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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1   **Oxygen availability and strain combination modulate yeast growth dynamics in mixed**

2   **culture fermentations of grape must with *Starmerella bacillaris* and *Saccharomyces***

3   ***cerevisiae***

4

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35 **ABSTRACT**

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37 *Starmerella bacillaris* (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast that has

38 been proposed as a co-inoculant of selected *Saccharomyces cerevisiae* strains in mixed

39 culture fermentations to enhance the analytical composition of the wines. In order to acquire

40 further knowledge on the metabolic interactions between these two species, in this study we

41 investigated the impact of oxygen addition and combination of *Starm. bacillaris* with *S.*

42 *cerevisiae* strains on the microbial growth and metabolite production. Fermentations were

43 carried out under two different conditions of oxygen availability. Oxygen availability and

44 strain combination clearly influenced the population dynamics throughout the fermentation.

45 Oxygen concentration increased the survival time of *Starm. bacillaris* and decreased the

46 growth rate of *S. cerevisiae* strains in mixed culture fermentations, whereas it did not affect

47 the growth of the latter in pure culture fermentations. This study reveals new knowledge

48 about the influence of oxygen availability on the successional evolution of yeast species

49 during wine fermentation.

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

52 Volatile metabolites

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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,

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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

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

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147 **2. Materials and methods**

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149 *2.1. Strains*

150

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

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160 *2.2. Fermentation trials*

161

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

174   inoculated 48 h after *Starm. bacillaris* inoculation. Mixed fermentations were carried out

175   using the 4 different combinations of *Starm. bacillaris* and *S. cerevisiae* strains (FC54 and

176   Uvaferm BC®, MUT 5705 and Uvaferm BC®, FC54 and IONYS WF®, MUT 5705 and

177   IONYS WF®). All strains were inoculated as active dry yeast (ADY) and rehydrated

178   according to manufacturer's instructions, except for strain MUT 5705 which was preadapted

179   in the same must for 48 h at 25 °C. Prior to inoculation, yeast cells were counted by a Thoma

180   hemocytometer chamber using methylene blue dye as a marker of cell viability. Then,

181   appropriate amounts of inoculum were used to reach an initial cell population of about 5.0 x

182   10^6 cells/mL, that corresponds to a dose of 25 g/hL of ADY.

183   Triplicate fermentations were performed without and with the addition of oxygen

184   (condition I and II respectively) in 1000 mL sterile glass bottles containing 800 mL Barbera

185   grape must at 25 °C without agitation. After inoculation the bottles were closed with air locks

186   containing sterile paraffin oil, to allow only the CO_2 to escape from the fermenting medium

187   and prevent external contamination. For oxygen addition, the fermenting musts were

188   saturated (about 7 mg/L of O_2) with pure oxygen (Rivoira, Milan, Italy) 24 and 48 hours after

189   yeast inoculation. To estimate the dissolution of oxygen during fermentation, another grape

190   must sample (inoculated with Uvaferm BC®) was micro-oxygenated and the oxygen content

191   was controlled using a Nomasense oxygen analyzer (Nomacorc, SA). In order to improve O_2

192   solubility, the must was maintained in medium/high agitation (about 150 rev min^{-1}) on a

193   rotary shaker (Velp Scientifica, Monza and Brianza, Italy) during oxygen addition. Samples

194   were micro-oxygenated with Ox-evolution and ceramic diffuser (Intec, Pramaggiore, VE,

195   Italy) with 10 mg/min oxygen flow rate for 10 minutes.

196   Fermentations were considered to be finished when the level of residual sugars was

197   below 2 g/L. At this time, wines produced under the two conditions were kept at 4 °C to

198   allow sedimentation of the solid parts. Wines were poured in 33cl glass bottles, supplemented

199   with SO_2 in order to achieve a final concentration of 50 mg/L of total SO_2 and kept at 4 °C

200   and analysed for chemical and volatile composition.

201

202   *2.3. Microbiological analysis*

203

204   The growth dynamics of the inoculated strains during the fermentation were

205   determined by counting the viable cell population on WLN medium. Aliquots of 1 mL were

206   periodically collected from each fermentation and serially diluted in sterile Ringer's solution

207   (Oxoid, Milan, Italy). Colony counting was performed after 3-5 days of incubation at 28 °C.

208   The bromocresol green present in WLN medium acts as a dye, which *Starm. bacillaris* strains

209   metabolize and therefore form flat, light to intense green colonies due to the acidogenic

210   nature of this species (Sipiczki, 2004). On the other hand, *S. cerevisiae* strains do not take up

211   this dye in the same way (strain dependent) and as a consequence generally form creamy

212   white colonies, with different light shades of green on the top, facilitating the concurrent

213   enumeration of the two species throughout the fermentation process.

214

215   *2.4. Calculation of yeast growth performance parameters*

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217   The maximum specific growth rate (μ_{max}), defined as the rate of increase in cell

218   number per time unit was calculated as follows: μ_{max}= (lnNf –lnN0)/(tf-t0), where Nf the

219   yeast concentration (cfu/mL) at the final time point considered (tf) and N0 the initial yeast

220   concentration, at the beginning of fermentation (t0). The generation number (g) defined as the

221   number of cell divisions was calculated as follows: g = (logNf – logN0)/Log2. Generation

222   time or doubling time (G) is called the time required for a cell to duplicate and divide itself

223   and was calculated using the following formula: G=ln(2)/μ_{max}. All equations were calculated

224   with the data from the exponential phase of growth for each strain. Strains were compared on

225   the basis of their maximum population production and the time employed to reach this value.

226

227   *2.5. Chemical analysis*

228

229   Extracellular glucose, fructose, glycerol, primary organic acids (g/L) and ethanol (%

230   v/v) concentrations were quantified after 2 days and at the end of fermentation, using an

231   Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) instrument,

232   equipped with an Aminex HPX-87H cation exchange column. The column was eluted with

233   0.0065 mol/L sulfuric acid (H_2SO_4) at a flow rate of 0.8 mL/min and a column temperature of

234   65 °C, using the protocols described by Rolle et al. (2012). The pH of the wines was

235   determined by using the InoLab 730 pH meter (WTW, Weilheim, DE), while total acidity

236   (TA) was determined and expressed in g/L of tartaric acid according to the official method

237   proposed by the International Organization of Vine and Wine (OIV, 2008). The initial YAN

238   concentration in the must, in terms of inorganic and organic nitrogen was determined

239   spectrophotometrically by using two enzymatic kits according to the manufacturer's

240   instructions (Megazyme International, Bray, Ireland).

241

242   *2.6. Volatile profile*

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244   Volatile metabolites were identified and subsequently quantified by HSPME-GC-MS

245   immediately after the end of fermentation, using the protocols reported by Englezos et al.

246   (2016b). Identification was carried out by matching the retention time of each compound

247   with either those registered in the NIST Spectra database

248   (http://webbook.nist.gov/chemistry/) or those of pure standards (Sigma-Aldrich, Milan, Italy)

249   analysed in the same conditions, whenever available. The identified compounds were further

250   verified, by calculating the Kováts retention index (KRI), using an alkane standard mixture

251   C10-C40 (Sigma, Milan, Italy) as a reference for the retention times. An internal standard (1-

252   heptanol) was added to each sample to semi-quantify the volatile compounds. Determinations

253   were obtained by measuring the relative peak area of the identified compounds with those of

254   the internal standard. Each replicate was analysed in duplicate.

255

256   *2.6. Statistical analyses*

257

258   Statistical analyses were performed using IBM SPSS Statistics software package

259   (version 19.0, IBM Corp., Armonk, NY, USA). Significant differences between samples

260   were determined using one-way Analysis of Variance (ANOVA). When significance was

261   reached, a Tukey-b post-hoc test comparison at $p < 0.05$ was performed. The effect and

262   interaction of oxygen addition, as well as the *S. cerevisiae* and *Starm. bacillaris* strain

263   combination were analyzed by factorial ANOVA.

264

265   **3. Results**

266

267   *3.1. Enumeration of yeast cell population*

268

269   The yeast growth dynamics in pure and mixed culture fermentations were estimated

270   using the plate count data and are illustrated in Figs. 1 and 2, respectively. *S. cerevisiae*

271   strains, grown under semi-anaerobic conditions (condition I) in pure culture fermentations

272   showed similar growth dynamics during the first two days of fermentation, reaching

273   population of about 1.0 x 10^8 cfu/mL (Fig. 1). Oxygen addition (condition II) influenced the

274   exponential growth rate of the cells, in a strain dependent manner, since only cell populations

275   of the laboratory-evolved strain IONYS WF$^®$ was positively affected (Table 1). The four

276   growth parameters (generation number, time, maximum specific growth rate and cell

277   viability) values registered for the strain IONYS WF$^®$ in the fermentations in which oxygen

278   was added were two fold higher in comparison with the fermentation performed under semi-

279   anaerobic conditions (respectively, 4.3 generations, 11.1, 0.063 h^{-1}, 11.1 and 1.0 x 10^8

280   cfu/mL for condition II and 2.1 generations, 23.8, 0.031 h^{-1} and 4.9 x 10^7 cfu/mL, for

281   condition I). The stationary phase was observed from the 2nd to the 7th day of fermentation. *S.*

282   *cerevisiae* strains showed different patterns of cell death after sugar exhaustion: strain

283   IONYS WF$^®$ maintained the same cell viability (1.0 x 10^7 cfu/mL in both conditions) at the

284   late stages of the fermentation, whereas Uvaferm BC$^®$ decreased to 1.0 x 10^5 cfu/mL.

285   The growth dynamics of the mixed culture fermentations using 4 different

286   combinations of *Starm. bacillaris* and *S. cerevisiae* strains, under the two conditions of

287   oxygen availability, are shown in Fig. 2. Conversely to that observed for *S. cerevisiae* strains

288   in pure culture fermentations, both *Starm. bacillaris* strains, showed an oxygen-addition-

289   dependent response, with significant differences between the two conditions (Fig. 2, Table 2).

290   As can be seen in Fig. 2, oxygen addition supported both *Starm. bacillaris* strains to grow

291   faster and reach a higher density at the beginning of the stationary phase with viable counts

292   well above 1.0 x 10^8 cfu/mL. Oxygen addition showed a clear positive effect on both growth

293   and fermentations parameters (Table 2) leading to a significant increase of 1.6 and 1.3 times

294   of the generation number and 3.2 and 1.3 times of the μ_{max} for the strains FC54 and MUT

295   5705, respectively. Accordingly, the doubling time was reduced 3.2 and 1.4 times,

296   respectively.

297   Concerning the coexistence of each of the two *Starm. bacillaris* strains in mixed

298   fermentations with Uvaferm BC$^®$, independently of the fermentation conditions applied, both

299   strains dominated the fermentation process, with values of 10^8 cfu/mL, in the first 7 days and

300   they became undetectable after 14 days. On the other hand, the survival time and dominance

301   of both *Starm. bacillaris* strains over *S. cerevisiae* in mixed fermentations with IONYS WF$^®$,

302   was extended up to day 14 only in the presence of higher levels of oxygen in the musts (with

303   cell viability above 1.0 x 10^7 cfu/mL).

304   The initial inoculation of the must with *Starm. bacillaris* strains in the mixed

305   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

339 fermentations independently of the *S. cerevisiae* used. The differences in these parameters

340 were higher in the wines produced from the evolved strain IONYS WF[®] in pure culture

341 fermentations. The aeration conditions altered the chemical composition of the wines,

342 especially the acetic acid content. In the presence of higher levels of dissolved oxygen in the

343 fermentation medium, *S. cerevisiae* strains showed a slight to moderate increase of acetic

344 acid (0.02-0.07 g/L), while in mixed fermentations the final content of this acid was almost

345 two-fold higher, except for the pairs with MUT5705.

346   Glycerol and ethanol yields were calculated using the data obtained at the end of the

347 fermentation. Pure and mixed culture fermentations with IONYS WF[®] strain, were clearly

348 differentiated from the fermentations performed with Uvaferm BC[®], on the basis of high

349 glycerol and low ethanol yields. Glycerol yield in pure culture fermentations with IONYS

350 WF[®] and mixed culture fermentations with FC54 was almost two times higher (about 0.0075

351 – 0.0078 g/g), than that registered for the Uvaferm BC[®] in pure culture fermentation (about

352 0.0059 – 0.0061 g/g). On the contrary, pure fermentations with IONYS WF[®] and mixed

353 fermentations independently of the *S. cerevisiae* strain used showed the lowest levels of

354 ethanol yield. Compared to Uvaferm BC[®], the ethanol yields were reduced by 0.002 and

355 0.004 in the mixed and pure culture fermentations with IONYS WF[®], respectively.

356

357 *3.3 Volatile composition*

358

359   A total of thirty-eight (38) volatile compounds were identified, semi-quantified using

360 an internal standard and subsequently subdivided into five chemical classes, namely alcohols,

361 fatty acids, esters, terpenes and other compounds. In order to uncover the influence of the

362 fermentation conditions and strain combination on the chemical and volatile composition a

363 univariate analysis was performed and the output is presented in Supplementary Table 2.

364 Esters was the most abundant group in the samples, followed by alcohols, fatty acids and

365 terpenes. Significant differences between pure and mixed culture fermentations were

366 registered for each aroma family and for the majority of the individual compounds,

367 independently of the oxygen addition. Pure fermented wines with IONYS WF[®], contained

368 higher concentrations of alcohols and esters compared to the strain Uvaferm BC[®]. Mixed

369 fermented wines contained significantly lower levels of volatile compounds relative to wines

370 produced with *S. cerevisiae* alone.

371   The total amount of alcohols in the wines was strongly associated with the

372 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

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

413

414 **4. Discussion**

415

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

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

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

441 showed that oxygen addition promoted the growth of the two *Starm. bacillaris* strains by

442 increasing their generation number and, as a consequence, the sugar consumption in the first

443 two days of fermentation. Thus, oxygen increased their survival and the coexistence for

444 longer period with *S. cerevisiae* strains in mixed culture fermentations. This result agrees

445 well with a previous study that demonstrated a decreased death rate of non-*Saccharomyces*

446 yeasts like *T. delbrueckii* and *L. thermotolerans*, in the presence of *S. cerevisiae*, at higher

447 levels of oxygen concentration (Hansen et al., 2001). It is generally acknowledged that the

448 death of non-*Saccharomyces* yeasts in wine fermentations is attributed to their sensitivity to

449 the increasing ethanol concentration in the must (Fleet, 2003). As a consequence, the non-

450 *Saccharomyces* species that are present until the middle-end stages of the fermentation, may

451 have also a higher tolerance to ethanol (Ciani and Comitini, 2015). Recent studies have

452 demonstrated that *Starm. bacillaris* is able to withstand and grow at relative medium-high

453 concentration of ethanol (Englezos et al, 2015; Tofalo et al., 2012). This fact led us to

454 speculate, that the earlier death of *Starm. bacillaris* in mixed culture fermentations without

455 oxygen addition, may be the result of the low oxygen levels in the medium. Further to the

456 importance of this parameter on growth and performance of non-S*accharomyces* yeasts

457 (Hansen et al., 2001), several authors demonstrated that *S. cerevisiae* produced unknown

458 metabolites that can negatively affect the performance of non-*Saccharomyces* in mixed

459 fermentations (Albergaria et al., 2016; Ciani and Comitini, 2015). Among these metabolites,

460 which are considered toxic for non-*Saccharomyces* yeasts, medium-chain fatty acids

461 (hexanoic, octanoic and decanoic acids), were found in higher levels in pure fermented wines

462 and probably influenced negatively the growth of *Starm. bacillaris* strains in the mixed

463 culture fermentations (Viegas et al., 1989).

464 The association of *Starm. bacillaris* and *S. cerevisiae* strains also influenced

465 significantly the fermentation kinetics resulting in wines with different compositions, in

466 agreement with previous reports (Englezos et al., 2016a). However, the concentration of the

467 conventional enological parameters in the sequentially inoculated wines were quite similar to

468 that of IONYS WF® in pure culture. As expected, pure fermented wines with IONYS WF®

469 had a marked increased glycerol production and decreased ethanol production than the

470 conventional *S. cerevisiae* strain, due to the ability of the former to divert carbon towards

471 glycerol and away from the production of ethanol (Tilloy et al., 2015). Mixed fermentations

472 led to the production of wine with significantly higher levels of glycerol, total acidity and

473 with reduced ethanol and pH, compared to the control wine fermented with Uvaferm BC® in

474 pure culture. Additionally, glycerol production was significantly higher in the wines

475   produced by FC54 and IONYS WF®, compared to wines produced by IONYS WF® in pure

476   fermentations without the addition of oxygen (condition I). These changes in mixed culture

477   compared to pure culture fermentations are in agreement with previous studies using a

478   conventional *S. cerevisiae* strain (Andorrà et al., 2010; Englezos et al., 2016a, Giaramida et

479   al., 2013). However, it should be underlined that mixed culture fermentations with IONYS

480   WF®, except the pair FC54 with IONYS WF® (condition I) ended up with residual sugar

481   more than 4 g/L. Such negative effect may be ascribed to nutrient limitation, presence of

482   growth-inhibitory compounds and cell-to-cell contact mechanism dependent on the presence

483   of viable *Starm. bacillaris* cells at high concentration (Ciani and Comitini, 2015). The results,

484   suggest that *S. cerevisiae* strain selection has a fundamental role on the fermentation of the

485   mixed fermentations with *Starm. bacillaris* and *S. cerevisiae*, as previously described by

486   Englezos et al. (2016a).

487   Additionally, in mixed fermentations using the conventional *S. cerevisiae* strain, pH

488   reduction and concomitant increase of the total acidity respectively was observed at a level

489   which could not be explained by the principal organic acid concentrations and/or any

490   secondary compound analyzed in this study (citric, tartaric, succinic, malic, and lactic acid)

491   (Supplementary Table S3). This character is probably related to the metabolic activity of

492   *Starm. bacillaris* strains, which are good producers of α-ketoglutaric and pyruvic acids

493   (Magyar et al., 2014). Thus, this acidification property could be exploited in winemaking, in

494   order to make wines produced in warm climate regions more acid and increase

495   microbiological stability at the end of the fermentation process.

496   For any yeast strain and inoculation protocol, the impact that it has on flavour and

497   aroma profile of the wines is of critical importance (Swiegers et al., 2005). The wines

498   produced from sequential inoculations contained significantly lower volatile compounds

499   compared to the respective controls, except for few individual compounds. For example,

500   mixed fermented wines, independently of the couple used significantly increased the

501   concentration of six aromatic compounds, namely 3-methylbenzaldehyde, benzaldehyde, γ-

502   butyrolactone, hexanol, 2-methyl-1-propanol and linalool, compared to pure *S. cerevisiae*

503   fermentation, indicating the presence of different metabolic pathways and interactions

504   between the two species that probably are involved in the formation of individual volatile

505   compounds.

506   Higher alcohols, are the most important group of volatile compounds produced by

507   yeast and are divided in two subgroups, the aromatic and branched-chain alcohols (Moreno-

508   Arribas et al., 2009). Among these alcohols, branched-chain higher alcohol, 2-methyl-1-

15

509   propanol is synthesized in the yeast cell through the Enrich-pathway, which involves the

510   transamination of the amino acid precursor valine to form the α-ketoisovaleric acid,

511   necessary for the formation of the corresponding alcohol (Swiegers et al., 2005). 2-methyl-1-

512   propanol production was significantly higher in the wines produced by mixed cultures,

513   compared to wines produced by pure Uvaferm BC$^®$ fermentation. This result agrees with

514   previous findings, indicating the ability of mixed fermentations to produce high levels of this

515   compound. However, in contrast to previous studies, low levels of the aromatic alcohol 2-

516   phenylethanol, were found in this study (Andorrà et al., 2012, Englezos et al., 2016b). The

517   use of different strains and/or fermentation conditions (such as, grape variety, temperature,

518   pH, YAN, degree of turbidity etc.) may explain the differences.

519   Fermentation derived esters is a group of volatile compounds that are largely

520   responsible for wine fruitiness and play a key role in the sensory composition of young red

521   wines (Moreno-Arribas et al., 2009). Fermentative esters are mainly produced by the yeast

522   metabolism through a reaction between alcohols with lipids and acetyl-CoA by

523   acetyltransferase enzymes. The fermentation esters associated with wine fruitiness are

524   divided in two groups: a. acetate esters (mainly: ethyl acetate, 2-phenyl ethyl acetate, 3-

525   methyl-1-butanol acetate (isoamyl acetate), hexyl acetate) and b. ethyl fatty acid esters

526   (mainly: ethyl butanoate, ethyl C_3 – ethyl C_{14}). Ester production was greatly influenced by the

527   inoculation strategy rather than the strain combination, since mixed fermented wines tended

528   to produce almost 3 times less esters compared the pure fermented wines. Ethyl decanoate,

529   ethyl dodecanoate, ethyl hexanoate, ethyl octanoate, 2-phenylethyl acetate, hexyl acetate and

530   3-methyl-1-butanol acetate were the most abundant esters in all fermentations, however, their

531   levels were significantly lower in sequentially inoculated wines compared to the control

532   wines. These results verify previous findings by Sadoudi et al. (2012) on lower levels of

533   acetate esters, however contradicting the levels of the major ethyl esters previously detected

534   (Andorra et al., 2010, 2012). Additionally, Andorra et al. (2010) reported that co-inoculation

535   of Macabeo must produced wines with increased concentration of ethyl esters, indicating that

536   factors such as grape variety and inoculation delay of *S. cerevisiae* are involved in the esters

537   formation in the mixed fermentations.

538   Terpenes concentration is a good parameter to reflect the fruity characteristics of the

539   wines, even those produced from non varietal attribute grapes, like Barbera wines. Their

540   levels were significantly higher in pure fermented wines with IONYS WF$^®$ and mixed

541   fermented wines independently of the strains used compared to pure fermented wines with

542   Uvaferm BC$^®$. Mixed fermented wines with FC54 and IONYS WF$^®$ without the addition of

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

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

570

571 **5. Conclusion**

572

573 In conclusion, the results obtained in this study demonstrated that oxygen addition,
574 promoted *Starm. bacillaris* growth parameters and in particular their persistence in mixed
575 fermentations. Nevertheless, this persistence did not influence greatly the chemical and
576 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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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.

720 **Table 2**

721 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		***	***	***

722 The values are means ± standard deviation of six independent experiments. Different Latin letters within the same column indicate significant differences (Sig[1]) between

723 *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

724 and with addition of oxygen, respectively.

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Table 3

739 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	***

740 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

741 independent experiments. Different superscript Latin letters within the same column indicate significant differences (Sig[1]) between pure and mixed culture fermentations

742 (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.*

743 *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

744 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®

745 (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 $p < 0.05$, $p <$

746 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

747 yield; Y (gly/sugar consumption) = glycerol yield.

748 **Figure captions**
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750 **Fig.1** Growth dynamics of pure culture fermentations inoculated with *S. cerevisiae* strains.
751 Fermentations were carried out in triplicate and the mean CFU/mL values ± standard
752 deviations are shown. Panel a and b indicates fermentations under condition I and II
753 respectively. Condition I, II: without and with addition of oxygen respectively.
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755 **Fig.2** Growth dynamics of mixed culture fermentations using different combinations of
756 *Starm. bacillaris* and *S. cerevisiae* strains. Fermentations were carried out in triplicate and the
757 mean CFU/mL values ± standard deviations are shown. Panel a and b indicates fermentations
758 under condition I and II respectively. Condition I, II: without and with addition of oxygen
759 respectively.
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761 **Fig.3** Principal component analysis of pure and mixed culture fermented wines. Loading plot
762 (panel a) and score plot (panel b) of the first two principal components corresponding to PCA
763 analysis of conventional enological parameters and volatile compounds. PFA, MFA: pure and
764 mixed culture fermentations respectively.

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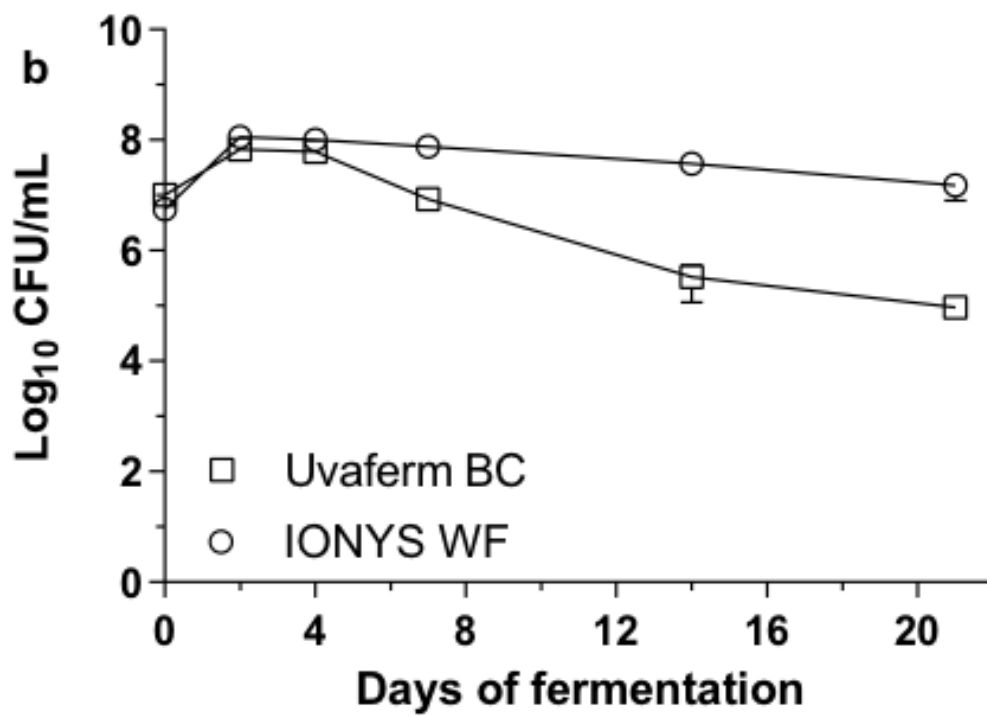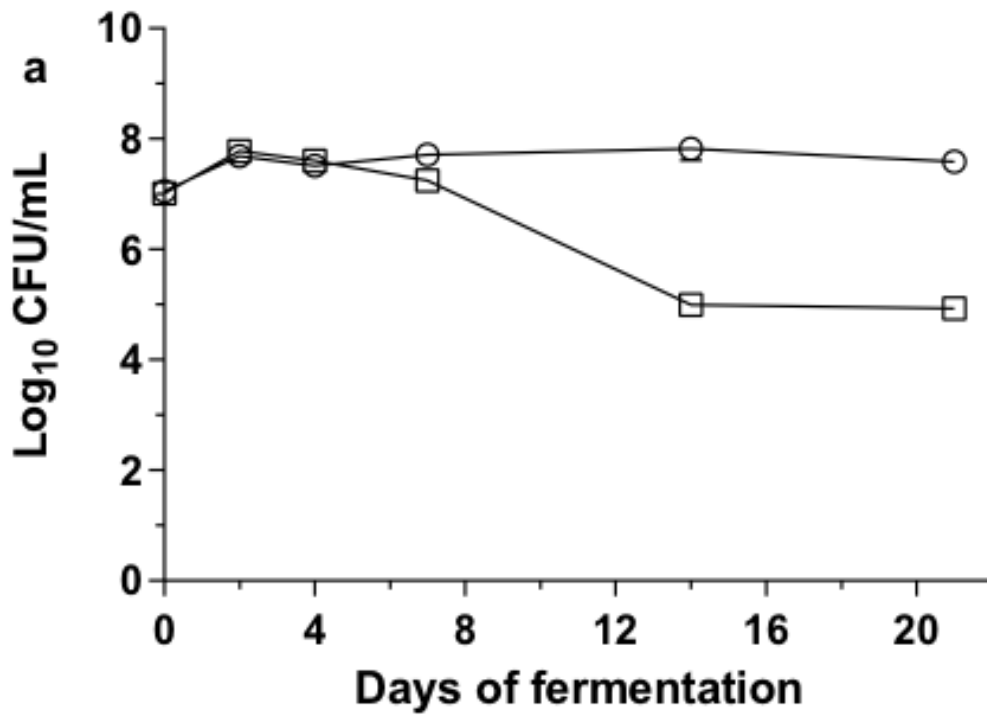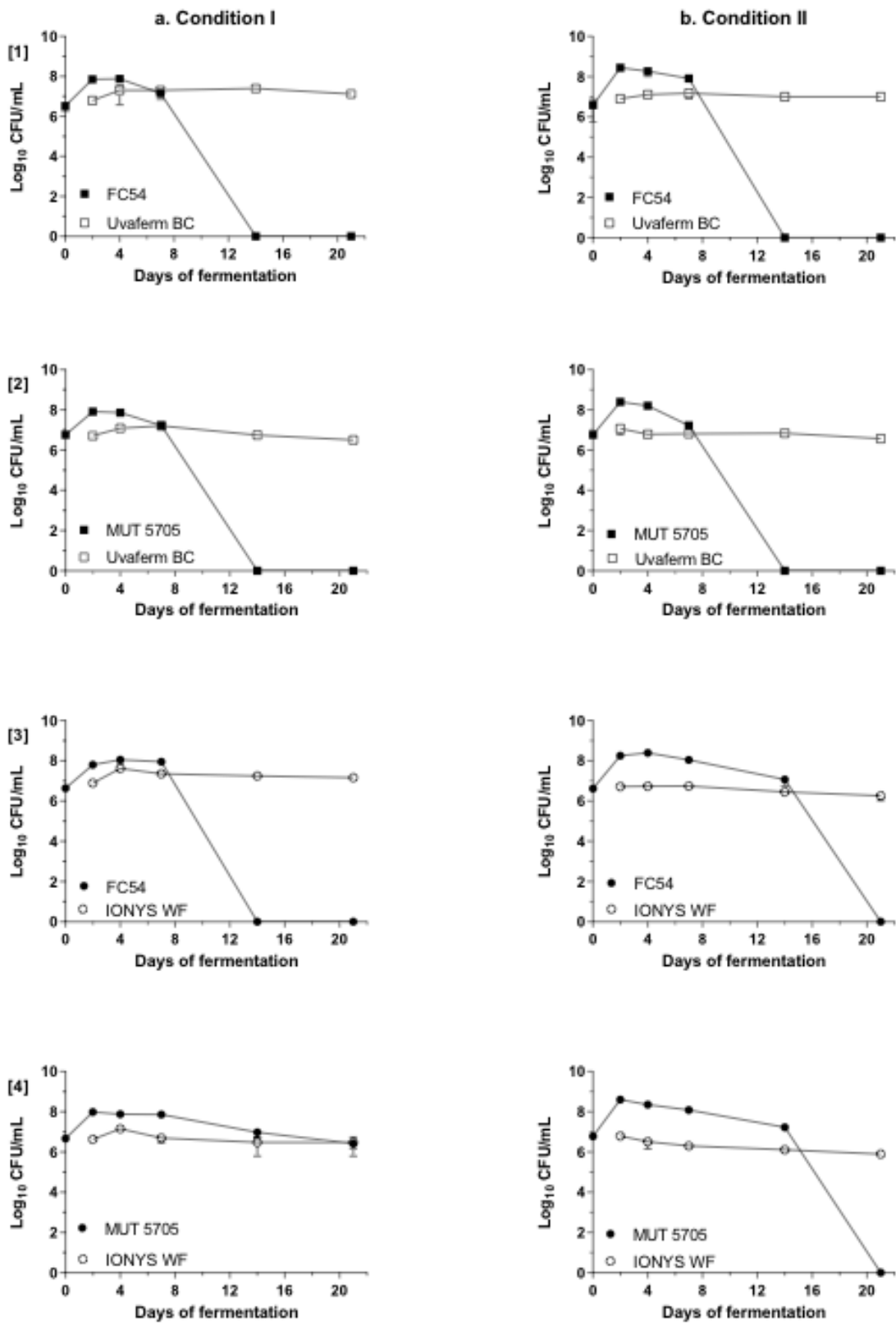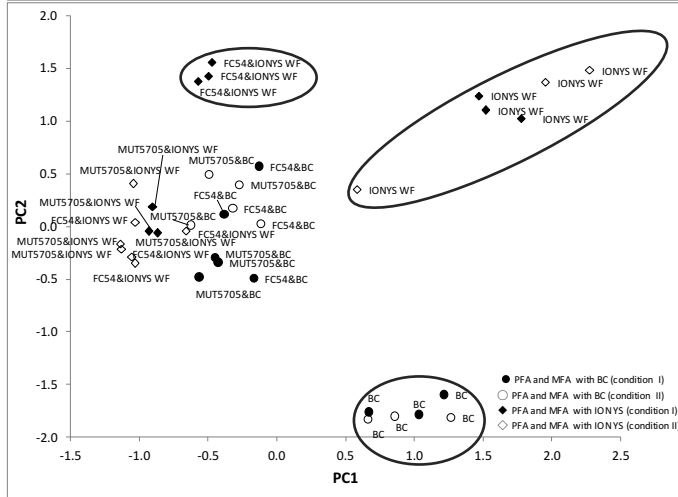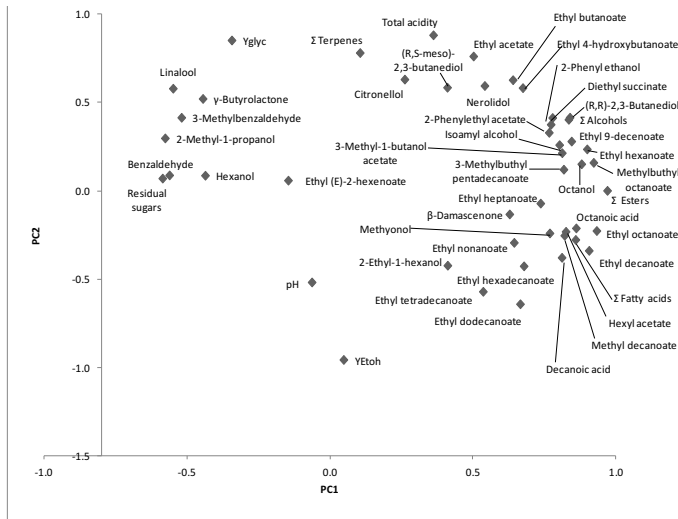769
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782 **Fig.1**

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