

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1647291> since 2018-01-03T11:54:29Z

*Published version:*

DOI:10.1016/j.fm.2017.08.007

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in FOOD MICROBIOLOGY, 69, 2017, 10.1016/j.fm.2017.08.007.

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

- (1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.
- (2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.
- (3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>), 10.1016/j.fm.2017.08.007

The publisher's version is available at:

<http://linkinghub.elsevier.com/retrieve/pii/S0740002017302605>

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/>

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  
5 Vasileios Englezos<sup>1</sup>, Francesco Cravero<sup>1</sup>, Fabrizio Torchio<sup>2</sup>, Kalliopi Rantsiou<sup>1</sup>, Anne Ortiz-  
6 Julien<sup>3</sup>, Milena Lambri<sup>2</sup>, Vincenzo Gerbi<sup>1</sup>, Luca Rolle<sup>1</sup>, Luca Cocolin<sup>1\*</sup>.

7  
8 <sup>1</sup>Università degli Studi di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari,  
9 Largo Braccini 2, 10095 Grugliasco, Italy

10  
11 <sup>2</sup>Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica del Sacro Cuore, Via  
12 Emilia Parmense 84, 29122 Piacenza, Italy.

13  
14 <sup>3</sup>Lallemand SAS, Blagnac, France.

15  
16 \*Corresponding author: Luca Cocolin, Fax: +39-011-6708553, email:  
17 lucasimone.cocolin@unito.it.

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.

50

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

53

54

55

56

57

58

59

60

61

62

63

64

65

66

67

68

69

70

71

## 72 1. Introduction

73

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 (SO<sub>2</sub>)  
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<sup>®</sup> and IONYS  
153 WF<sup>®</sup>, 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<sup>®</sup> (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<sup>®</sup>, MUT 5705 and Uvaferm BC<sup>®</sup>, FC54 and IONYS WF<sup>®</sup>, MUT 5705 and  
177 IONYS WF<sup>®</sup>). 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<sup>6</sup> 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<sub>2</sub> 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<sub>2</sub>) 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<sup>®</sup>) was micro-oxygenated and the oxygen content  
191 was controlled using a Nomasense oxygen analyzer (Nomacorc, SA). In order to improve O<sub>2</sub>  
192 solubility, the must was maintained in medium/high agitation (about 150 rev min<sup>-1</sup>) 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<sub>2</sub> in order to achieve a final concentration of 50 mg/L of total SO<sub>2</sub> 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 ( $\mu_{\max}$ ), 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  $1.0 \times 10^8$  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<sup>®</sup> 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<sup>®</sup> 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,  $0.063 \text{ h}^{-1}$ , 11.1 and  $1.0 \times 10^8$   
280 cfu/mL for condition II and 2.1 generations, 23.8,  $0.031 \text{ h}^{-1}$  and  $4.9 \times 10^7$  cfu/mL, for  
281 condition I). The stationary phase was observed from the 2<sup>nd</sup> to the 7<sup>th</sup> day of fermentation. *S.*  
282 *cerevisiae* strains showed different patterns of cell death after sugar exhaustion: strain  
283 IONYS WF<sup>®</sup> maintained the same cell viability ( $1.0 \times 10^7$  cfu/mL in both conditions) at the  
284 late stages of the fermentation, whereas Uvaferm BC<sup>®</sup> decreased to  $1.0 \times 10^5$  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  $1.0 \times 10^8$  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  $\mu_{\text{max}}$  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<sup>®</sup>, 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<sup>®</sup>,  
302 was extended up to day 14 only in the presence of higher levels of oxygen in the musts (with  
303 cell viability above  $1.0 \times 10^7$  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  $5.0 \times 10^7$  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<sup>®</sup> (condition II) and MUT 5705 with IONYS WF<sup>®</sup> (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<sup>®</sup> 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<sup>®</sup> (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<sup>®</sup> (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<sup>®</sup>.  
334 On the other hand, mixed fermented wines using Uvaferm BC<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> 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<sup>®</sup> strain, were clearly  
348 differentiated from the fermentations performed with Uvaferm BC<sup>®</sup>, on the basis of high  
349 glycerol and low ethanol yields. Glycerol yield in pure culture fermentations with IONYS  
350 WF<sup>®</sup> 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<sup>®</sup> in pure culture fermentation (about  
352 0.0059 – 0.0061 g/g). On the contrary, pure fermentations with IONYS WF<sup>®</sup> and mixed  
353 fermentations independently of the *S. cerevisiae* strain used showed the lowest levels of  
354 ethanol yield. Compared to Uvaferm BC<sup>®</sup>, the ethanol yields were reduced by 0.002 and  
355 0.004 in the mixed and pure culture fermentations with IONYS WF<sup>®</sup>, 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<sup>®</sup>, contained  
368 higher concentrations of alcohols and esters compared to the strain Uvaferm BC<sup>®</sup>. 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<sup>®</sup>  
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<sup>®</sup> 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<sup>®</sup> 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,  $\gamma$ -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<sup>®</sup> on the upper part of the plot, while wines from Uvaferm BC<sup>®</sup> on  
408 the bottom. Wines with Uvaferm BC<sup>®</sup> were characterized by high pH values and high ethanol  
409 yields, on the other hand wines with IONYS WF<sup>®</sup> 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<sup>®</sup> "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<sup>®</sup> in pure culture. As expected, pure fermented wines with IONYS WF<sup>®</sup>  
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<sup>®</sup> in  
474 pure culture. Additionally, glycerol production was significantly higher in the wines



475 produced by FC54 and IONYS WF<sup>®</sup>, compared to wines produced by IONYS WF<sup>®</sup> 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<sup>®</sup>, except the pair FC54 with IONYS WF<sup>®</sup> (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  $\alpha$ -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,  $\gamma$ -  
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  $\alpha$ -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<sup>®</sup> 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<sub>3</sub> – ethyl C<sub>14</sub>). 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<sup>®</sup> and mixed  
541 fermented wines independently of the strains used compared to pure fermented wines with  
542 Uvaferm BC<sup>®</sup>. Mixed fermented wines with FC54 and IONYS WF<sup>®</sup> 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  $\beta$ -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  $\beta$ -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<sup>®</sup> 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

## 582 **Acknowledgements**

583 The authors would like to thank Sylvie Dequin for providing IONYS WF<sup>®</sup> for this work.

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 ( $\mu_{max}$ , h <sup>-1</sup> )
<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 <sup>1</sup>		***	***	***
Sign <sup>2</sup>		***	***	***
Sign <sup>3</sup>		***	***	***

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

711  
 712  
 713  
 714  
 715  
 716  
 717  
 718  
 719



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 ( $\mu_{max}$ . h <sup>-1</sup> )
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<sup>1</sup>) 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.

725

726

727

728

729

730

731

732

733

734

735

736

737

738  
739

**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 <sub>(g/s)</sub> (g/g)	Y <sub>(eth/s)</sub> (g/g)	pH	TA (g/L)
Uvaferm BC <sup>®</sup>	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 <sup>®</sup>	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 <sup>®</sup>	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 <sup>®</sup>	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 <sup>®</sup>	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 <sup>®</sup>	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 <sup>1</sup>		***	***	***	***	***	***	***	***	***
Sign <sup>2</sup>		***	***	***	***	***	***	***	*	***
Sign <sup>3</sup>		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<sup>1</sup>) 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<sup>2</sup>) between pure and mixed fermentations performed with *S.*  
743 *cerevisiae* Uvaferm BC<sup>®</sup> (Tukey-b test,  $p < 0.05$ ). Different Greek letters within the same column indicate significant differences (Sig<sup>3</sup>) pure and mixed fermentations  
744 performed with *S. cerevisiae* IONYS WF<sup>®</sup> (Tukey-b test,  $p < 0.05$ ). Mixed fermentations with FC54 and IONYS WF<sup>®</sup> (condition II) and MUT and IONYS WF<sup>®</sup>  
745 (conditions I, II) were excluded from the statistical analysis due to high concentration of residual sugars. Sign<sup>1,2,3</sup>: \*, \*\*, \*\*\* 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**

749

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.

754

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.

765

766

767

768

769

770

771

772

773

774

775

776

777

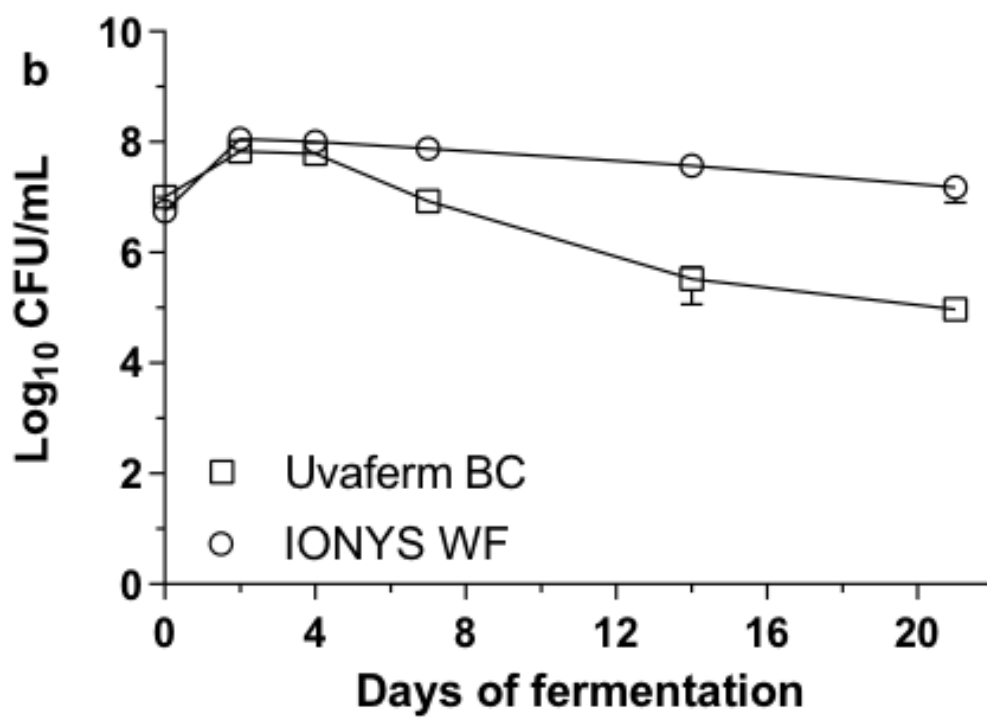
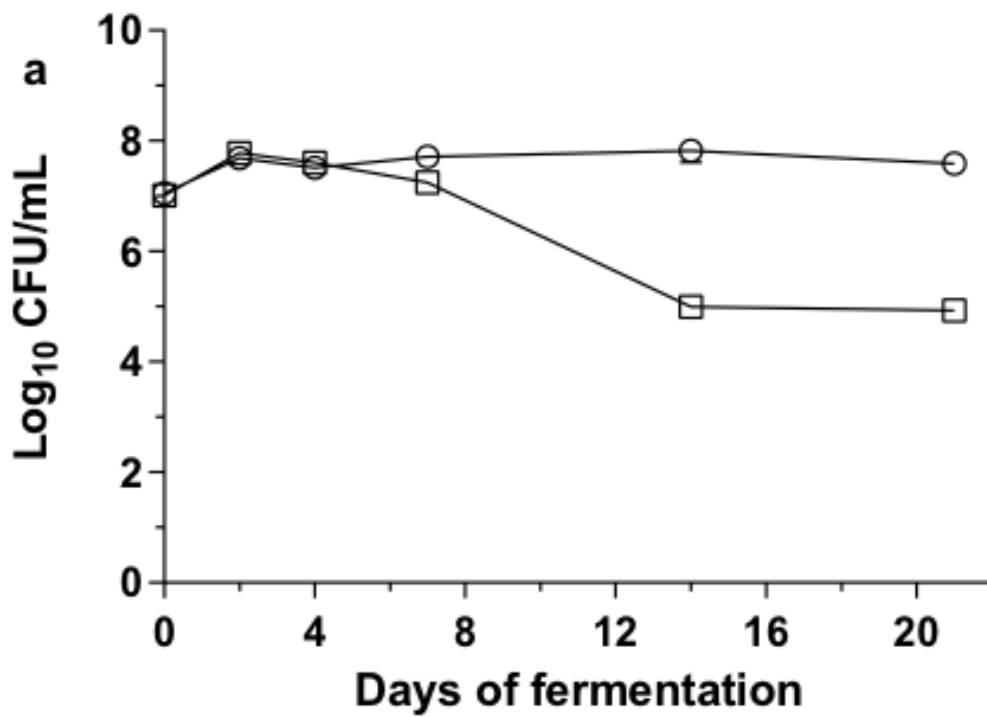
778

779

780

781

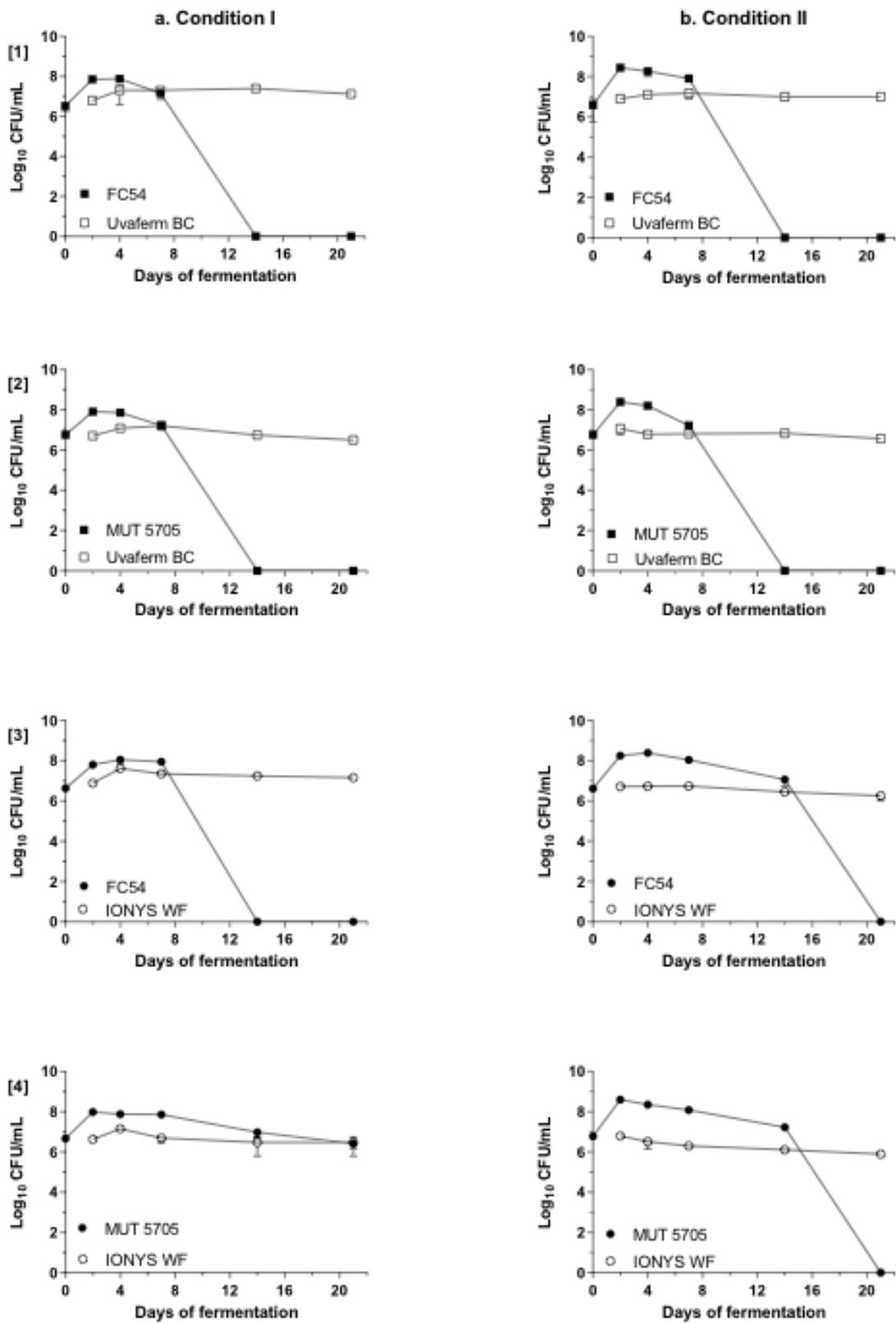
782 Fig.1



783

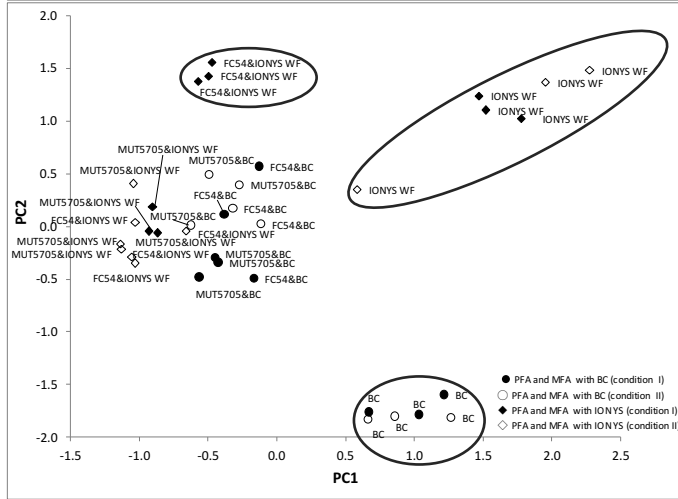
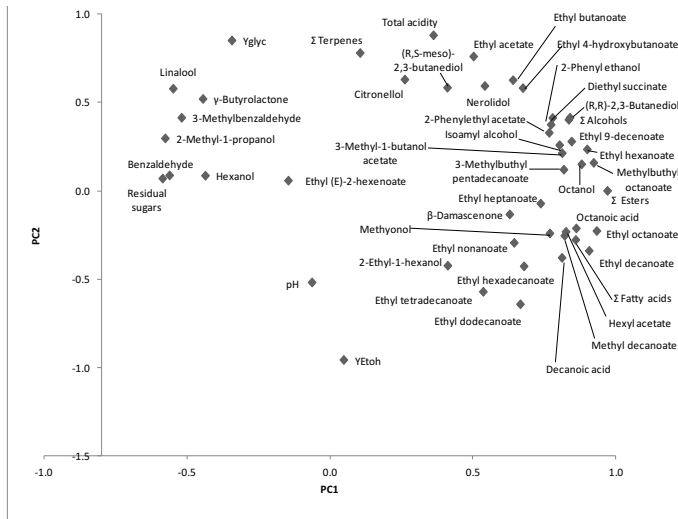
784

785



789 **Fig.3**

790



791