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1 **Volatile profiles and chromatic characteristics of red wines produced with *Starmerella***
2 ***bacillaris* and *Saccharomyces cerevisiae***

3

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18 **ABSTRACT**

19

20 The use of mixed fermentations with *Starmerella bacillaris* and *Saccharomyces cerevisiae* is
21 gaining attention in recent years due to their ability to modulate the metabolites production of
22 enological interest. In the present study, four of the most popular planted red grape varieties
23 (Cabernet sauvignon, Merlot, Pinot noir and Shiraz) were fermented using the
24 aforementioned species and two different inoculation protocols (inoculation of *S. cerevisiae*
25 after 24 and 48 h from the *Starm. bacillaris* inoculation), in order to evaluate their impact on
26 the volatile composition and chromatic characteristics of wines. Analysis from chemical
27 composition showed that titratable acidity and glycerol content exhibited marked differences
28 among wines after fermentation. For volatile compounds, mixed fermented wines using an
29 inoculation delay of 48 h led to reduction of volatile compounds (mainly esters). A shorter
30 24 h delay produced wines with higher values of color intensity than pure fermented wines.
31 The differences observed between the inoculation protocols can be explained by the growth
32 dynamics of both species during fermentation. These findings suggest that mixed
33 fermentations posed a great potential in reducing metabolites which are considered negative
34 for wine quality (mainly ethyl acetate and volatile fatty acids) and with an improvement of
35 the chromatic profile of the wines.

36

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

39

40 1. Introduction

41

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

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

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

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

83 Among non-*Saccharomyces* yeasts, *Starmerella bacillaris* (synonym *Candida*
84 *zemplanina*) has been described as a yeast with a positive effect on wine quality. Generally,
85 strains of this species are known as high producers of glycerol, pyruvic acid and low
86 producers of ethanol (Magyar, Nyitrai-Sárdy, Leskó, Pomázi, & Kállay, 2014; Mestre,
87 Maturano, Combina, Mercado, Toro, & Vasquez, 2017; Rantsiou et al., 2017, Zara et al.
88 2014). However, contradictory results were observed for acetic acid production, indicating
89 intraspecific variation (Englezos, Giacosa, Rantsiou, Rolle, & Cocolin, 2017). These
90 phenotypic characteristics and its ability to tolerate relatively high concentrations of ethanol
91 enable the use of this non-*Saccharomyces* yeast in mixed fermentations with selected *S.*
92 *cerevisiae* strains. In the last years, several studies have made significant progresses in many
93 aspects including the importance of inoculation density, timing, and combination of strains
94 in the organoleptic properties of wines (Comitini et al., 2011, Englezos et al., 2016a,
95 Sadoudi et al., 2012). However, several efforts must be undertaken in order to establish a
96 link between an inoculation protocol and chemical composition of wines using the same
97 couple of strains and fermentation conditions. Understanding the nature and origins of wine
98 volatile metabolites may provide the potential to manipulate yeast ecology towards the
99 production of wines with flavour, aroma, and chromatic characteristics desired by targeted
100 consumer groups.

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

106

107 **2. Materials and methods**

108

109 2.1. Strains

110

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

118

119 2.2. Must preparation

120

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

134

135 2.3. Fermentation trials

136

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

142 semi-anaerobic conditions, using air-locks to maintain semi-anaerobic conditions during
143 fermentation. Pure and mixed culture fermentations were inoculated with 5.0×10^6 cells/mL,
144 which corresponds to a dose of 25 g/hL of active dry yeast (ADY) (Lallemand SAS,
145 Toulouse, France), previously activated in a sterile glucose solution (5 %), incubated at 37
146 °C. After inoculation, the musts were incubated at 25 °C without agitation. The fermentation
147 process was tracked by plate counting and chemical analysis described below. Fermentations
148 were considered finished when the residual sugars were less than 2 g/L. Afterwards, the
149 chemical composition, chromatic characteristics and volatile profiles of wines was analysed.

150

151 2.4. Microbiological analysis

152

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

163

164 2.5. Must and wine analysis

165

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

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

176 Space Solid Phase Micro-Extraction (HS-SPME) followed by Gas Chromatography-Mass
177 Spectroscopy (GC-MS). Briefly, 5 mL of sample was placed in a 20 mL headspace glass vial,
178 containing 2 g of NaCl and 200 μ L of internal standard (prepared by adding 15.5 mg/L of 1-
179 heptanol (analytical grade, 99.95%, Sigma, Milan, Italy) in a 10 % v/v ethanol solution).
180 Afterwards, the vials were tightly sealed with 18-mm diameter screw caps with silicon
181 septum (Supelco, Bellefonte, PA, USA) and shaken carefully to dissolve NaCl. Sample vials
182 were placed onto a Gerstel MPS2 XL (Gerstel, Baltimore, MD, USA) auto sampling device.
183 The chromatographic conditions were as those reported by Englezos et al. (2018). Briefly,
184 the program consisted of heating the vial at 40 °C for 10 min, inserting the fiber into the
185 headspace of the sample vials for 20 min at 40 °C and desorbed in the GC inlet in splitless
186 mode for 5 min at 250 °C, the ion source temperature was 150 °C and interface was 280 °C.
187 Analyses were performed on an Agilent 7890C gas chromatograph (Little Falls, DE, USA)
188 coupled to an Agilent 5975 mass selective detector and a DB-WAX capillary column (30 m x
189 0.25 mm inner diameter, 0.25 mm film thickness, J&W Scientific Inc., Folsom, CA, USA).
190 The software used was Agilent G1702-90057 MSD ChemStation. The oven temperature was
191 started at 40 °C, held for 5 min, increased to 200 °C at 2°C/min, held at that temperature for
192 10 min and increased to 220 °C at 5 °C/min. The carrier gas was Helium with a flow rate of 1
193 mL/min in constant flow mode. Mass spectra detection was carried out in total ion current
194 mode (TIC mode) with a scan range of 33-330 m/z. The detection of the volatile compounds
195 was carried by matching the retention time of each compound with either reported in the
196 literature and in the online database (<http://webbook.nist.gov/chemistry/>) and pure standards,
197 whenever available (2,3-butanediol isomers mixture, 2-methyl-1-propanol, 1-octanol, 2-
198 phenylethanol, diethyl succinate, ethyl acetate, ethyl decanoate, ethyl dodecanoate, ethyl
199 heptanoate, ethyl hexanoate, ethyl nonanoate, ethyl octanoate, ethyl phenylacetate, hexanol,
200 hexanoic acid, hexyl acetate, linalool, methyl decanoate, octanoic acid and β -damascenone,
201 all from Sigma). Concentration of each identified compound was calculated by a calibration
202 with standard solutions analysed under the same conditions as the wine samples. Each
203 replicate was analysed in duplicate.

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

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

213

214 2.7. Statistical analyses

215

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

222

223 3. Results and discussion

224

225 3.1. Yeast growth during fermentation

226

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

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

244 concentration of around 1.0×10^8 CFU/mL, which is almost 50 % higher than those of 24 h
245 delay (around 5.0×10^7 CFU/mL). This increase in *Starm. bacillaris* viable cells led to a
246 reduction in the number of viable cells of *S. cerevisiae* and almost 50% lower viable cell
247 population was registered compared to pure culture fermentations with *S. cerevisiae*. This
248 finding was in agreement with previous studies that demonstrated that the early growth of
249 *Starm. bacillaris* negatively influenced the growth of *S. cerevisiae* (Englezos et al., 2016a,
250 Sadoudi et al., 2012). Such negative effect may be ascribed to the enhanced competition of
251 non-*Saccharomyces*, which was probably caused by completion of nutrients or cell-to-cell
252 contact mechanisms as previously noted by Ciani & Comitini (2015), Albergaria & Arneborg
253 (2016). Fermentation kinetics were in accordance with growth kinetics, in particular the sugar
254 consumption of mixed fermentations with 24h delay was comparable to that of *S. cerevisiae*
255 in pure culture. Conversely, the dominance of *Starm. bacillaris* on *S. cerevisiae* resulted in
256 significantly lower sugar consumption rates.

257

258 3.2. Basic oenological parameters

259

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

272 As a result, the inoculation protocol and in particular the inoculation time of *S.*
273 *cerevisiae* influenced greatly the chemical composition of the wines. Compared to wines
274 produced by *S. cerevisiae* in pure culture, the use of mixed cultures produced wines with
275 more glycerol and less ethanol. Wines fermented using an inoculation delay of 24 and 48 h
276 always contained higher levels of glycerol (1.1–5.9 g/L more glycerol) and lower ethanol
277 (0.2–0.6 % v/v less ethanol). These differences were lower for the 24h inoculation delay.

278 Glycerol and ethanol yields were calculated using the data of sugar consumption and glycerol
279 and ethanol production, respectively, at the end of fermentation. Mixed fermentations were
280 distinguished by a relatively high glycerol and low ethanol yields. These differences were
281 higher for the 48h inoculation delay. Therefore, the time of *S. cerevisiae* inoculation affected
282 the production of metabolites, confirming earlier findings (Englezos et al., 2016a,b). In
283 particular, glycerol could have a favourable impact on wine sensory perception. Due to its
284 non-aromatic nature, it can significantly contribute to wine structure/body perception. In
285 wines, levels between 7 and 15 g/L are frequently encountered and higher levels are thought
286 to contribute also to the smoothness and viscosity of wine (Scanes, Hohmann, & Priori,
287 1998). Therefore, high glycerol levels contribute to wine traits and indicate that the
288 overproduction of glycerol by *Starm. bacillaris* in mixed fermentations could improve the
289 sensory qualities of the wine (Swiegers et al., 2005). Conversely, acetic acid significantly
290 increased by increasing the delay of *S. cerevisiae*, although all the wines contained less than
291 0.43 g/L.

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

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

324 Chromatic characteristics of wines produced by pure and mixed culture fermentations
325 are presented in Table 2. CIEL*a*b* color measurements indicated that mixed fermented
326 wines with 24 and 48 hours delay had a lower degree of lightness (L*) compared to pure
327 fermented wines. Wines produced by mixed starter cultures also had the highest amount of
328 redness (a*), yellowness (b*), and color intensity. These changes may be explained by the
329 reduction of pH in wines due to the metabolic activity of *Starm. bacillaris* which is a good
330 producer of organic acids (Magyar et al., 2014). Furthermore, in Merlot and Shiraz trials a
331 significant decrease of the color hue parameter was observed, which is negatively influenced
332 by the red color contribution in relation to the yellow component. Lower values were found
333 in the mixed trials, with a significant effect also of the *S. cerevisiae* inoculation delay (24 or
334 48 h), thus meaning a higher red color contribution. This effect was not significantly
335 observed in C. sauvignon and Pinot noir samples. Together with the evaluation of the color
336 components for each produced wine, the ΔE^* color difference was assessed between pure
337 fermentations and each mixed fermentation sample (Table 2). When considering red wines, a
338 ΔE^* value of 3 was assessed as the general color tolerance perceptible by the human eye
339 (Martínez, Melgosa, Pérez, Hita, & Negueruela, 2001). In our experiment, all the mixed
340 fermentation wines evidenced a perceptible color difference (ΔE^* higher than 3) when
341 compared with pure fermentations, with the exception of the mixed 24h experiment in Pinot
342 noir. For all the varieties considered the mixed 24h samples evidenced less overall color
343 differences than mixed 48h samples (lower ΔE^* parameter) both in relation to pure
344 fermentations.

345

346 3.3 Volatile composition

347

348 Aroma compounds give the wine its typical odour. Yeast species represents one of the
349 most important factors affecting wine fermentative volatile composition. Esters and alcohols
350 mainly influence the general volatile composition of young red wines, while varietal
351 components such as terpenes and norisoprenoids are present depending by the grape content
352 (López, Ferreira, Hernández, & Cacho, 1999). Therefore, the fermentation process has an
353 important role in defining the key aroma components of a young wine (Hirst & Richter,
354 2016). Table 3 lists the volatile compounds identified in wines fermented with different
355 inoculation protocols. Thirty-five volatile compounds were listed and grouped in 4 aroma
356 families, including alcohols, esters, fatty acids, terpenes and C₁₃-norisoprenoids and other
357 compounds. Esters were the most abundant group in the wines, followed by alcohols, while
358 fatty acids, terpenes and C₁₃-norisoprenoids were found to have smaller figures. In general,
359 the content of the most volatile compounds varied significantly in function of the
360 employment of *Starm. bacillaris* and of the inoculation delay of *S. cerevisiae*, while it was
361 not influenced by the grape variety used. Wines fermented with mixed yeast cultures were
362 distinct for their general lack of volatile compounds compared to pure fermented wines with
363 *S. cerevisiae*. This reduction was particularly evident in wines in which *S. cerevisiae* was
364 inoculated with a delay of 48 h with respect to the inoculation of *Starm. bacillaris*.

365

366 3.3.1 Higher alcohols

367

368 In addition to ethanol, yeasts also produce a large number of long-chain alcohols.
369 These alcohols, called higher alcohols (also known as fusel alcohols) are secondary yeast
370 metabolites produced from amino acid catabolism via the Ehrlich pathway (Hazelwood et al.
371 2008). Excessive concentrations of higher alcohols are strongly correlated with strong and
372 pungent smell and taste, whereas optimal levels can impart fruity character in wines
373 (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Both pure and mixed fermentations,
374 independently of the grape variety used, produced the same levels of higher alcohols, at
375 concentrations ranging from 17.8 mg/L to 21.8 mg/L, well below the threshold of 300 mg/L
376 which have been found to contribute positively to wine complexity (Rapp & Mandery, 1986).
377 This was true except Pinot noir wines, in which mixed fermented wine with a sequential
378 delay of 48 h was distinguished from the other inoculation protocols by lower amounts of
379 total higher alcohols (18.5 mg/L vs 22.6 mg/L).

380 Due to the strict correlation with yeast metabolism, the concentration of each higher
381 alcohol in wine represents an important variable for yeast differentiation (Swiegers et al.,
382 2005). A total of 7 alcohols were identified across the wines, with isoamylic alcohol, 2,3-
383 butanediol (1), 2-methyl-1-propanol (isobutanol) and 2-phenyl ethanol as the major
384 representatives. However, none of them surpassed their odour threshold (Cullere, Escudero,
385 Cacho & Ferrerira, 2004; Ferreira, Lopez & Cacho, 2000; Guth, 1997; Li, 2006). Isobutanol
386 and isoamylic alcohol are produced by yeasts during alcoholic fermentation through the
387 conversion of leucine and isoleucine, respectively via Ehrlich pathway (Hazelwood et al.,
388 2005). Mixed fermentations led to a lower production of isoamylic alcohol (herbaceous
389 notes) and octanol (fruity notes) for all the grape varieties used. Similar results have been
390 observed by Sadoudi et al. (2012) in Sauvignon blanc must fermented with *Starm. bacillaris*
391 and *S. cerevisiae*, using 24 h delay. To the contrary, isobutanol production, which contributes
392 to wine aroma with further herbaceous notes, tended to increase in sequential mixed
393 fermented wines with increasing the inoculation delay of *S. cerevisiae*. The concentration of
394 2-phenyl ethanol, an aromatic compound associated with pleasant floral and rose notes, was
395 not significantly different between pure and mixed fermented wines produced from Shiraz
396 and Merlot grapes, in agreement with recent studies (Sadoudi et al., 2012; Zara et al., 2014).
397 C. sauvignon wines produced from mixed culture fermentation with 48 h delay contained
398 significant higher level of this metabolite. Conversely, Pinot noir wines fermented with the
399 above mentioned inoculation protocol contained significant lower level of this metabolite.
400 Finally, (R,R; R,S-meso) 2,3-butanediol was the only higher alcohol that didn't respond to
401 yeast inoculation protocol, except for C. sauvignon wines in which the concentration of R,S-
402 meso, 2,3-butanediol increased in wines fermented with mixed cultures, using an
403 inoculation delay of 48 h. These results let us hypothesize that both species have different
404 preference on amino acid consumption and, as a result, the formation of individual higher
405 alcohols is strictly correlated to the concentration of the respective amino acids in must.

406

407 3.3.2 Esters

408

409 Yeast derived esters are a class of volatile compounds with positive contribution to
410 wine aroma, through the introduction of fruity and floral notes (Swiegers et al., 2005). Even
411 small changes in the concentration of these secondary metabolites can have tangible effects
412 on the sensory evaluation of the final product. Two classes of esters are synthesized by yeasts
413 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 synthesize 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

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

483

484 3.3.4 Fatty acids

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

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

505

506 3.3.5 Terpenes and C₁₃-norisoprenoids

507

508 The last class of volatile compounds terpenes and C₁₃-norisoprenoids have a
509 significant influence on the fruity and floral character of wines. Terpenes and C₁₃-
510 norisoprenoids contribute to the varietal character of many wines, especially aromatic
511 cultivars (Swiegers et al., 2005). During fermentation, this group of compounds also present
512 in grapes in glycoside form can be released through acid-induced hydrolysis by grape
513 endogenous and yeast hydrolytic enzymes (Moreno-Arribas & Polo, 2009). Yeast species
514 have been shown to have different expression levels and activities of these enzymes (Strauss,

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

528

529 **4. Conclusion**

530

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

540

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542

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546

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693

694 **Table 1**

695 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. | | | *** | *** | *** | *** | *** | *** | *** | *** | *** |

696 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
697 the three wines produced. TA: titratable acidity, Ygly (glycerol/sugar consumption): glycerol yield and Yeth (ethanol/sugar consumption): ethanol yield.

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706 **Table 2**

707 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. | | *** | *** | ** | *** | * | |

708 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,
709 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
710 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

| Metabolites | Cabernet Sauvignon (Cs) | | | Merlot (M) | | | Pinot noir (Pn) | | | Shiraz (S) | | | Statistical differences | | | | | | |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|-------------------------|----------------------------|---------------------------|---------------------------|---------------------------|---------------------------|-------------------------|-------------------------|---------|-------|-----|-----|-----|-----|
| | 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 |
| <i>Alcohols</i> | | | | | | | | | | | | | | | | | | | |
| Benzyl 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 | ** | ** | *** |
| Isoamyl 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 |
| <i>Esters</i> | | | | | | | | | | | | | | | | | | | |
| 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 | *** | *** | *** | *** | *** | *** | *** |

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|--|---------------------------|---------------------------|--------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|----------------------------|---------------------------|-----|-----|-----|-----|-----|-----|-----|
| 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 | *** | *** | *** | *** | *** | *** | *** |
| <i>Fatty acids</i> | | | | | | | | | | | | | | | | | | | |
| 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 | *** | *** | *** | *** | *** | *** | *** |
| <i>Terpenes and C13-norisoprenoids</i> | | | | | | | | | | | | | | | | | | | |
| 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 | *** |
| <i>Other metabolites</i> | | | | | | | | | | | | | | | | | | | |
| 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 |

713 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: *, **,

714 *** and ns indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively. a: variety and b: interaction

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719 **Figure captions**

720

721 **Fig.1**

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

728

729 **Fig.2**

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

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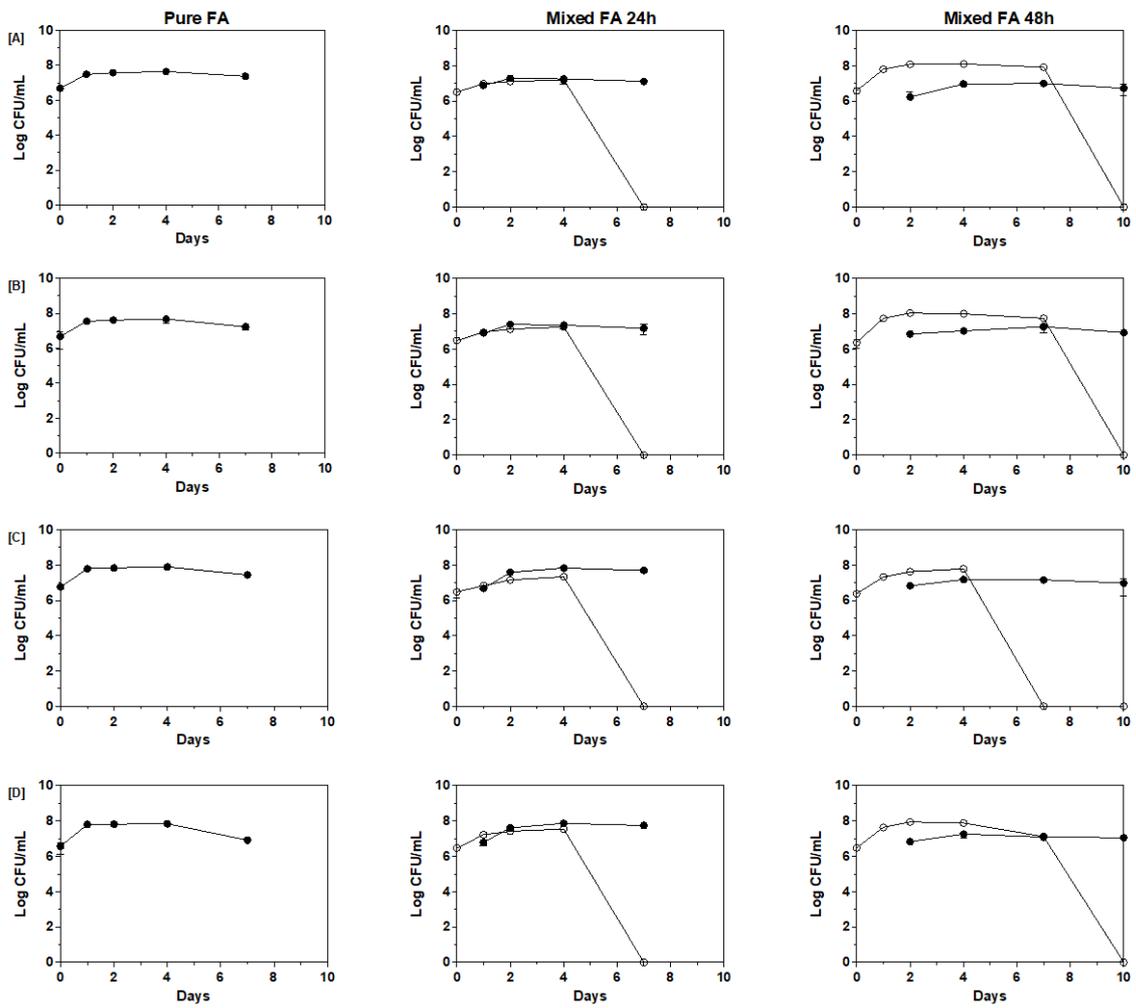
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756 **Figures**

757 **Fig.1**



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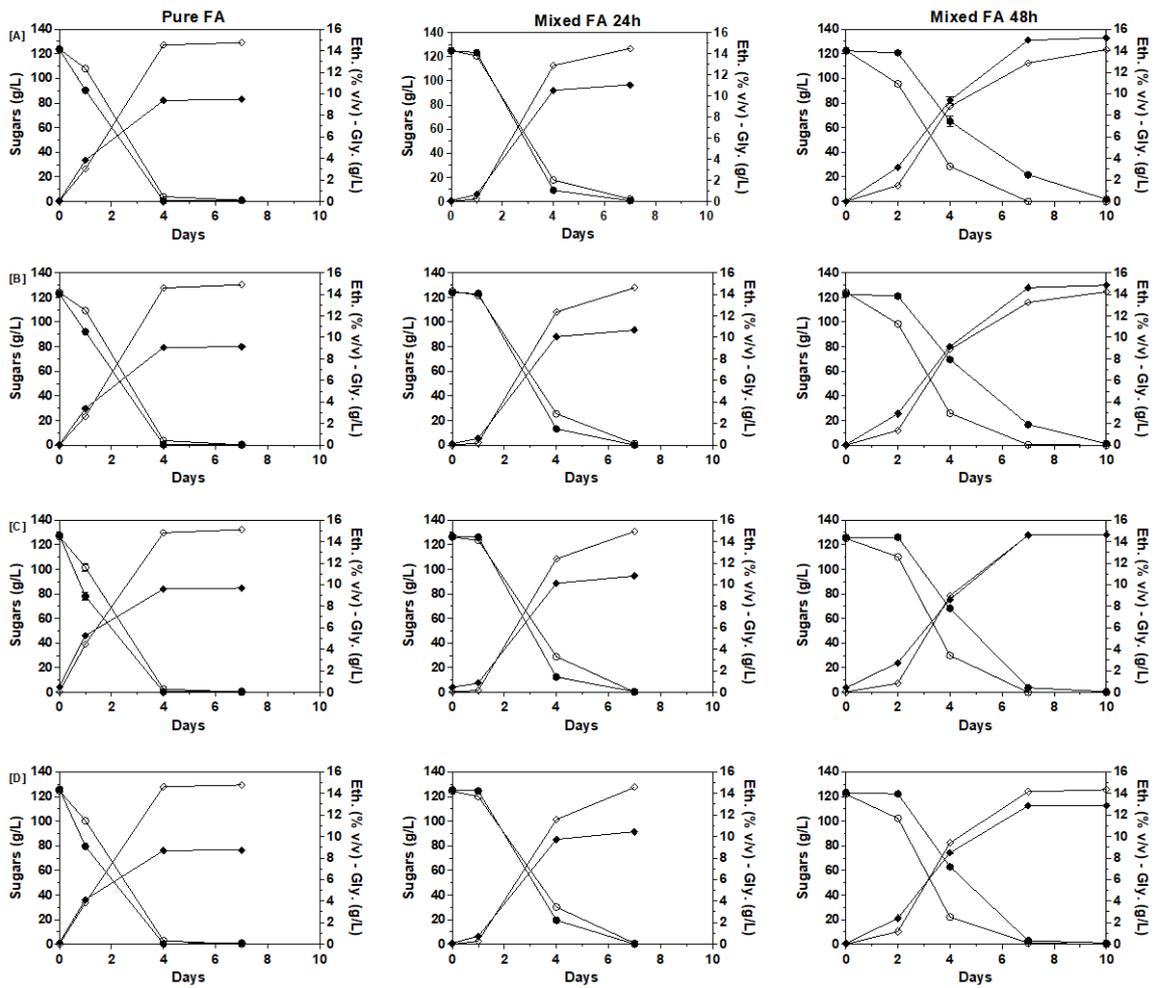
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