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(Article begins on next page)

1 **Effect of mixed fermentations with *Starmerella bacillaris* and *Saccharomyces cerevisiae***  
2 **on management of malolactic fermentation**

3

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29 **Abstract**

30 This work aims to improve the management of the malolactic fermentation (MLF) in red  
31 wines by elucidating the interactions between *Starmerella bacillaris* and malolactic bacteria.  
32 Two *Starm. bacillaris* strains were individually used in mixed fermentations with a  
33 commercial *Saccharomyces cerevisiae*. MLF was performed using two autochthonous  
34 *Lactobacillus plantarum* and one commercial *Oenococcus oeni* inoculated following a  
35 simultaneous (together with *S. cerevisiae*) or sequential (at the end of alcoholic fermentation)  
36 approach. The impact of yeast inoculation on the progress of MLF was investigated by  
37 monitoring the viable microbial populations and the evolution of the main oenological  
38 parameters, as well as the volatile organic composition of the wines obtained in mixed and  
39 pure micro-scale winemaking trials. Our results indicated that MLF was stimulated, inhibited,  
40 or unaffected in mixed fermentations depending on the strains and on the regime of  
41 inoculation. *O. oeni* was able to perform MLF under all experimental conditions, and it  
42 showed a minimal impact on the volatile organic compounds of the wine. *L. plantarum* was  
43 unable to perform MLF in sequential inoculation assays, and strain-dependent interactions  
44 with *Starm. bacillaris* were indicated as factor affecting the outcome of MLF. Moreover,  
45 uncompleted MLF were related to a lower aromatic complexity of the wines. Our evidences  
46 indicate that tailored studies are needed to define the appropriate management of non-  
47 *Saccharomyces* and malolactic starter cultures in order to optimize some technological  
48 parameters (i.e. reduction of vinification time) and to improve qualitative features (i.e.  
49 primary and secondary metabolites production) of red wines.

50

51 **Key words:** *Starmerella bacillaris*; non-*Saccharomyces*; mixed fermentation; *Oenococcus*  
52 *oeni*; *Lactobacillus plantarum*; malolactic fermentation; wine.

53

## 54 **1. Introduction**

55 Wine fermentations are complex microbiological processes in which yeasts and bacteria play  
56 a pivotal role carrying out alcoholic fermentation (AF) and malolactic fermentation (MLF),  
57 respectively. Although final stages of AF are dominated by strains of *Saccharomyces*  
58 *cerevisiae*, many other species of yeasts are known to occur in grape must and contribute to  
59 the early-middle phases of fermentation (Fleet, 2008; Garofalo et al., 2016; Tristezza et al.,  
60 2013). In the last years, the use of non-*Saccharomyces* yeasts in winemaking was re-proposed  
61 with the aim to solve specific technological issues and/or to improve the organoleptic  
62 complexity of wines (Berbegal et al., 2017; Ciani & Comitini, 2011). In particular, these non-  
63 conventional yeasts have been proposed to produce wines with specific characteristics  
64 (reduced alcohol content and volatile acidity, colour stabilization etc.) and modulate some  
65 sensory quality attributes by producing high levels of glycerol, mannoproteins,  
66 polysaccharides, and volatile organic compounds (Ciani et al., 2016; Contreras et al., 2014;  
67 Jolly et al., 2014 Medina et al., 2018). According with this trend, commercial starter  
68 formulations containing non-*Saccharomyces* yeasts are increasingly available on the market  
69 (Roudil et al., 2019).

70 Among non-conventional oenological yeasts, *Starmerella bacillaris* (synonym *Candida*  
71 *zemplanina*) has been reported to affect the chemical composition of the musts and wines by  
72 lowering ethanol production and producing various metabolites of oenological interest  
73 (Englezos et al., 2017; Masneuf-Pomarede et al., 2015), which contribute to the mouth-feel  
74 and flavour complexity of wines (Magyar & Tóth, 2011; Tofalo et al., 2012). With respect to  
75 other non-*Saccharomyces* yeasts, the major interest in the application of *Starm. bacillaris* in  
76 winemaking is related mainly to its fructophilic character and tolerance to relative high levels  
77 of ethanol, playing an active role in the biochemical modifications of wine until the end of  
78 AF (Rantsiou et al., 2017). Therefore, some recent studies investigated the exploitation of

79 *Starm. bacillaris* and *S. cerevisiae* in mixed culture fermentations to enhance the chemical  
80 composition of the wines (Englezos, Rantsiou, Cravero, Torchio, Giacosa, et al., 2018;  
81 Englezos, Rantsiou, Cravero, Torchio, Pollon, et al., 2018), and to improve the knowledge on  
82 the successional evolution of yeast species during wine fermentation (Englezos, Cravero,  
83 Torchio, Rantsiou, Ortiz-Julien, et al., 2018).

84 *Oenococcus oeni* is the main lactic acid bacterium (LAB) responsible for MLF, the metabolic  
85 decarboxylation of L-malic acid in grape must into L-lactic acid and carbon dioxide. This  
86 desired bacterial development positively impacts on deacidification, flavour modification and  
87 microbial stability of wine (Bartowsky & Borneman, 2011). In the last few years,  
88 *Lactobacillus plantarum* strains have also been reported to survive in winemaking conditions  
89 and possess many favourable biological properties that would make them suitable candidates  
90 as MLF starter cultures (du Toit et al., 2011). Moreover, different MLF inoculation strategies  
91 (i.e. simultaneous or sequential inoculation of LAB and yeasts) can differently impact on the  
92 outcome of fermentation and on the quality of wine (Knoll et al., 2012; Tristezza et al., 2016;  
93 Zapparoli et al., 2009). Despite the extensive information on the interactions between the  
94 abovementioned LAB species and *S. cerevisiae* isolates, little is known about the effect of  
95 mixed fermentations with non-*Saccharomyces* and *S. cerevisiae* on LAB behavior during  
96 MLF (Balmaseda et al., 2018). Except few studies that investigated the impact of mixed  
97 fermentations on the MLF performed by *O. oeni* (Capozzi et al., 2019; Du Plessis et al.,  
98 2017a; du Plessis et al., 2017b; Nardi et al., 2019) and *L. plantarum* (Du Plessis et al., 2019;  
99 Englezos et al., 2019).

100 To get an insight to these interactions, the present study was performed with the aim to  
101 further investigate the effect of two *Starm. bacillaris* strains in mixed fermentations with *S.*  
102 *cerevisiae* on the progress of MLF carried out by three different LAB strains (two  
103 autochthonous *L. plantarum* and one commercial *O. oeni*) in simultaneous or sequential

104 inoculation. Finally, the impact of their interactions on chemical and volatile profile of the  
105 wines was evaluated.

106

## 107 **2. Materials and Methods**

### 108 *2.1 Microbial strains and growth conditions*

109 *Lactobacillus plantarum* strains of oenological origin, namely *L. plantarum* UFG44 (Lp44)  
110 and *L. plantarum* UFG87 (Lp87) previously characterized for their ability to perform MLF  
111 (Berbegal et al., 2016), were available at the collection of Industrial Microbiology of the  
112 University of Foggia (Foggia, Italy). *L. plantarum* strains were routinely grown in MRS broth  
113 (Biogenetics, Ponte San Nicolò, Italy) at 30 °C and maintained on MRS plates at 4 °C. The  
114 commercial *Oenococcus oeni* Lalvin VP41<sup>®</sup> (Lallemand, Montreal, Canada) was used as  
115 MLF reference strain.

116 *Starmerella bacillaris* FC54 and *Starm. bacillaris* MUT5705, both strains of oenological  
117 origin, and previously extensively characterized (Englezos et al., 2015), were provided by the  
118 Turin University Culture Collection (TUCC, Torino, Italy). The commercial strain *S.*  
119 *cerevisiae* Lalvin ICV D254<sup>®</sup> (Lallemand Inc. Montreal, Canada) was used to perform the  
120 AF. Oenological yeasts were grown in YPD broth (Biogenetics) at 28 °C and maintained on  
121 YPD plates at 4 °C.

### 122 *2.2 Must preparation and inoculum*

123 *Vitis vinifera* L. cv. Barbera red grapes (Barbaresco, North-West Italy) were used for micro-  
124 scale winemaking assays. Grapes were manually pressed, and solid parts (skins and seeds)  
125 were separated from the juice using a stainless-steel sieve. Then, the must was exposed to  
126 heat treatment (60 °C for 60 min) to inactivate the indigenous microbiota. Pasteurization  
127 efficacy was checked by plating on Wallerstein Laboratory (WL) Nutrient and MRS agar  
128 (Biogenetics). Must was then aliquoted (200 mL) in sterile Erlenmeyer flasks, and again

129 submitted to the abovementioned heat treatment. The Barbera must used for the trials had the  
130 following characteristics: 251 g/L of sugars, pH 3.5, total acidity of 7.4 g/L (expressed as g/L  
131 of tartaric acid), and 230 mg/L of YAN, composed by 60 mg/L of inorganic and 170 mg/L of  
132 organic nitrogen.

133 In order to pre-adapt the microbes to the must conditions, single colonies of each strain  
134 previously grown on YPD and MRS plates were inoculated in 5 mL of pasteurized grape  
135 must and incubated at 28 °C for 24 h. Then, the 5-mL must were added to 50 mL of must and  
136 incubated at 28 °C for 24 h. These cultures were used to inoculate 200 mL of must samples in  
137 order to obtain approximately the following initial cell populations: *Starm. bacillaris* ( $1 \times 10^6$   
138 CFU/mL), *S. cerevisiae* ( $1 \times 10^6$  CFU/mL), and *L. plantarum* ( $8 \times 10^6$  CFU/mL). *O. oeni* was  
139 rehydrated by suspending 0.1 g of commercial formulation in 20 mL of commercial sterile  
140 mineral water. After incubation at room temperature for 20 min, 400 µL of the suspension  
141 were used to inoculate 200 mL of grape must in order to achieve an initial population of  
142 about  $5 \times 10^5$  CFU/mL. The microbial populations of inocula was in line with those  
143 previously reported by Englezos et al. (2019).

### 144 2.3 Micro-scale winemaking assays

145 Mixed culture fermentations were performed by inoculating *S. cerevisiae* 48 h after *Starm.*  
146 *bacillaris* inoculation, according to Englezos et al. (2016). While, pure culture fermentations  
147 were performed by inoculating only *S. cerevisiae*. LAB strains were inoculated by using two  
148 different strategies, namely co-inoculation (simultaneously with *S. cerevisiae* inoculation)  
149 and sequential inoculation (when residual sugars were below 2.0 g/L) in mixed (with *Starm.*  
150 *bacillaris*) and pure (without *Starm. bacillaris*) fermentations. The corresponding control  
151 samples were *Starm. bacillaris* – *S. cerevisiae* AF (without LAB, no MLF). An additional  
152 control sample inoculated only with *S. cerevisiae* was performed. Within the examined  
153 experimental modes, both *Starm. bacillaris* strains were separately tested with each LAB

154 strain. After inoculation, Erlenmeyer flasks were closed with air locks containing sterile  
155 paraffin oil, to allow only the CO<sub>2</sub> loss from the fermenting must and to prevent external  
156 contamination, according to the procedure reported by Englezos et al. (2016). Fermentations  
157 were carried out at 25 °C and Erlenmeyer flasks were manually shaken each 12 h. AF was  
158 monitored until complete depletion of glucose and fructose (< 2.0 g/L), as determined by  
159 HPLC. Malolactic fermentation was monitored until complete depletion of malic acid (0.1  
160 g/L), as determined by HPLC, or until viable cells of LAB (more than 1 Log CFU/mL) were  
161 enumerated. Each fermentation condition was tested by performing three simultaneous  
162 independent biological repetitions.

#### 163 2.4 Microbiological analysis

164 To enumerate the microbial viable populations, tenfold serial dilutions in sterile Ringer's  
165 solution (Biogenetics Diagnostics, Padova, Italy) were plated as follows. Yeast growth  
166 dynamics were monitored by enumeration of viable cells on WL Nutrient agar after  
167 incubation at 30 °C for 3–5 days, which allowed to discriminate *Starm. bacillaris* and *S.*  
168 *cerevisiae* colonies. Viable *L. plantarum* were enumerated on MRS agar, after incubation for  
169 48 h at 30 °C. *O. oeni* viable cells were quantified after inclusion in MRS plates at pH 5.0,  
170 containing L-malic acid (10 g/L) (Sigma Aldrich, St Louis, MO, USA). After solidification,  
171 plates were covered with an additional layer of the medium in order to favour anaerobic  
172 conditions. Counting was performed after 20 days of incubation at 30 °C. Both MRS agar  
173 plates were supplemented with 25 mg/mL Delvocid (DSM Specialties, Heerlen, The  
174 Netherlands) to avoid yeast growth.

#### 175 2.5 Must and wine analysis

176 Ethanol, glycerol, and organic acids, as well as glucose and fructose, were quantified in grape  
177 juice, during and at the end of the alcoholic and/or malolactic fermentation by means of  
178 HPLC using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA)



179 equipped with an Aminex HPX-87H cation exchange column (300 mm x 7.8 mm i.d.), a UV  
180 detector set to 210 nm and a refractive index detector. The eluent was 0.0065 mol/L sulfuric  
181 acid (H<sub>2</sub>SO<sub>4</sub>) at a flow rate of 0.7 mL/min and the column temperature was 65 °C, as  
182 previously described Englezos et al. (2018c). The concentrations of D- and L-lactic acid were  
183 determined spectrophotometrically at 340 nm using specific enzymatic kit (product code: K-  
184 DLATE; Megazyme International) and according to the manufacturer's instructions, using an  
185 UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) in grape juice and at the  
186 end MLF. The concentration of yeast available nitrogen (YAN), was determined in grape  
187 juice using specific enzymatic kits (product codes: K-LARGE and K-PANOPA; Megazyme  
188 International) and according to the manufacturer's instructions, using the above-mentioned  
189 spectrophotometer at 340 nm. The pH in grape juice and at the end of the  
190 alcoholic/malolactic fermentations was registered using the InoLab 730 pH meter (WTW,  
191 Weilheim, DE), while total acidity (TA) was determined and expressed in g/L of tartaric acid  
192 according to the official protocol described by the International Organization of Vine and  
193 Wine (OIV, 2015).

#### 194 *2.6 Determination of volatile organic compounds*

195 Volatile organic compounds in wines were identified and subsequently quantified by HS-  
196 SPME-GC-MS, immediately after the end of the AF or MLF using the protocol reported by  
197 Englezos et al. (2018c). Briefly, an aliquot of internal standard (1-heptanol) was introduced  
198 in 20 mL glass headspace vial with a headspace screw cap, containing 2 g of sodium  
199 chloride, 5 mL of water and 5 mL of wine sample. Analyses were carried using the apparatus  
200 and chromatic conditions reports by Englezos et al. (2018). Each volatile compound was  
201 identified by matching the retention time and mass spectra with those of pure standards  
202 analysed under the same conditions and those available on NIST database  
203 (<http://webbook.nist.gov/chemistry/>). Quantification of each compound was performed by

204 external calibration with pure standards when available using the abovementioned internal  
205 standard. While semi-quantification was carried out, by calculating the area of the 1-heptanol  
206 internal standard.

207

## 208 *2.7 Statistical analysis*

209 Statistical analysis was carried out using the statistical software package IBM SPSS Statistics  
210 (IBM Corp., Armonk, NY, USA). The Tukey's HSD *post-hoc* test was used to compare  
211 different levels of the factor in the case of ANOVA null hypothesis rejection ( $p$  value  $< 0.05$ ).  
212 Principal Component Analysis (PCA) was carried out for both technological parameters and  
213 volatile compounds on normalized data (standard deviation of each variable equal to one).

214

## 215 **3. Results**

### 216 *3.1 Grape must chemical parameters and microbial growth dynamics*

217 The growth dynamics in the control micro-scale winemaking trials (without LAB  
218 inoculation) are reported in Fig. 1. After two days in pure culture fermentation, *S. cerevisiae*  
219 achieved the stationary phase ( $2 \times 10^8$  CFU/mL) and remained stable for further two days  
220 before declining. In mixed fermentations, the growth of *S. cerevisiae* was at least 1 Log  
221 CFU/mL lower than that of pure fermentation, while both *Starm. bacillaris* were dominant  
222 during the microvinification assays increasing their population to approximately  $10^8$  CFU/mL  
223 after two days, which remained constant until the end of the AF (Fig. 1).

224 In pure culture fermentations with addition of LAB, the growth of *S. cerevisiae* was not  
225 affected by the simultaneous occurrence of *L. plantarum* or *O. oeni* (Fig. 2). The growth  
226 kinetics of both *L. plantarum* strains showed a similar pattern. In co-inoculation, after a slight  
227 increase up to about  $2.0 \times 10^7$  CFU/mL at the second day, their concentration drastically  
228 dropped by 3 Log in the following two days, leading to a level lower than  $1 \times 10^2$  CFU/mL

229 after seven days. When inoculated at the end of AF, the viability constantly decreased in the  
230 following two weeks down to less than 1 Log CFU/mL. Instead, *O. oeni*, although inoculated  
231 at an initial concentration about 1 Log CFU/mL lower than *L. plantarum*, increased gradually  
232 during the co-inoculum assay. Interestingly, in the sequential approach, *O. oeni* viability  
233 decreased slightly and steadily by about 1 Log CFU/mL during the first seven days, but it  
234 significantly increased up to  $2 \times 10^6$  CFU/mL in the next week of fermentation.

235 The microbial population dynamics in mixed fermentations with the addition of LAB are  
236 represented in Fig. 3. The growth of *S. cerevisiae* was similar to what observed in mixed  
237 control fermentation. Likewise, both *Starm. bacillaris* strains were dominant until the end of  
238 AF regardless of the presence of malolactic starter. Interestingly, after AF, a very different  
239 pattern was observed in growth kinetics of non-*Saccharomyces*. Indeed, FC54 population was  
240 about  $1 \times 10^8$  CFU/mL after nine days when co-inoculated with *L. plantarum*, and it further  
241 declined until about  $2 \times 10^4$  CFU/mL after two weeks of fermentation with Lp44. By  
242 contrast, no culturable cells ( $< 10$  CFU/mL) of MUT5705 strain were detected after the same  
243 period regardless of the co-inoculated *L. plantarum* strains. Interestingly, the viability of *L.*  
244 *plantarum* strain Lp87 after one week from its inoculation was 2 Log CFU/mL higher in  
245 wines fermented by MUT5705 than in those by FC54. Another intriguing difference was  
246 observed in the sequential approach. In these assays, a complete loss of viability of both  
247 *Starm. bacillaris* strains was noted after seven days from the sequential inoculation with *O.*  
248 *oeni* (14 days of fermentation), while, at the same experimental time, a concentration of about  
249  $3 \times 10^4$  and  $3 \times 10^5$  CFU/mL was found in samples inoculated with Lp44 and Lp87,  
250 respectively (Fig. 3).

### 251 3.2 Kinetics of main oenological parameters

252 The evolution of the main oenological parameters during fermentation are shown in Fig 1-3. .  
253 Generally, AF was completed in seven days. In pure control fermentations, no differences

254 were detected in the depletion rate of glucose and fructose. In contrast, during mixed control  
255 fermentations fructose was metabolized faster than glucose (about 10 and 60 g/L after four  
256 days, respectively). The kinetics of glucose and fructose consumption as well as of ethanol  
257 and glycerol production in pure and mixed fermentations with the presence of LAB were  
258 very close to those observed in control fermentations, and therefore only shown in Fig. 1.

259 When MLF was performed (Fig. 2 and 3), different times were required for completion  
260 (Table 1). Among pure fermentations, co-inoculum with Lp44 seems the best combination to  
261 perform MLF, since after 48 h malic acid was almost completely depleted (0.2 g/L), and  
262 MLF finished within four days, while *O. oeni* required seven days. However, the difference  
263 in the inoculated populations of *L. plantarum* and *O. oeni* may have affected the duration of  
264 MLF. By contrast, longer time was required to complete MLF in co-inoculum with Lp87.  
265 Indeed, although malic acid was metabolized faster than *O. oeni* in the first two days (to  
266 about 1 g/L), its complete fermentation needed twelve days. When sequentially inoculated,  
267 both *L. plantarum* strains were unable to perform MLF, unlike *oeni*, that required 12 days to  
268 complete MLF with malic acid depletion mainly occurring in the last five days. (Fig. 2). In  
269 mixed fermentations, with both *Starm. bacillaris* strains, *O. oeni* was able to perform MLF in  
270 two days when co-inoculated (Fig. 3). Contrarily to what observed in pure assays, MLF was  
271 only partially performed by Lp44, since malic acid was degraded only in the first two days  
272 and subsequently remained constant at levels of about 2.5 and 1.5 g/L in mixed fermentations  
273 with FC54 and MUT5705 strains, respectively. Instead, Lp87 finished the MLF in seven and  
274 twelve days in co-inoculum with strains FC54 and MUT5705, respectively. When LAB were  
275 sequentially inoculated in wines produced from mixed cultures, the same kinetics of MLF  
276 were observed with those reported in pure fermentations.

### 277 3.3 Standard chemical parameters of wines

278 The main chemical parameters of the wines at the end of the vinification are reported in  
279 Table 2. In order to highlight relationships among wine chemical compounds and  
280 fermentation conditions investigated; data were submitted to a principal component analysis  
281 (PCA) as shown in Fig. 4A. The first principal component (PC1, 45.6% of explained  
282 variance) was positively correlated mainly to malic acid, acetic acid and glucose, and  
283 negatively correlated mainly to lactic acid, fructose, and ethanol concentrations (Fig. 4B).  
284 The second principal component (PC2, 29.7% of explained variance) was positively  
285 correlated mainly to glycerol and lactic acid, and negatively correlated to ethanol, fructose,  
286 and malic acid concentrations (Fig. 4C).  
287 Generally, pure fermentations negatively correlated to PC2, resulting in a wine containing  
288 about 14.2% v/v of ethanol and 9.3 g/L of glycerol, while wines obtained from mixed  
289 fermentations had a lower alcohol content (about 13.7% v/v of ethanol) and a glycerol  
290 concentration almost two-fold higher with respect to pure fermentations (> 16.2 g/L).  
291 Additionally, MUT5705 was a higher glycerol producer than FC54. When MLF was not  
292 carried out, mixed fermented wines clustered very close among them and were characterized  
293 by the highest positive values of PC1 (i.e. high malic and acetic acid levels), while pure  
294 fermented wine was identified by a negative value of PC1 due the low levels of D-lactic  
295 acid..  
296 Interestingly, wines co-inoculated with *Starm. bacillaris* and Lp87 were clearly characterized  
297 by negative PC1 and the highest positive values of PC2, which implies complete AF and  
298 MLF with low contents of acetic acid and ethanol as well as high production of glycerol.  
299 Higher concentrations of L-lactic acid were detected in pure fermentations co-inoculated with  
300 *L. plantarum* (Table 2). Differently, D-lactic acid production was approximately 2.5-fold  
301 lower in pure than mixed fermentations, and its concentration seems to be further increased  
302 by co-inoculation with Lp87 (Table 2).

### 303 3.4 Volatile organic compounds

304 A total of 42 volatile organic compounds were identified and determined, including 11  
305 alcohols, 21 esters (15 ethyl and 4 acetate esters), 5 acids, 1 lactone, and 4 among terpenes  
306 and norisoprenoids (Table S1). Pure fermentations were clearly characterized by a  
307 concentration at least 1.5-fold higher of some alcohols such as 1-octanol, methionol, and 2-  
308 phenylethanol, and a lower level of isobutanol, 2,3-butanediol, and hexadecanol than mixed  
309 fermentations. In general, the addition of LAB had a negative impact on the alcohol  
310 concentration in mixed fermentations. In particular, samples without MLF showed the lowest  
311 amount of the above mentioned higher alcohol, some of which were not detected under these  
312 conditions (1-butanol, 1-octanol, and hexadecanol), while. By contrast, 1-hexanol and  
313 isobutanol concentrations were higher in mixed fermentation sequentially inoculated with *L.*  
314 *plantarum* when compared with all other tests. Some strain-specific features were also  
315 observed: Lp87 seemed to be related to high levels of 2,3-butanediol, and *O. oeni* to 1-  
316 butanol. In general, samples fermented by FC54 showed a lower content in higher alcohols  
317 than fermentations carried out in the same conditions but fermented by MUT5705.

318 Pure fermentations were clearly identified by a higher richness in acetate esters (except for  
319 ethyl acetate) and some ethyl esters (i.e. ethyl hexanoate, ethyl decanoate, ethyl octanoate,  
320 diethyl succinate, ethyl-9-decenoate, ethyl-3-methylbutylpentadecanoate, ethyl-3-  
321 methylbutyloctanoate, ethyl hexadecanoate), while only ethyl-2-hexenoate was higher in  
322 mixed fermentations. Overall, MLF weakly affected the ester composition of pure  
323 fermentation when co-inoculated, while the sequential inoculation regime reduced their  
324 concentration. A similar pattern was observed more pronounced in mixed fermentations  
325 because co-inoculation with LAB was associated to a significant reduction of these since  
326 some compounds but some of which were not detected in fermentations sequentially  
327 inoculated (i.e. methyl octanoate, isopentyl hexanoate, ethyl 3-methylbutyloctanoate, ethyl 3-

328 methylbutylpentadecanoate, ethyl 9-decenoate). However, the sequential approach increased  
329 the level of ethyl acetate in all the experimental conditions, and diethyl succinate by *O. oeni*  
330 particularly in pure and mixed fermentations and sequential inoculation. Most of samples  
331 inoculated with LAB were characterized by the production of ethyl lactate. This compound  
332 was not detected in control fermentations or when MLF did not begin in mixed fermentations  
333 and diminished in sequential inoculation. In particular, in wines co-inoculated with *O. oeni*  
334 the ethyl lactate production was about 4.5-fold higher than in those co-inoculated with *L.*  
335 *plantarum* strains. The level of ethyl lactate always detected in lower levels in sequential  
336 inoculation of LAB, compared to the respective co-inoculated FML.

337 Four major volatile fatty acids were identified, namely hexanoic, octanoic, decanoic, and  
338 dodecanoic acid. These compounds were found in lower amounts in mixed fermentations,  
339 particularly in samples fermented by FC54. However, the addition of LAB always reduced  
340 the concentration of these volatile compounds, especially in sequentially inoculated wines.  
341 Interestingly, in mixed fermentations without MLF, levels 4-fold lower of hexanoic acid and  
342 10-fold lower of octanoic and decanoic acids were detected.

343 Among terpenes, linalool and citronellol were more abundant in mixed fermentation, being  
344 linalool especially richer in mixed fermentation with FC54 and positively affected by the  
345 occurrence of both *L. plantarum*. Instead, geraniol was not clearly related with the  
346 experimental condition. The concentration of  $\gamma$ -butyrolactone was higher in pure and mixed  
347 fermentation without LAB inoculation and when *O. oeni* was co-inoculated. This last  
348 experimental condition seems also to slightly increase the level of  $\beta$ -damascenone detected.

349 The identified volatile compounds were submitted to PCA as shown in Fig. 5. PC1 explained  
350 51.3% of the total variance and was positively correlated mainly to ethyl esters, hexanoic  
351 acid and decanoic acid, while it was negatively correlated with linalool, citronellol, and 2,3-  
352 butanediol (Fig. 5B). PC2 explained 13.0% of the total variance and was positively correlated

353 with ethyl-2-hexenoate, linalool,  $\gamma$ -butyrolactone, and  $\beta$ -damascenone, while it was  
354 negatively correlated to ethyl esters, mainly ethyl acetate and diethyl succinate (Fig. 5C).  
355 Pure fermented wines co-inoculated with Lp44 or *O. oeni* were characterized by a positive  
356 correlation with both PC1 and PC2, and their volatile profile was close to the wine produced  
357 by *S. cerevisiae* in pure fermentation without LAB. However, the respective wine co-  
358 inoculated with Lp87 was remarkably different and characterized by high negative PC2  
359 values. Generally, mixed fermented wines with FC54 and LAB inoculation were  
360 characterized by positive PC2 and negative PC1 values, except wines that underwent MLF  
361 with *O. oeni*. In contrast, control mixed fermented wines with MUT5707 were characterized  
362 by both positive PC1 and PC2 values, with the PC2 decreasing to different extent depending  
363 on the co-inoculated LAB strain. Volatile compounds were strongly affected by the  
364 sequential approach. Thereby, wines obtained by sequential inoculation of malolactic starters  
365 were almost characterized by negative PC1 values, except for pure fermented wines  
366 inoculated with *O. oeni* that presented positive PC1. Interestingly, a LAB strain-depending  
367 contribution to the volatome was observed. Thus, MLF performed with *O. oeni* were always  
368 more positive on the PC1 than those carried out under same conditions with *L. plantarum*  
369 strains, while most of samples inoculated with Lp87 were always more negative on the PC2  
370 than the other trials.

#### 371 **4. Discussion**

372 In the last decades, several studies investigated the interactions between *S. cerevisiae* and  
373 LAB (Alexandre et al., 2004). The selection of compatible *S. cerevisiae* and LAB strain  
374 couple is fundamental in order to ensure a successfully AF and MLF, as certain strains have  
375 been found to have stimulatory, inhibitory and neutral impact on LAB and vice versa. To date  
376 few studies aimed to understand the interactions between non-*Saccharomyces* yeasts and  
377 LAB and their impact on wine quality (Belsamada et al., 2018). To this end in the present



378 study we have investigated the effect of mixed fermentations with *Starm. bacillaris* and *S.*  
379 *cerevisiae* on the overall performance of two LAB namely *L. plantarum* and *O. oeni*.  
380 During the first days of AF, a reduced growth of *S. cerevisiae* was observed in mixed trials  
381 with respect to pure assays, as previously observed by (Englezos et al., 2016). Moreover, the  
382 populations of LAB and yeasts were reciprocally affected in a strain-dependent way. These  
383 interactions were more evident some days after co-inoculation, as already reported (Lucio et  
384 al., 2018). Interestingly, the occurrence of LAB modulated the growth kinetics of *Starm.*  
385 *bacillaris* in a strain-dependent way. In particular, co-inoculation with *L. plantarum*, strongly  
386 affected only MUT5705 since after one week of coexistence no viable cells were detected.  
387 However, despite the LAB growth, only in some combinations, slightly affected in a negative  
388 way by the simultaneous occurrence of *Starm. bacillaris*, important differences were detected  
389 in their ability to carry out the MLF. Indeed, while co-inoculation with Lp44 was the fastest  
390 strategy to perform MLF among pure fermentations, the same strain was unable to complete  
391 MLF under mixed assays or sequential inoculation. Nonetheless, malic acid was depleted at  
392 higher extent in samples fermented with MUT5705 than in those with FC54, probably due to  
393 the inhibition of the MUT5705 growth. In contrast, this *Starm. bacillaris* strain had no effect  
394 on MLF when inoculated with Lp87. Some compounds including medium-chain fatty acids,  
395 organic acids, and peptides could have inhibitory effect against LAB (Balmaseda et al.,  
396 2018), and *Starm. bacillaris* has been demonstrated to possess antifungal activity associated  
397 to the production of volatile compounds (Nadai et al., 2018). Interestingly, co-inoculation  
398 with FC54 and Lp87, resulted in a gain of five days in completing MLF compared to the pure  
399 assays, indicating that positive interactions could take place among these strains. This  
400 beneficial effect was even more evident in both mixed fermentations co-inoculated with *O.*  
401 *oeni* that finished MLF after only two days. A specific feature of *Starm. bacillaris* strains was  
402 the low assimilation of nitrogen sources during the early phase of AF (Englezos, Cocolin,

403 Rantsiou, Ortiz-Julien, Bloem, et al., 2018). Therefore, we can hypothesize that in  
404 simultaneous inoculation LAB found more available nitrogen sources in mixed than pure  
405 fermentations.

406 Concerning the main oenological parameters, it is well known that *Starm. bacillaris* strains  
407 are high producers of glycerol and pyruvic acid as result of their high glyceropyruvic  
408 fermentation activity (Jolly et al., 2014 Magyar & Tóth, 2011). To date, no evidences have  
409 been reported on how glycerol affects MLF. In contrast, pyruvic acid can enter into citric acid  
410 pathway that sustains longer viability and provides energy, as well it could act as external  
411 electron acceptor, thus improving MLF performance (Balmaseda et al., 2018; Maicas, Sergi  
412 et al., 2002). Interestingly, it has been previously reported that both FC54 and MUT5705  
413 exhibited higher yields of pyruvic acid than *S. cerevisiae* on synthetic must, being this  
414 metabolite produced about two-fold more by FC54 than MUT5705 (Englezos, Cocolin,  
415 Rantsiou, Ortiz-Julien, Bloem, et al., 2018). Thus, we may hypothesize that this biochemical  
416 trait could partially explain the faster MLF observed in the co-inoculum of Lp87 with FC54.  
417 Finally, some non-*Saccharomyces* spp., including *Schizosaccharomyces pombe* and *C.*  
418 *zemplinina*, showed mentionable malic acid degradation ability (du Plessis et al., 2017b),  
419 suggesting that non-conventional oenological yeasts could actively contribute to the fast  
420 completion of MLF. However, few available studies seem to indicate that interactions among  
421 oenological resources are species and strain specific, as well as depending from the  
422 fermentation protocol (Wang et al., 2016).

423 Indeed, unlike simultaneous inoculation, MLF was not affected by mixed fermentations in  
424 sequential approach. The harsh environment probably mainly due to negative impact of  
425 ethanol on the expression of malolactic enzyme of *L. plantarum* has been demonstrated,  
426 providing evidences that this species should be better applied in co-inoculation (Miller et al.,  
427 2011). Du Plessis et al. (2017b) found that *Starm. bacillaris* strains did not have any

428 inhibitory effect on MLF performed by *O. oeni* in sequential inoculation, and that some  
429 strain-dependent delays in MLF could be alleviated by nutrient supplementation. It is well  
430 known that at the end of AF the wine is characterized by harsh conditions, including  
431 nutritional starvation, and high ethanol concentrations, combined with low pH. In this  
432 environment only *O. oeni* was able to perform MLF, employing the same time as in the pure  
433 fermentation.

434 Interestingly, a higher loss of viability of both *Starm. bacillaris* was observed in samples  
435 sequentially inoculated with *O. oeni* than with *L. plantarum*. It is conceivable that molecular  
436 mechanisms of adaptation, including malate utilization, allowed *O. oeni* more than *L.*  
437 *plantarum* to face the stressful environment of wine, thus encouraging some competitiveness  
438 against *Starm. bacillaris* (Bebegal et al., 2016; Grandvalet et al., 2005; Olguín et al., 2010).  
439 Therefore, the importance of malolactic starter acclimation to induce molecular responses  
440 that would allow better adaptation to the wine should be recommended in sequential  
441 inoculation (Costantini et al., 2015).

442 The evolution of primary metabolites typical of *Starm. bacillaris* mixed fermentations (i.e.  
443 fructose consumption, high glycerol production, and lower ethanol yields with respect to *S.*  
444 *cerevisiae*) have been here confirmed and never influenced by malolactic bacteria. In the  
445 management of MLF, co-inoculum with *L. plantarum* is preferred to *O. oeni* because it  
446 catabolizes hexoses homofermentatively preventing acetic acid from increasing (Lucio et al.,  
447 2018). Interestingly, it was detected an increase of acetic acid in mixed fermentations  
448 inoculated with *L. plantarum* when MLF was not completed, indicating that a longer time of  
449 vinification can affect the main oenological parameters and the volatile organic compounds  
450 by stimulating unwanted metabolic pathways.

451 It is well known that wines that underwent MLF generally show a significant increase in  
452 volatile compounds improving the sensory properties and quality of wines (Maicas et al.,

1999; Pozo-Bayón et al., 2005; Ugliano & Moio, 2005). In this study, it was found that, except for the production of ethyl lactate, the addition of malolactic starter in co-inoculation does not affect significantly the volatile profile of the control pure fermentation, while the sequential inoculation reduced the concentration of almost all secondary metabolites. Accordingly, it was reported that wines with sequential MLF had the lowest concentration of acetate and ethyl esters, which might result in decreased fruitiness (Abrahamse & Bartowsky, 2012; Knoll et al., 2012). Two exceptions are ethyl acetate and diethyl succinate whose increase in sequential approach was probably due to the longer time needed to complete the vinification (Ugliano & Moio, 2006). Interestingly, the addition of LAB in mixed fermentations resulted in a general strong decrease of almost all the identified volatile compounds in comparison to the corresponding pure assay. These differences were further increased when MLF was not completed, regardless of the inoculation regime. Intriguingly, lower levels of aromatic compounds were also detected in trials showing a faster MLF than pure assays, suggesting that yeast-bacteria competition for nutritional sources could divert some compounds from the metabolic pathways responsible for the biosynthesis of secondary metabolites.

Differences in the volatile profiles depending on the LAB species were also observed. Interestingly, higher alcohols and esters produced by *O. oeni* was generally greater than that derived from *L. plantarum*. In particular, wines resulting from mixed fermentations and inoculated with *O. oeni* were richer in these compounds than the corresponding wines fermented by *L. plantarum*. According to these findings, *O. oeni* and *L. plantarum* have shown to possess two different enzyme activities for ethyl ester biosynthesis (Costello et al., 2013). By contrast, isobutanol, 1-hexanol, and 2,3-butanediol were more enhanced by *L. plantarum* than by *O. oeni*, confirming what was observed by Lee et al. (2009).

## 5. Conclusion

478 In conclusion, the employment of *Starm. bacillaris* in mixed fermentation is a consolidated  
479 biotechnological strategy to obtain reduced-ethanol and high-glycerol wines. However, this  
480 work evidences the different impact of this non-conventional yeast on the progress of MLF  
481 and the importance of strain-dependent interactions, which could influence positively or  
482 negatively some technological aspects (i.e. vinification time) and compositional parameters  
483 (i.e. primary and secondary metabolites production). *O. oeni* was the best species to perform  
484 MLF in combination with *Starm. bacillaris* strains tested in this study. Co-inoculation  
485 approach was useful to reduce the time of MLF without negative impact on the volatile  
486 organic compound's complexity of the wine. Therefore, a careful selection of the strains to  
487 conduct AF and MLF and inoculation strategy could help to produce wines with established  
488 criteria. Moreover, further studies should be addressed to clarify the metabolites and  
489 molecular mechanisms underlying the observed interactions among non-conventional yeasts  
490 and malolactic bacteria.

491

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496

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720 **Legend to the figures**

721 **Fig. 1.** Growth dynamics of yeasts, and evolution of malic acid, lactic acid, fructose, glucose,  
722 glycerol, and ethanol during control pure (only *S. cerevisiae*) and mixed (*S. cerevisiae* with  
723 FC54 or MUT5705) microvinification. The assays were performed in triplicate and standard  
724 deviations are indicated.

725 **Fig. 2.** Growth dynamics of *S. cerevisiae* and malolactic LAB, and evolution of malic acid,  
726 and lactic acid during pure microvinification obtained by simultaneous or sequential  
727 inoculation of LAB. The assays were performed in triplicate and standard deviations are  
728 indicated.

729 **Fig. 3.** Growth dynamics of *S. cerevisiae*, *Starm. bacillaris* and malolactic LAB, and  
730 evolution of malic acid and lactic acid during mixed microvinification obtained by  
731 simultaneous or sequential inoculation of LAB. The assays were performed in triplicate and  
732 standard deviations are indicated.

733 **Fig. 4.** Principal component analysis of main enological parameters in pure and mixed  
734 microvinification (A). The first principal component (PC1) and the second principal  
735 component (PC2) descriptors are also showed (B and C, respectively).

736 **Fig. 5.** Principal component analysis of volatile organic compounds in pure and mixed  
737 microvinification (A). The first principal component (PC1) and the second principal  
738 component (PC2) descriptors are also showed (B and C, respectively). \*These volatile  
739 compounds were semi-quantified in relation to the area of the 1-heptanol internal standard.  
740 The rest of volatile compounds were quantified by a calibration with standard solutions  
741 analyzed under the same conditions as the wine samples.

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745 **Supplementary Table 1.** Mean concentration of volatile compounds in pure and mixed  
746 culture fermentations. Assays were performed in triplicate and standard deviations are  
747 reported.

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769 **Table 1.** Time required to complete MLF and vinification (AF and MLF) in pure and mixed  
 770 fermentations in microvinifications performed by co-inoculation or sequential inoculation of  
 771 malolactic bacteria.

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Inoculation protocol		Co-inoculation		Sequential inoculation	
		Days to complete MLF	Days to complete vinification	Days to complete MLF	Days to complete vinification
Pure culture ferm.	<i>S. cerevisiae</i> and <i>O. oeni</i>	7	7	12	19
	<i>S. cerevisiae</i> and Lp44	4	7	NC	NC
	<i>S. cerevisiae</i> and Lp87	12	12	NC	NC
Mixed culture ferm.	( <i>S. cerevisiae</i> and FC54) and <i>O. oeni</i>	2	7	12	19
	( <i>S. cerevisiae</i> and FC54) and Lp44	NC	NC	NC	NC
	( <i>S. cerevisiae</i> and FC54) and Lp87	7	9	NC	NC
	( <i>S. cerevisiae</i> and MUT 5705) and <i>O. oeni</i>	2	7	12	19
	( <i>S. cerevisiae</i> and MUT 5705) and Lp44	NC	NC	NC	NC
	( <i>S. cerevisiae</i> and MUT 5705) and Lp87	12	14	NC	NC

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774 Days to complete MLF: time of lactic acid bacteria (LAB) to complete malolactic  
 775 fermentation (MLF). Days to complete vinification: time from yeast inoculation to  
 776 completion of alcoholic and MLF. MLF was considered finished when malic acid  
 777 concentration was below 0.1 g/L. NC: MLF not completed.

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**Table 2.** Mean concentration of the main oenological parameters in pure and mixed culture fermentations. Assays were performed in triplicate and standard deviations are reported.

	Sample	Malic acid (g/L)	D-Lactic acid (g/L)	L-lactic acid (g/L)	D+L Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Glucose (g/L)	Fructose (g/L)	Ethanol (% v/v)
	<b>Pure culture fermentation</b>									
	<i>S. cerevisiae</i>	2.92 ± 0.02Dbcd	0.12 ± 0.00a	0.02 ± 0.01Aab	0.14 ± 0.01Aabc	0.31 ± 0.00Bde	9.35 ± 0.01Ca	0.13 ± 0.01Aab	0.70 ± 0.06AB	14.25 ± 0.01a
Coinoculum	<i>S. cerevisiae</i> and <i>O. oeni</i>	0.03 ± 0.00Aa	0.10 ± 0.01a	2.04 ± 0.01Bcd	2.14 ± 0.01Bdef	0.31 ± 0.01Be	9.37 ± 0.04Ca	0.12 ± 0.01Aabcd	0.71 ± 0.04AB	14.26 ± 0.01a
	<i>S. cerevisiae</i> and Lp44	0.02 ± 0.01Aa	0.16 ± 0.01ab	2.62 ± 0.04Cd	2.78 ± 0.05Cef	0.31 ± 0.03Bcde	9.32 ± 0.03Bca	0.31 ± 0.26Aabc	0.52 ± 0.14A	14.25 ± 0.03a
	<i>S. cerevisiae</i> and Lp87	0.02 ± 0.00Aa	0.12 ± 0.00a	2.71 ± 0.00Ccd	2.83 ± 0.00Cdef	0.23 ± 0.00Abcde	9.08 ± 0.00Aa	0.24 ± 0.00Aabc	0.64 ± 0.00AB	14.26 ± 0.00a
Sequential	<i>S. cerevisiae</i> and <i>O. oeni</i>	0.03 ± 0.02Aab	0.13 ± 0.01a	2.05 ± 0.04Bbc	2.18 ± 0.05Bbcd	0.32 ± 0.00Be	9.23 ± 0.03Ba	0.06 ± 0.00Aabc	0.72 ± 0.00ABC	14.27 ± 0.01a
	<i>S. cerevisiae</i> and Lp44	2.83 ± 0.01Ccd	0.11 ± 0.01a	0.11 ± 0.02Aa	0.21 ± 0.03Aa	0.31 ± 0.01Be	9.41 ± 0.05Ca	0.66 ± 0.05Bcd	0.79 ± 0.01BC	14.23 ± 0.03a
	<i>S. cerevisiae</i> and Lp87	2.67 ± 0.06Bcd	0.10 ± 0.08a	0.11 ± 0.01Aa	0.21 ± 0.09Aa	0.3 ± 0.03Bde	9.35 ± 0.03Ca	0.77 ± 0.07Bcd	0.93 ± 0.04C	14.24 ± 0.03a
	Sign. <sup>1</sup>	***	NS	***	***	***	***	***	***	NS
	<b>Mixed culture fermentation</b>									
	<i>S. cerevisiae</i> and FC54	2.85 ± 0.05Ccd	0.31 ± 0.00Cde	0.02 ± 0.01Aa	0.33 ± 0.01Aab	0.16 ± 0.02Aa	16.23 ± 0.13b	0.11 ± 0.03Aab	0.67 ± 0.10BC	13.67 ± 0.01b
Coinoculum	( <i>S. cerevisiae</i> and FC54) and <i>O. oeni</i>	0.02 ± 0.01Aa	0.13 ± 0.01Aa	2.23 ± 0.06Dcd	2.36 ± 0.07Ddef	0.31 ± 0.03Bde	16.54 ± 0.18bcd	0.52 ± 0.23Babcd	0.78 ± 0.11C	13.71 ± 0.02b
	( <i>S. cerevisiae</i> and FC54) and Lp44	2.54 ± 0.01Bbcd	0.18 ± 0.01Aabc	0.62 ± 0.03Cab	0.80 ± 0.04Cabc	0.63 ± 0.04Cf	16.41 ± 0.24bc	1.34 ± 0.13De	0.51 ± 0.11AB	13.68 ± 0.03b
	( <i>S. cerevisiae</i> and FC54) and Lp87	0.08 ± 0.02Aa	0.87 ± 0.02Dg	2.40 ± 0.00Ecd	3.27 ± 0.02Ef	0.20 ± 0.00Aabc	16.32 ± 0.11b	0.03 ± 0.05Aa	0.67 ± 0.07BC	13.68 ± 0.02b
Sequential	( <i>S. cerevisiae</i> and FC54) and <i>O. oeni</i>	0.02 ± 0.01Aa	0.23 ± 0.01Bbcd	2.22 ± 0.02Dcd	2.46 ± 0.02Ddef	0.22 ± 0.02Aabcd	16.71 ± 0.13bcd	0 ± 0Aa	0.72 ± 0.01BX	13.67 ± 0.02b
	( <i>S. cerevisiae</i> and FC54) and Lp44	2.84 ± 0.01Ccd	0.33 ± 0.02Cde	0.14 ± 0.03Ba	0.47 ± 0.04Bab	0.64 ± 0.02Cf	16.44 ± 0.02bc	0.86 ± 0.02Cde	0.52 ± 0.12AB	13.7 ± 0.02b
	( <i>S. cerevisiae</i> and FC54) and Lp87	2.92 ± 0.06Ccd	0.31 ± 0.04Cde	0.14 ± 0.03Ba	0.45 ± 0.05Bab	0.62 ± 0.02Cf	16.27 ± 0.15b	0.84 ± 0.06Cde	0.39 ± 0.02A	13.67 ± 0.01b
	Sign. <sup>1</sup>	***	***	***	***	***	NS	***	***	NS
	<i>S. cerevisiae</i> and MUT5705	2.64 ± 0.01Ccd	0.31 ± 0.01Abde	0.02 ± 0.00Aa	0.32 ± 0.01Aab	0.20 ± 0.02Ababc	17.49 ± 0.25NCfg	0.27 ± 0.24acd	0.58 ± 0.27	13.68 ± 0.01b
Coinoculum	( <i>S. cerevisiae</i> and MUT 5705) and <i>O. oeni</i>	0.04 ± 0.01Aa	0.24 ± 0.02Abcd	2.39 ± 0.05Dcd	2.62 ± 0.05Ddef	0.28 ± 0.02Bcde	17.45 ± 0.11Bcefg	0.59 ± 0.33abcd	0.60 ± 0.42	13.69 ± 0.02b
	( <i>S. cerevisiae</i> and MUT 5705) and Lp44	1.4 ± 0.02Babc	0.40 ± 0.07Be	1.43 ± 0.02Cbcd	1.83 ± 0.07Ccde	0.18 ± 0.02Aab	16.45 ± 0.31Abc	0.63 ± 0.51bcd	0.57 ± 0.30	13.67 ± 0.06b
	( <i>S. cerevisiae</i> and MUT 5705) and Lp87	0.01 ± 0.01Aa	0.55 ± 0.00Cf	2.50 ± 0.01Ecd	3.05 ± 0.01Ef	0.19 ± 0.01Ababc	16.86 ± 0.07Acd	0.22 ± 0.02abc	0.68 ± 0.11	13.66 ± 0.03b
Sequential	( <i>S. cerevisiae</i> and MUT 5705) and <i>O. oeni</i>	0.03 ± 0.01Aa	0.26 ± 0.04Acd	2.38 ± 0.04Dcd	2.64 ± 0.02Ddef	0.20 ± 0.02Ababc	17.62 ± 0.28Cg	0 ± 0a	0.72 ± 0.01	13.67 ± 0.01b
	( <i>S. cerevisiae</i> and MUT 5705) and Lp44	2.93 ± 0.05Dcd	0.32 ± 0.02Abde	0.14 ± 0.00Ba	0.46 ± 0.02Bab	0.65 ± 0.06Cf	17.00 ± 0.19Abdef	0.74 ± 0.04cd	0.45 ± 0.09	13.67 ± 0.03b
	( <i>S. cerevisiae</i> and MUT 5705) and Lp87	3.10 ± 0.01Ed	0.30 ± 0.00Ad	0.02 ± 0.01Aa	0.32 ± 0.01Aab	0.64 ± 0.06Cf	16.99 ± 0.13Abde	0.66 ± 0.01bcd	0.45 ± 0.11	13.69 ± 0.02b
	Sign. <sup>1</sup>	***	***	***	***	***	***	NS	NS	NS

Sign. <sup>2</sup>	***	***	***	***	***	***	***	NS	***
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784 Data are expressed as average value  $\pm$  standard deviation ( $n=3$ ).

785 Capital letters indicate significant differences among pure and mixed fermentations (Sign.<sup>1</sup>). Lowercase letters indicate significant differences within pure fermentations, and  
 786 within both mixed fermentations (Sign.<sup>2</sup>)

787 Sign.<sup>1,2</sup>:\*, \*\*, \*\*\*, and NS indicate significance at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ , and not significant respectively

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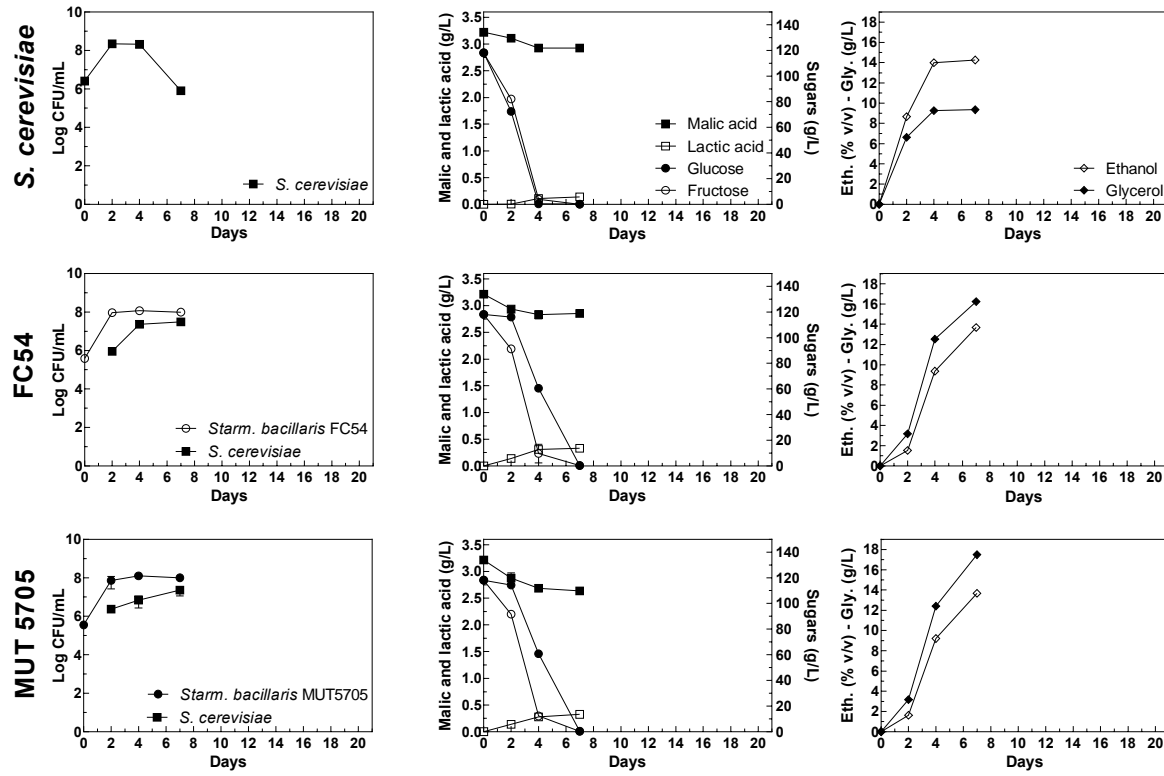
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803 FIGURES

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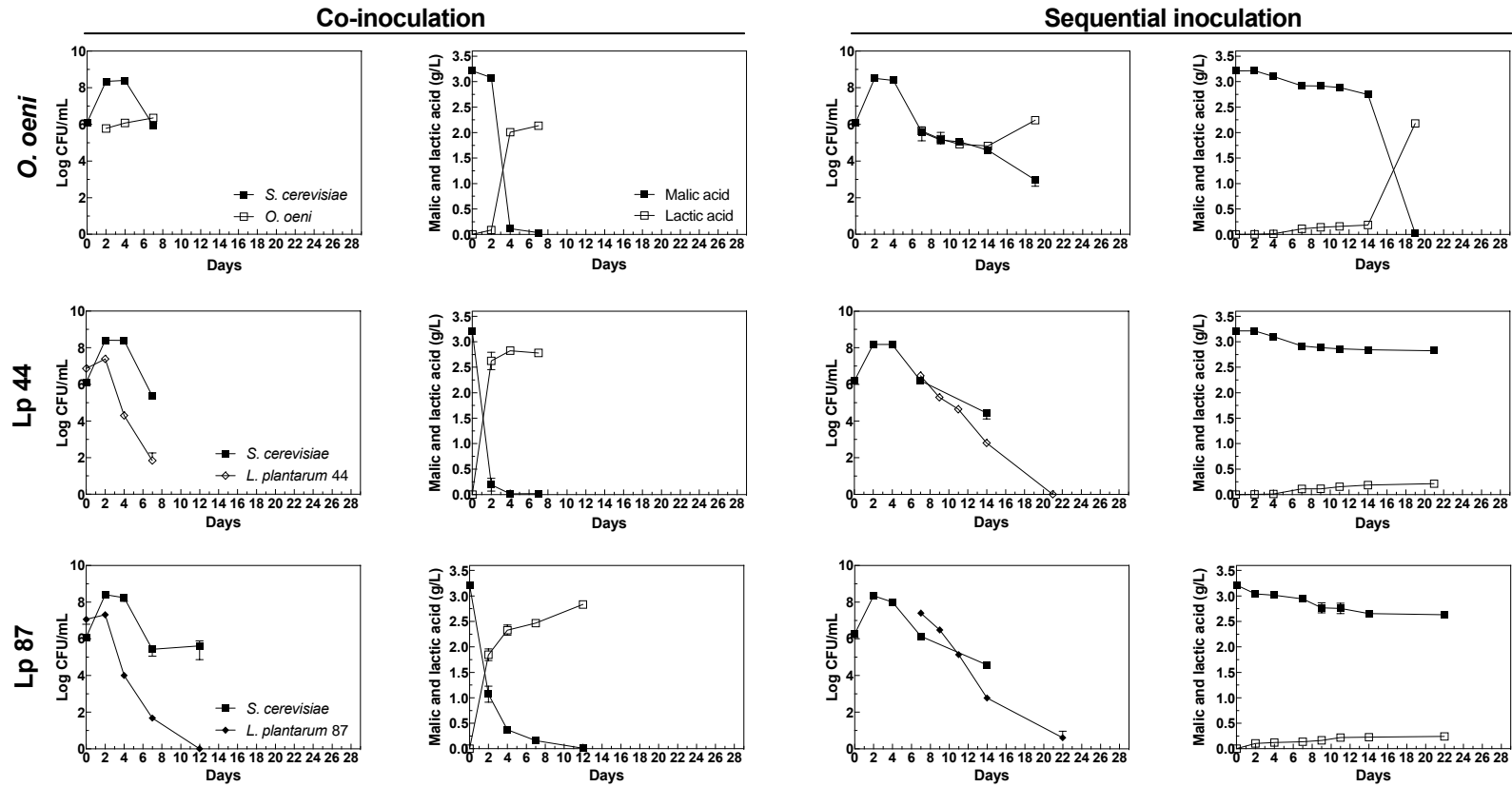
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