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

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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***

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35 **ABSTRACT**

36

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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72 1. Introduction

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

92 To this end, several techniques are being developed, targeting various steps of the
93 winemaking process, starting from the vineyard to the winery, including grapevine and clonal
94 selection, pre-fermentation, fermentation and post-fermentation strategies (Longo et al.,
95 2016, Pickering, 2000; Varela et al. 2015). Among the available strategies, the choice should
96 be economically relevant and at the same time, should not compromise organoleptic balance
97 and other sensory characteristics of wine (Varela et al. 2015). The selection of yeasts able to
98 convert glucose and fructose towards multiple secondary metabolites rather than ethanol,
99 seems to be best suited for this purpose, since they do not require specific equipment (Tilloy
100 et al., 2015). Indigenously isolated *Saccharomyces cerevisiae* strains exhibit similar ethanol
101 yield values and as a consequence the research is focusing on developing *S. cerevisiae* and
102 isolating non-*Saccharomyces* strains with improved phenotypes, able to divert carbon away
103 from ethanol production (Ciani et al., 2016, Tilloy et al., 2015). Non-*Saccharomyces* yeasts
104 are an integral part of the indigenous mycobiota present on grapes and at least at the initial
105 stages of most spontaneous or inoculated grape must fermentations (Cravero et al., 2016,

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

114 Mixed fermentations and the employment of non-*Saccharomyces* species have
115 received growing attention over the recent years from the winemaking community. They
116 reflect yeast biodiversity of indigenous wine microbiota and modulate the production of
117 specific chemical compounds, as a consequence of the early growth of non-*Saccharomyces*
118 species (Ciani et al., 2010; Fleet, 2008; Jolly et al., 2014). Their efficiency is associated with
119 the promotion of the growth and metabolic activity of the selected non-*Saccharomyces* yeasts
120 by outcompeting or reducing the activity of the *S. cerevisiae* strain (Varela, 2016b). To this
121 end, numerous winemaking variables could be manipulated to encourage non-*Saccharomyces*
122 growth rate and contribution to the chemical composition and sensory quality of the wine.
123 These variables, include sugar concentration, fermentation temperature, inoculum density,
124 nitrogen and oxygen availability, inhibitory or stimulatory substances produced by the
125 growth of yeasts or bacteria, fungicide residues from the grapes and sulphur dioxide (SO₂)
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.

146

147 **2. Materials and methods**

148

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).

159

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
182 10⁶ 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 CO₂ 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 O₂) 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 O₂
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 SO₂ in order to achieve a final concentration of 50 mg/L of total SO₂ 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

216

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: $\mu_{\max} = (\ln N_f - \ln N_0) / (t_f - t_0)$, where N_f the
219 yeast concentration (cfu/mL) at the final time point considered (t_f) and N_0 the initial yeast
220 concentration, at the beginning of fermentation (t_0). The generation number (g) defined as the
221 number of cell divisions was calculated as follows: $g = (\log N_f - \log N_0) / \log 2$. 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: $G = \ln(2) / \mu_{\max}$. 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*

243

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 1.0×10^8 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 1.0×10^8
280 cfu/mL for condition II and 2.1 generations, 23.8, 0.031 h^{-1} and 4.9×10^7 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 (1.0×10^7 cfu/mL in both conditions) at the
284 late stages of the fermentation, whereas Uvaferm BC[®] decreased to 1.0×10^5 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 1.0×10^8 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 1.0×10^7 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 5.0×10^7 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-*Saccharomyces* 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-

509 propanol is synthesized in the yeast cell through the Ehrlich-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 C₃ – ethyl C₁₄). 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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584

585 **References**

586

587 Albergaria, H., Arneborg, N., 2016. Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation
588 processes: role of physiological fitness and microbial interactions. *Appl. Microbiol. Biotechnol.* 100
589 (5), 2035-2046.

590 Andorrà, I., Berradre, M., Rozès, N., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B., 2010. Effect of pure and
591 mixed cultures of the main wine yeast species on grape must fermentations. *Eur. Food Res. Technol.*
592 231, 215-224.

593 Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B., Guillamón, J.M., 2012. Effect of mixed culture
594 fermentations on yeast populations and aroma profile. *LWT-Food Sci. Technol.* 49 (1), 8-13.

595 Bely, M., Renault, P., Da Silva, T., Masneuf-Pomerade, I., Albertin, W., Moine, V., Coulon, J., Sicard, D., De
596 Vienne, D., Marullo, P., 2013. Nonconventional yeasts and alcohol level reduction, in: Teissedre, P.L.,
597 (Ed), *Alcohol level reduction in wine*. Vigne et Vin Publications Internationales, Villenave d'Ormon,
598 France.

599 Canonico, L., Comitini, F., Oro, L., Ciani, M., 2016. Sequential fermentation with selected immobilized non-
600 *Saccharomyces* yeast for reduction of ethanol content in wine. *Front. Microbiol.* 7.

601 Ciani, M., Comitini, F., Mannazzu, I., Domizio, P., 2010. Controlled mixed culture fermentation: a new
602 perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* 10 (2), 123-133.

603 Ciani, M., Comitini, F., 2015. Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* 1, 1-
604 6.

605 Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, J.A., Oro, L., Rodrigues A.J, Gonzalez,
606 R., 2016. Non-conventional yeast species for lowering ethanol content of wines. *Front. Microbiol.*, 7.

607 Contreras, A., Curtin, C., Varela, C., 2015a. Yeast population dynamics reveal a potential “collaboration”
608 between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol
609 wines during Shiraz fermentation. *Appl. Microbiol. Biotechnol.* 99, 1885–1895.

610 Contreras, A., Hidalgo, C., Schmidt, S., Henschke, P.A., Curtin, C., Varela, C., 2015b. The application of non-
611 *Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine
612 with reduced alcohol content. *Int. J. Food Microbiol.* 205, 7-15.

613 Cravero, F., Englezos, V., Torchio, F., Giacosa, S., Segade, S.R., Gerbi, V., Rantsiou, K., Rolle, L., Cocolin, L.,
614 2016. Post-harvest control of wine-grape mycobiota using electrolyzed water. *Innov. Food Sci. Emerg.*
615 *Technol.* 35, 21-28.

616 Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin, L., 2015. Exploitation of the non-
617 *Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation:
618 physiological and molecular characterizations. *Int. J. Food Microbiol.* 199, 33-40.

619 Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., Cocolin, L., 2016a.
620 *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in
621 wine. *Appl. Microbiol. Biotechnol.* 100, 5515-5526

622 Englezos, V., Torchio, F., Cravero, F., Marengo, F., Giacosa, S., Gerbi, V., Rantsiou, K., Rolle, L., Cocolin, L.,
623 2016b. Aroma profile and composition of Barbera wines obtained by mixed fermentations of
624 *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae*. *LWT-Food*
625 *Sci. Technol.* 73, 567-575.

626 Fleet, G.H., Heard, G., 1993. *Wine Microbiology and Biotechnology*. Harwood Academic Publishers, Chur,
627 Switzerland.

628 Fleet, G.H., 2008. Wine yeasts for the future. *FEMS Yeast Res.* 8, 979-995.

629 Giaramida, P., Ponticello, G., Di Maio, S., Squadrito, M., Genna, G., Barone, E., Scacco, A., Corona, O.,
630 Amore, G., Di Stefano, R., Oliva, D., 2013 *Candida zemplinina* for production of wines with less
631 alcohol and more glycerol. *S. Afr. J. Enol. Vitic.* 34, 204–211.

632 Goldner, M.C., Zamora, M.C., Di Leo Lira, P., Gianninoto, H., Bandoni, A., 2009. Effect of ethanol level in the
633 perception of aroma attributes and the detection of volatile compounds in red wine. *J. Sens. Stud.* 24,
634 243-257.

635 Hansen, E.H., Nissen, P., Sommer, P., Nielsen, J.C., Arneborb, N., 2001. The effect of oxygen on the survival of
636 non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces*
637 *cerevisiae*. *J. Appl. Microbiol.* 91, 541-547.

638 Henderson, C.M., Block, D.E., 2014. Examining the role of membrane lipid composition in determining the
639 ethanol tolerance of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 80, 2966-2972.

640 Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine
641 production uncovered. *FEMS Yeast Res.* 14(2), 215-237.

642 Longo, R., Blackman, J.W., Torley, P.J., Rogiers, S.Y., Schmidtke, L.M., 2016. Changes in volatile
643 composition and sensory attributes of wines during alcohol content reduction. *J. Sci. Food Agric.* 97, 8-
644 16.

645 Magyar, I., Nyitrai-Sárdy, D., Leskó, A., Pomázi, A., Kállay, M., 2014. Anaerobic organic acid metabolism of
646 *Candida zemplinina* in comparison with *Saccharomyces* wine yeasts. *Int. J. Food Microbiol.* 178, 1-6.

647 Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F., Alexandre, H., Capozzi,
648 V., Cocolin, L., Colonna-Ceccaldi, B., Englezos, V., Girard, P., Gonzalez, B., Lucas, P., Mas, A.,
649 Nisiotou, A., Sipiczki, M., Spano, G., Tassou, C., Bely, M., Albertin, W.: The yeast *Starmerella*
650 *bacillaris* (synonym *Candida zemplinina*) shows high genetic diversity in winemaking environments.
651 *FEMS Yeast Res* 2015, 15:fov045. doi: 10.1093/femsyr/fov045

652 Mira de Orduña, R., 2010. Climate change associated effects on grape and wine quality and production. *Food*
653 *Res. Int.* 43, 1844-1855.

654 Moreno-Arribas, M.V., Polo, M.C., 2009. *Wine chemistry and biochemistry*, first ed. Springer-Verlag, New
655 York.

656 OIV (2008). Recueil international des méthodes d'analyse des vins et des moûts. Paris, France: Organisation
657 Internationale de la Vigne et du Vin

658 Pickering, G.J., 2000. Low and reduced-alcohol wine: A review. *J. Wine Res.* 11, 129-144.

659 Quirós, M., Rojas, V., Gonzalez, R., Morales, P., 2014. Selection of non-*Saccharomyces* yeast strains for
660 reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* 181, 85-91.

661 Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., Cocolin, L., 2012.
662 *Candida zemplinina* can reduce acetic acid production by *Saccharomyces cerevisiae* in sweet wine
663 fermentations. *Appl. Environ. Microbiol.* 78, 1987-1994.

664 Ribéreau Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2006. The microbiology of wine and
665 vinifications. *Handbook of enology*, vol. 1, second ed. Wiley, Chichester, England.

666 Rolle, L., Giordano, M., Giacosa, S., Vincenzi, S., Río Segade, S., Torchio, F., Perrone, B., Gerbi, V., 2012.
667 CIEL*a*b* parameters of white dehydrated grapes as quality markers according to chemical
668 composition volatile profile and mechanical properties. *Anal. Chim. Acta* 732, 105-112.

669 Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J., Ballester, J., Vichi, S.,
670 Guérin-Schneider, R., Caixach, J., Alexandre, H., 2012. Yeast-yeast interactions revealed by aromatic
671 profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and
672 *Saccharomyces* yeasts. *Food Microbiol.* 32, 243-253.

673 Sharma, A., Vandenberg, B., Hollingsworth, B., 2014. Minimum pricing of alcohol versus volumetric taxation:
674 Which policy will reduce heavy consumption without adversely affecting light and moderate
675 consumers. *PLoS One* 9, e80936.

676 Sipiczki, M., 2004. Species identification and comparative molecular and physiological analysis of *Candida*
677 *zemplinina* and *Candida stellata*. *J. Basic Microbiol.* 44 (6), 471-479.

678 Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S., 2005. Yeast and bacterial modulation of wine
679 aroma and flavour. *Aust. J. Grape Wine Res.* 11 (2), 139-173.

680 Tilloy, V., Ortiz-Julien, A., Dequin, S., 2014. Reduction of ethanol yield and improvement of glycerol formation
681 by adaptive evolution of the wine yeast *Saccharomyces cerevisiae* under hyperosmotic conditions.
682 *Appl. Environ. Microbiol.* 80, 2623-2632.

683 Tilloy, V., Cadière, A., Ehsani, M., Dequin, S., 2015. Reducing alcohol levels in wines through rational and
684 evolutionary engineering of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 213, 49-58.

685 Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., Suzzi, G., 2012. Diversity of
686 *Candida zemplinina* strains from grapes and Italian wines. *Food Microbiol.* 29, 18-26.

687 Tofalo, R., Patrignani, F., Lanciotti, R., Perpetuini, G., Schirone, M., Di Gianvito, P., Pizzoni, D., Arfelli, G.,
688 Suzzi, G., 2016. Aroma profile of Montepulciano d' Abruzzo wine fermented by single and co-culture
689 starters of autochthonous *saccharomyces* and non-*Saccharomyces* yeasts. *Front. Microbiol.* 7, 610.

690 Varela, C., Dry, P.R., Kutyna, D.R., Francis, I.L., Henschke, P.A., Curtin, C.D., Chambers, P.J., 2015.
691 Strategies for reducing alcohol concentration in wine. *Aust. J. Grape Wine Res.* 21, 670-679.

692 Varela, C., Borneman, A.R., 2016a. Yeasts found in vineyards and wineries. *Yeast*, doi: 10.1002/yea.3219.

693 Varela, C., 2016b. The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. *Appl.*
694 *Microbiol. Biotechnol.* 100 (23), 9861-9874.

- 695 Varela, C., Sengler, F., Solomon, M., Curtin, C., 2016c. Volatile flavour profile of reduced alcohol wines
696 fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces*
697 *uvarum*. Food Chem. 209, 57-64.
- 698 Viegas, C.A, Rosa, M.F, Sá-Correia, I., Novais, J.M., 1989. Inhibition of yeast growth by octanoic and decanoic
699 acids produced during alcoholic fermentation. Appl. Environ. Microbiol. 55, 21-28.
- 700 Zapparoli, G., Tosi, E., Azzolini, M., Vagnoli, P., Krieger, S., 2009. Bacterial inoculation strategies for the
701 achievement of malolactic fermentation in high-alcohol wines. S. Afr. J. Enol. Vitic. 30 (1), 49-55.
702

703 **Table 1**
 704 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 (μ_{max} , h ⁻¹)
<i>Pure culture fermentations</i>				
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
<i>Mixed culture fermentations</i>				
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		***	***	***

705
 706 The values are means ± standard deviation of three independent experiments. Different superscript Latin letters within the same column indicate significant differences (Sig¹)
 707 between *S. cerevisiae* strains independent the inoculation strategy applied (Tukey-b test, P < 0.05). Different Upper Latin letters indicate significant differences (Sig²) between
 708 *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
 709 significant differences (Sig³) between *S. cerevisiae* strains in mixed fermentations performed with *S. cerevisiae* IONYS WF® (Tukey-b test, p < 0.05). Sign^{1,2,3}: *** indicate
 710 significance at p < 0.001. Condition I, II: without and with addition of oxygen, respectively.

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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 (μ_{\max} . 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 (Sig¹) 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
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 ± 0.001b,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 (Sig¹) between pure and mixed culture fermentations
742 (Tukey-b test, $p < 0.05$). Different Upper Latin letters within the same column indicate significant differences (Sig²) 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 (Sig³) 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 \pm 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 \pm 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.

760

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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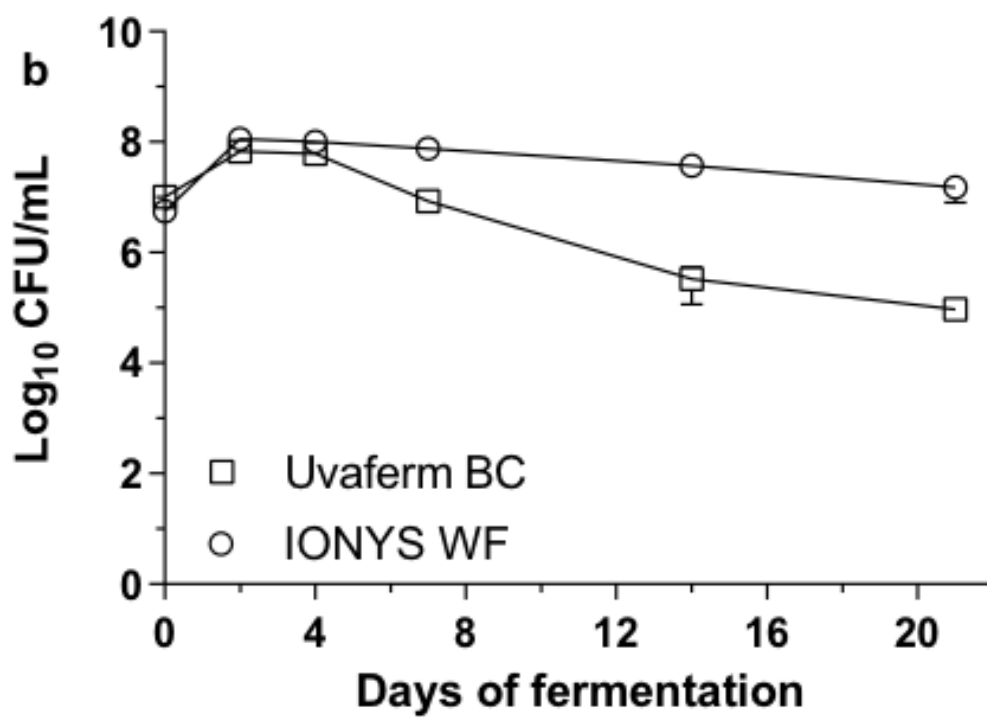
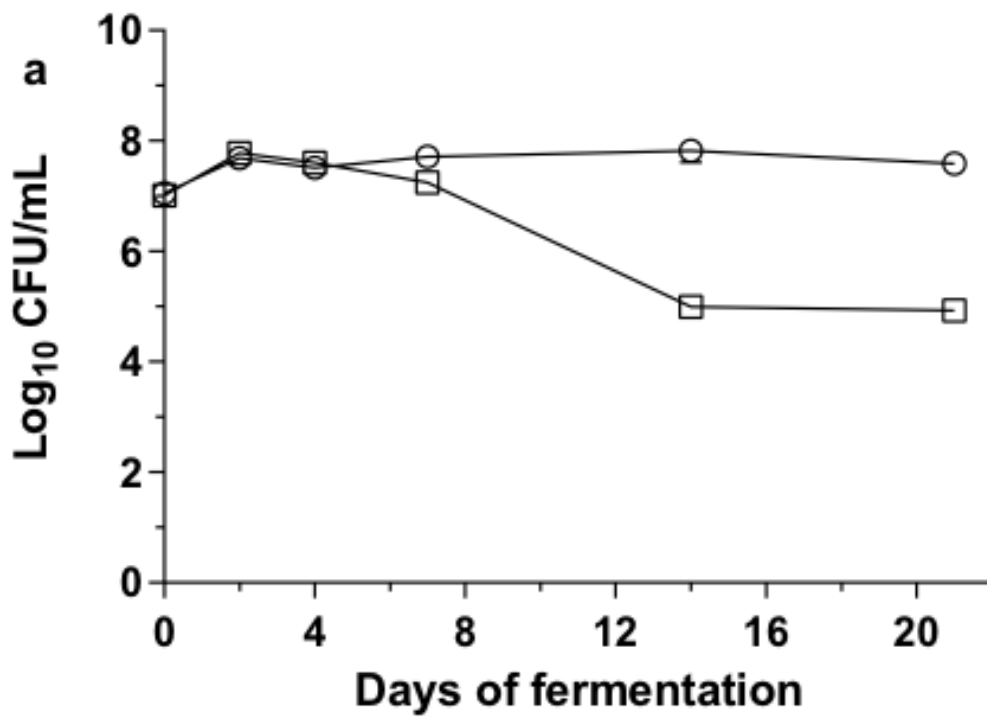
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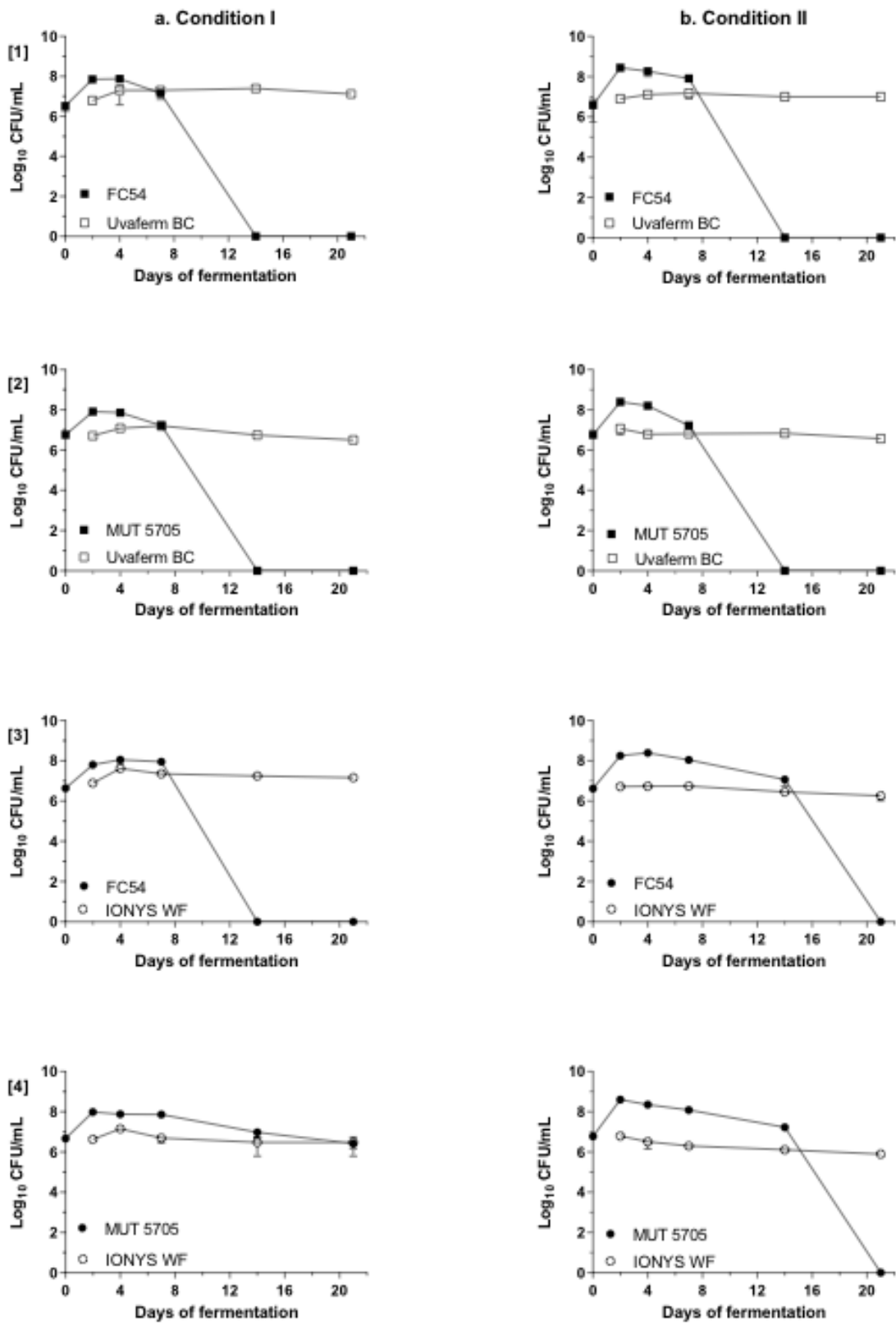
782 Fig.1



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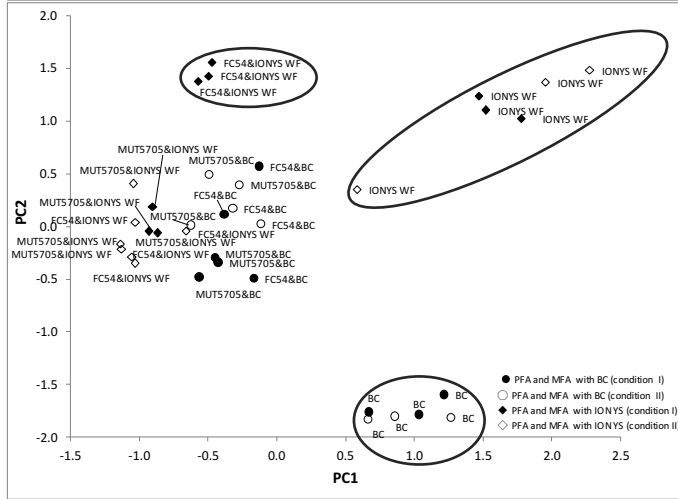
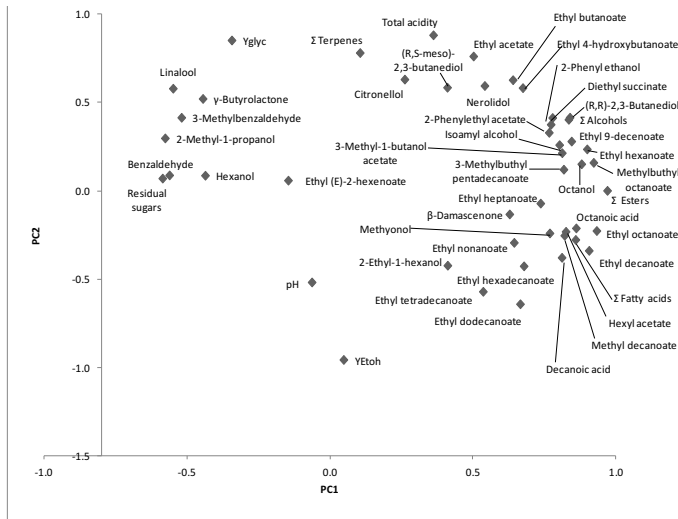
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789 **Fig.3**

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