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

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1 **Effect of mixed fermentations with *Starmerella bacillaris* and *Saccharomyces cerevisiae***

2 **on management of malolactic fermentation**

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Abstract

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

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

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1. Introduction

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

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

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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® (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® (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 x 10^6

138   CFU/mL), *S. cerevisiae* (1 x 10^6 CFU/mL), and *L. plantarum* (8 x 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 x 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_2 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_2SO_4) 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.

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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 (2×10^8 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 2.0×10^7 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 1×10^2 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 x 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 x 10^8 CFU/mL after nine days when co-inoculated with *L. plantarum*, and it further

241 declined until about 2 x 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 x 10^4 and 3 x 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-

13

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 γ-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 β-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, γ-butyrolactone, and β-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,

16

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* (Berbegal 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.,

453   1999; Pozo-Bayón et al., 2005; Ugliano & Moio, 2005). In this study, it was found that,

454   except for the production of ethyl lactate, the addition of malolactic starter in co-inoculation

455   does not affect significantly the volatile profile of the control pure fermentation, while the

456   sequential inoculation reduced the concentration of almost all secondary metabolites.

457   Accordingly, it was reported that wines with sequential MLF had the lowest concentration of

458   acetate and ethyl esters, which might result in decreased fruitiness (Abrahamse & Bartowsky,

459   2012; Knoll et al., 2012). Two exceptions are ethyl acetate and diethyl succinate whose

460   increase in sequential approach was probably due to the longer time needed to complete the

461   vinification (Ugliano & Moio, 2006). Interestingly, the addition of LAB in mixed

462   fermentations resulted in a general strong decrease of almost all the identified volatile

463   compounds in comparison to the corresponding pure assay. These differences were further

464   increased when MLF was not completed, regardless of the inoculation regime. Intriguingly,

465   lower levels of aromatic compounds were also detected in trials showing a faster MLF than

466   pure assays, suggesting that yeast-bacteria competition for nutritional sources could divert

467   some compounds from the metabolic pathways responsible for the biosynthesis of secondary

468   metabolites.

469   Differences in the volatile profiles depending on the LAB species were also observed.

470   Interestingly, higher alcohols and esters produced by *O. oeni* was generally greater than that

471   derived from *L. plantarum*. In particular, wines resulting from mixed fermentations and

472   inoculated with *O. oeni* were richer in these compounds than the corresponding wines

473   fermented by *L. plantarum*. According to these findings, *O. oeni* and *L. plantarum* have

474   shown to possess two different enzyme activities for ethyl ester biosynthesis (Costello et al.,

475   2013). By contrast, isobutanol, 1-hexanol, and 2,3-butanediol were more enhanced by *L.*

476   *plantarum* than by *O. oeni*, confirming what was observed by Lee et al. (2009).

477   **5. Conclusion**

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

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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.	*S. cerevisiae* and *O. oeni*	7	7	12	19
	S. cerevisiae and Lp44	4	7	NC	NC
	S. cerevisiae and Lp87	12	12	NC	NC
Mixed culture ferm.	(*S. cerevisiae* and FC54) and *O. oeni*	2	7	12	19
	(*S. cerevisiae* and FC54) and Lp44	NC	NC	NC	NC
	(*S. cerevisiae* and FC54) and Lp87	7	9	NC	NC
	(*S. cerevisiae* and MUT 5705) and *O. oeni*	2	7	12	19
	(*S. cerevisiae* and MUT 5705) and Lp44	NC	NC	NC	NC
	(*S. cerevisiae* and MUT 5705) and Lp87	12	14	NC	NC

773

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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781 **Table 2.** Mean concentration of the main oenological parameters in pure and mixed culture fermentations. Assays were performed in triplicate and
782 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)
	Pure culture fermentation									
	S. cerevisiae	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	*S. cerevisiae* and *O. oeni*	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
	S. cerevisiae 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
	S. cerevisiae 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	*S. cerevisiae* and *O. oeni*	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
	S. cerevisiae 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
	S. cerevisiae 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.[1]	***	NS	***	***	***	***	***	***	NS
	Mixed culture fermentation									
	S. cerevisiae 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	(*S. cerevisiae* and FC54) and *O. oeni*	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
	(*S. cerevisiae* 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
	(*S. cerevisiae* 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	(*S. cerevisiae* and FC54) and *O. oeni*	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
	(*S. cerevisiae* 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
	(*S. cerevisiae* 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.[1]	***	***	***	***	***	NS	***	***	NS
	S. cerevisiae 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	(*S. cerevisiae* and MUT 5705) and *O. oeni*	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
	(*S. cerevisiae* 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
	(*S. cerevisiae* 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	(*S. cerevisiae* and MUT 5705) and *O. oeni*	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
	(*S. cerevisiae* 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
	(*S. cerevisiae* 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.[1]	***	***	***	***	***	***	NS	NS	NS

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| Sign.[2] | *** | *** | *** | *** | *** | *** | *** | NS | *** |

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Data are expressed as average value ± standard deviation (*n*=3).

Capital letters indicate significant differences among pure and mixed fermentations (Sign.[1]). Lowercase letters indicate significant differences within pure fermentations, and within both mixed fermentations (Sign.[2])

Sign.[1,2]:*, **, ***, and NS indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$, and not significant respectively

FIGURES

FIG.1

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811   **Fig. 2**

Fig. 3

Fig. 5