7	0.63 ± 0.03	1.05 ± 0.06	14.2 ± 0.1	10.9 ± 0.7	0.075 ± 0.005	0.058 ± 0.001	3.26 ± 0.08	7.65 ± 0.21
$Sign^1$		***	***	***	***	***	***	***	***	***
$Sign^2$		***	***	***	***	***	***	***	*	***
$Sign^3$		NS	**	***	**	NS	*	NS	NS	***

The concentration of sugar at the beginning of experiment was 246.4 g/L (121.5 g/L glucose and 124.9 g/L fructose). The values are means ± standard deviation of three independent experiments. Different superscript Latin letters within the same column indicate significant differences (Sig^1) between pure and mixed culture fermentations (Tukey-b test, $p < 0.05$). Different Upper Latin letters within the same column indicate significant differences (Sig^2) between pure and mixed fermentations performed with *S. cerevisiae* Uvaferm BC® (Tukey-b test, $p < 0.05$). Different Greek letters within the same column indicate significant differences (Sig^3) pure and mixed fermentations performed with *S. cerevisiae* IONYS WF® (Tukey-b test, $p < 0.05$). Mixed fermentations with FC54 and IONYS WF® (condition II) and MUT and IONYS WF® (conditions I, II) were excluded from the statistical analysis due to high concentration of residual sugars. $Sign^{1,2,3}$: *, **, *** and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and no significant differences respectively. Condition I, II: without and with addition of oxygen. TA: titratable acidity; Y (eth/sugar consumption) = ethanol yield; Y (gly/sugar consumption) = glycerol yield.

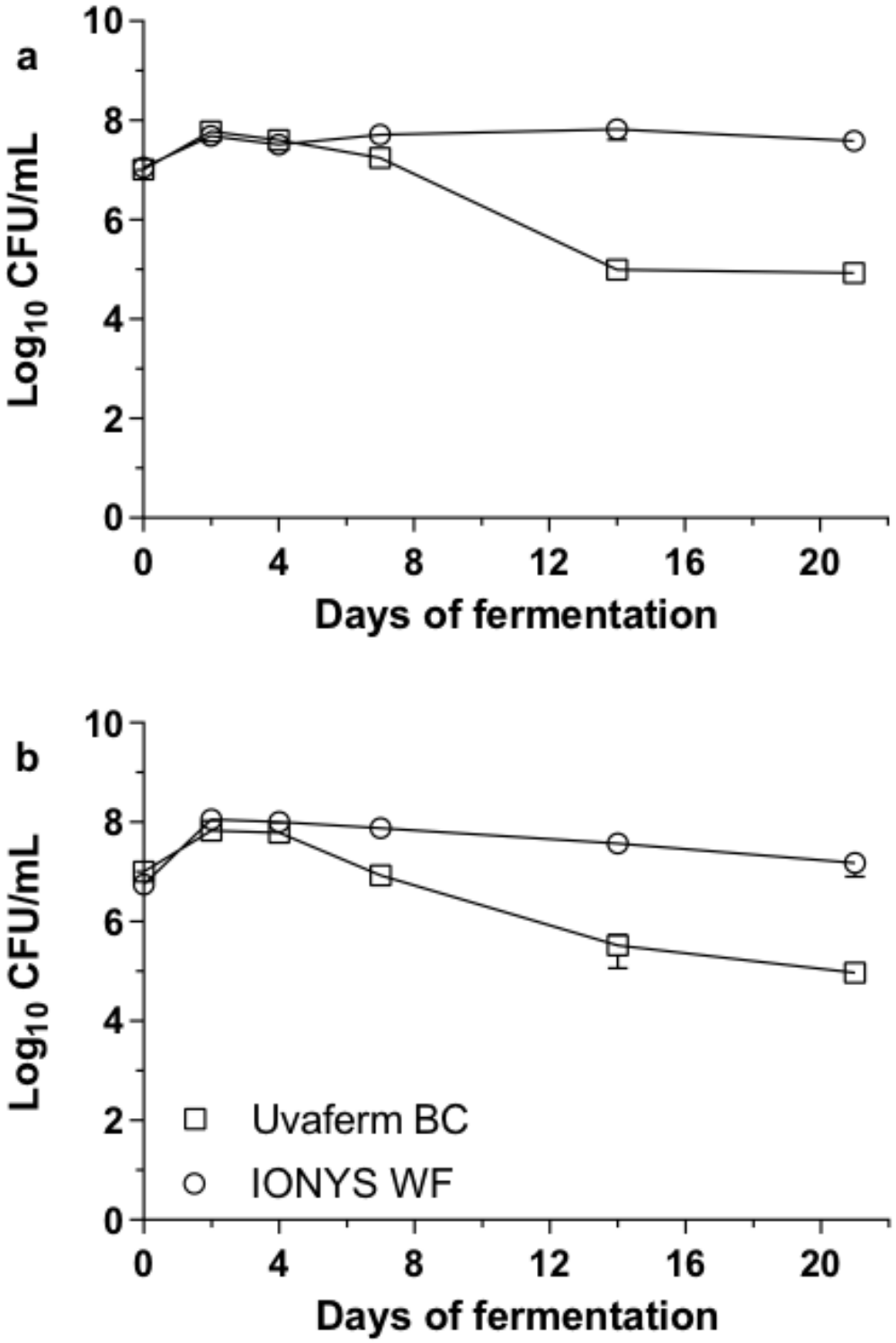
Figure captions

Fig.1 Growth dynamics of pure culture fermentations inoculated with *S. cerevisiae* strains. Fermentations were carried out in triplicate and the mean CFU/mL values ± standard deviations are shown. Panel a and b indicates fermentations under condition I and II respectively. Condition I, II: without and with addition of oxygen respectively.

Fig.2 Growth dynamics of mixed culture fermentations using different combinations of *Starm. bacillaris* and *S. cerevisiae* strains. Fermentations were carried out in triplicate and the mean CFU/mL values ± standard deviations are shown. Panel a and b indicates fermentations under condition I and II respectively. Condition I, II: without and with addition of oxygen respectively.

Fig.3 Principal component analysis of pure and mixed culture fermented wines. Loading plot (panel a) and score plot (panel b) of the first two principal components corresponding to PCA analysis of conventional enological parameters and volatile compounds. PFA, MFA: pure and mixed culture fermentations respectively.

782 **Fig.1**



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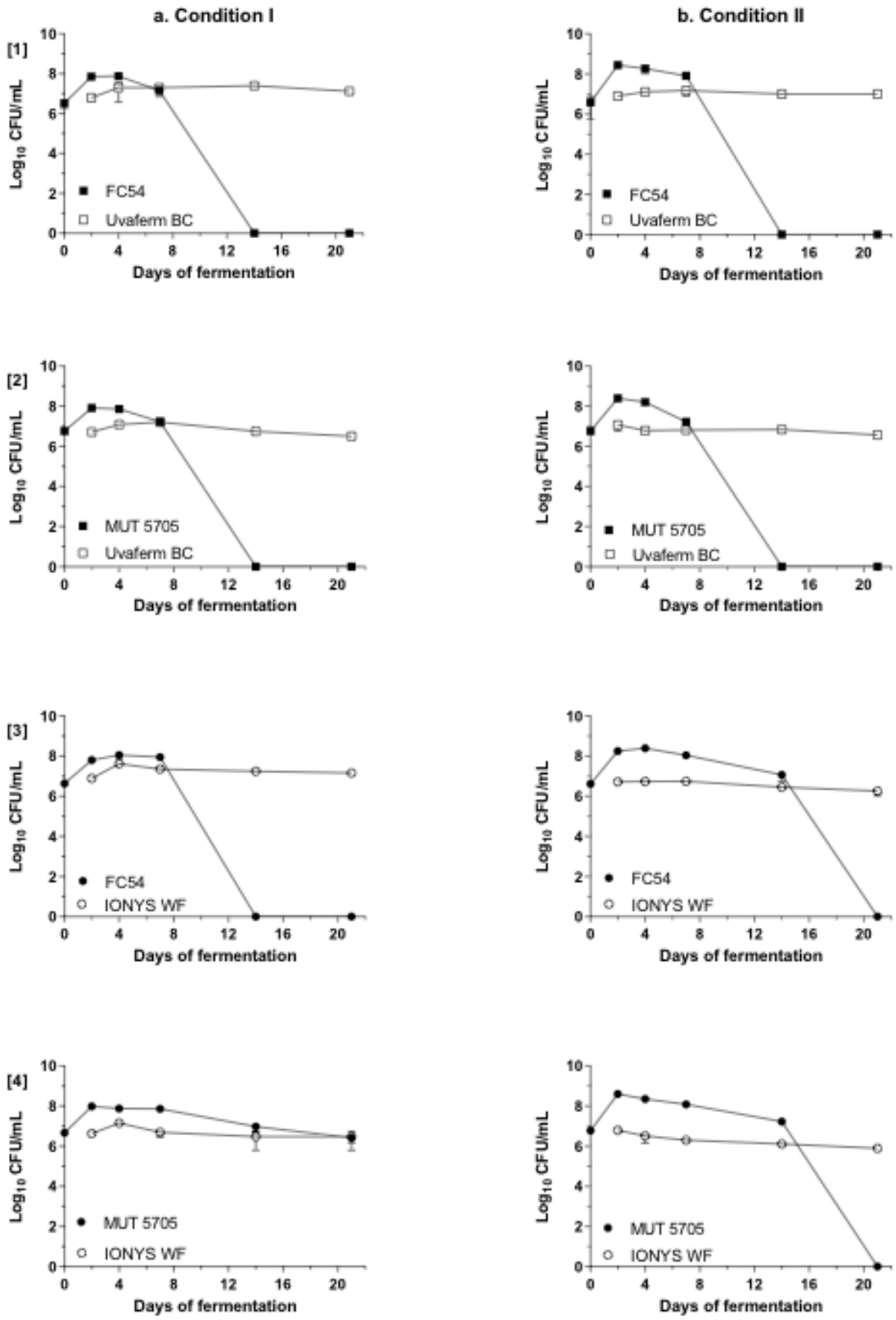
785

786 **Fig.2**



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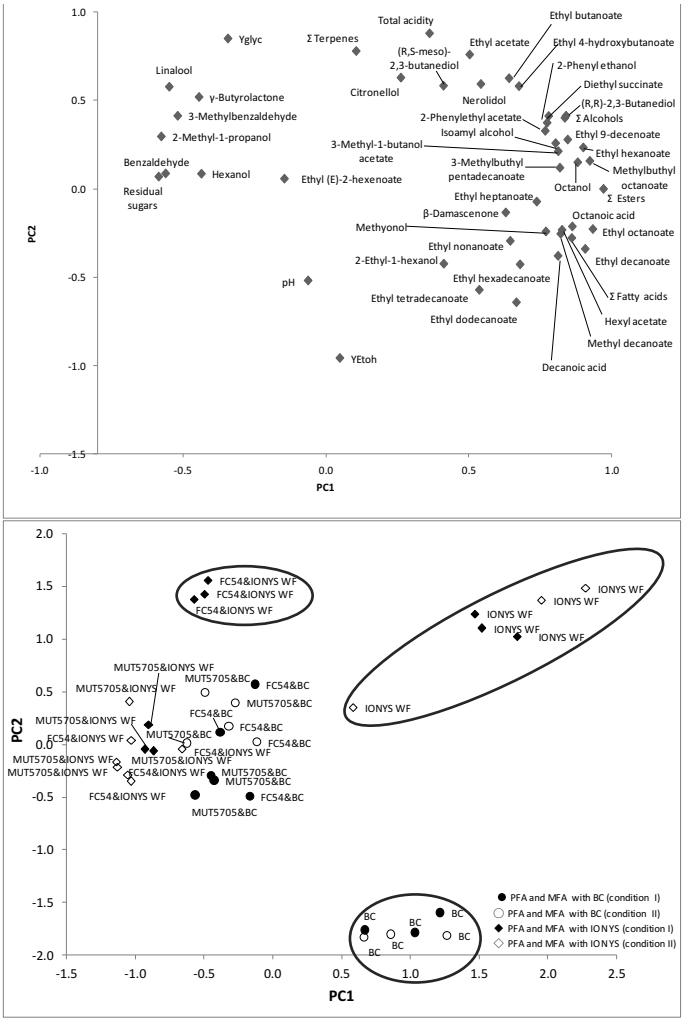
788

Fig.3


Yglyc
Σ Terpenes
Total acidity
Ethyl butanoate
Ethyl acetate
Ethyl 4-hydroxybutanoate
(R,S-meso)-2,3-butanediol
2-Phenyl ethanol
Linalool
Citronellol
Diethyl succinate
γ-Butyrolactone
Nerolidol
(R,R)-2,3-Butanediol
3-Methylbenzaldehyde
2-Phenylethyl acetate
Σ Alcohols
2-Methyl-1-propanol
Isoamyl alcohol
Ethyl 9-decenoate
3-Methyl-1-butanol acetate
Ethyl hexanoate
Benzaldehyde
3-Methylbuthyl pentadecanoate
Methylbuthyl octanoate
Hexanol
Ethyl (E)-2-hexenoate
Octanol
Σ Esters
Residual sugars
Ethyl heptanoate
β-Damascenone
Octanoic acid
Methyonol
Ethyl octanoate
Ethyl nonanoate
2-Ethyl-1-hexanol
Ethyl decanoate
pH
Ethyl hexadecanoate
Σ Fatty acids
Ethyl tetradecanoate
Hexyl acetate
Ethyl dodecanoate
Methyl decanoate
YEtoh
Decanoic acid
PC1
PC2
FC54&IONYS WF
IONYS WF
MUT5705&IONYS WF
MUT5705&BC
FC54&BC
BC
PFA and MFA with BC (condition I)
PFA and MFA with BC (condition II)
PFA and MFA with IONYS (condition I)
PFA and MFA with IONYS (condition II)
PC1
PC2