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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.

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

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

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

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

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

2. Materials and Methods

2.1 Microbial strains and growth conditions

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

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

2.2 Must preparation and inoculum

Vitis vinifera L. cv. Barbera red grapes (Barbaresco, North-West Italy) were used for micro-scale winemaking assays. Grapes were manually pressed, and solid parts (skins and seeds) were separated from the juice using a stainless-steel sieve. Then, the must was exposed to heat treatment (60 °C for 60 min) to inactivate the indigenous microbiota. Pasteurization efficacy was checked by plating on Wallerstein Laboratory (WL) Nutrient and MRS agar (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* (1×10^6
138 CFU/mL), *S. cerevisiae* (1×10^6 CFU/mL), and *L. plantarum* (8×10^6 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 5×10^5 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

strain. After inoculation, Erlenmeyer flasks were closed with air locks containing sterile paraffin oil, to allow only the CO_2 loss from the fermenting must and to prevent external contamination, according to the procedure reported by Englezos et al. (2016). Fermentations were carried out at 25 °C and Erlenmeyer flasks were manually shaken each 12 h. AF was monitored until complete depletion of glucose and fructose (< 2.0 g/L), as determined by HPLC. Malolactic fermentation was monitored until complete depletion of malic acid (0.1 g/L), as determined by HPLC, or until viable cells of LAB (more than 1 Log CFU/mL) were enumerated. Each fermentation condition was tested by performing three simultaneous independent biological repetitions.

2.4 Microbiological analysis

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

2.5 Must and wine analysis

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

equipped with an Aminex HPX-87H cation exchange column (300 mm x 7.8 mm i.d.), a UV detector set to 210 nm and a refractive index detector. The eluent was 0.0065 mol/L sulfuric acid (H_2SO_4) at a flow rate of 0.7 mL/min and the column temperature was 65 °C, as previously described Englezos et al. (2018c). The concentrations of D- and L-lactic acid were determined spectrophotometrically at 340 nm using specific enzymatic kit (product code: K-DLATE; Megazyme International) and according to the manufacturer's instructions, using an UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) in grape juice and at the end MLF. The concentration of yeast available nitrogen (YAN), was determined in grape juice using specific enzymatic kits (product codes: K-LARGE and K-PANOPA; Megazyme International) and according to the manufacturer's instructions, using the above-mentioned spectrophotometer at 340 nm. The pH in grape juice and at the end of the alcoholic/malolactic fermentations was registered using the InoLab 730 pH meter (WTW, Weilheim, DE), while total acidity (TA) was determined and expressed in g/L of tartaric acid according to the official protocol described by the International Organization of Vine and Wine (OIV, 2015).

2.6 Determination of volatile organic compounds

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

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

2.7 Statistical analysis

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

3. Results

3.1 Grape must chemical parameters and microbial growth dynamics

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

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

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

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

3.2 Kinetics of main oenological parameters

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

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

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

3.3 Standard chemical parameters of wines

The main chemical parameters of the wines at the end of the vinification are reported in Table 2. In order to highlight relationships among wine chemical compounds and fermentation conditions investigated; data were submitted to a principal component analysis (PCA) as shown in Fig. 4A. The first principal component (PC1, 45.6% of explained variance) was positively correlated mainly to malic acid, acetic acid and glucose, and negatively correlated mainly to lactic acid, fructose, and ethanol concentrations (Fig. 4B). The second principal component (PC2, 29.7% of explained variance) was positively correlated mainly to glycerol and lactic acid, and negatively correlated to ethanol, fructose, and malic acid concentrations (Fig. 4C).

Generally, pure fermentations negatively correlated to PC2, resulting in a wine containing about 14.2% v/v of ethanol and 9.3 g/L of glycerol, while wines obtained from mixed fermentations had a lower alcohol content (about 13.7% v/v of ethanol) and a glycerol concentration almost two-fold higher with respect to pure fermentations (> 16.2 g/L). Additionally, MUT5705 was a higher glycerol producer than FC54. When MLF was not carried out, mixed fermented wines clustered very close among them and were characterized by the highest positive values of PC1 (i.e. high malic and acetic acid levels), while pure fermented wine was identified by a negative value of PC1 due the low levels of D-lactic acid..

Interestingly, wines co-inoculated with *Starm. bacillaris* and Lp87 were clearly characterized by negative PC1 and the highest positive values of PC2, which implies complete AF and MLF with low contents of acetic acid and ethanol as well as high production of glycerol. Higher concentrations of L-lactic acid were detected in pure fermentations co-inoculated with *L. plantarum* (Table 2). Differently, D-lactic acid production was approximately 2.5-fold lower in pure than mixed fermentations, and its concentration seems to be further increased 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 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

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

4. Discussion

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

study we have investigated the effect of mixed fermentations with *Starm. bacillaris* and *S. cerevisiae* on the overall performance of two LAB namely *L. plantarum* and *O. oeni*.

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

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

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

Indeed, unlike simultaneous inoculation, MLF was not affected by mixed fermentations in sequential approach. The harsh environment probably mainly due to negative impact of ethanol on the expression of malolactic enzyme of *L. plantarum* has been demonstrated, providing evidences that this species should be better applied in co-inoculation (Miller et al., 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.,

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

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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References

Abrahamse, C. E., & Bartowsky, E. J. (2012). Timing of malolactic fermentation inoculation in Shiraz grape must and wine: influence on chemical composition. *World Journal of Microbiology and Biotechnology*, *28*(1), 255–265. https://doi.org/10.1007/s11274-011-0814-3

Alexandre, H., Costello, P. J., Remize, F., Guzzo, J., & Guilloux-Benatier, M. (2004). *Saccharomyces cerevisiae–Oenococcus oeni* interactions in wine: current knowledge and perspectives. *International Journal of Food Microbiology*, *93*(2), 141–154. https://doi.org/10.1016/j.ijfoodmicro.2003.10.013

Augagneur, Y., Ritt, J.-F., Linares, D. M., Remize, F., Tourdot-Maréchal, R., Garmyn, D., & Guzzo, J. (2007). Dual effect of organic acids as a function of external pH in *Oenococcus oeni*. *Archives of Microbiology*, *188*(2), 147–157. https://doi.org/10.1007/s00203-007-0230-0

Balmaseda, A., Bordons, A., Reguant, C., & Bautista-Gallego, J. (2018). Non-*Saccharomyces* in Wine: Effect Upon *Oenococcus oeni* and Malolactic Fermentation. *Frontiers in Microbiology*, *9*, 534. https://doi.org/10.3389/fmicb.2018.00534

Bartowsky, E. J., & Borneman, A. R. (2011). Genomic variations of *Oenococcus oeni* strains and the potential to impact on malolactic fermentation and aroma compounds in wine. *Applied Microbiology and Biotechnology*, *92*(3), 441–447. https://doi.org/10.1007/s00253-011-3546-2

Berbegal, C., Peña, N., Russo, P., Grieco, F., Pardo, I., Ferrer, S., Spano, G., & Capozzi, V. (2016). Technological properties of *Lactobacillus plantarum* strains isolated from grape must fermentation. *Food Microbiology*, *57*, 187–194. https://doi.org/10.1016/j.fm.2016.03.002

Berbegal, C., Spano, G., Tristezza, M., Grieco, F., & Capozzi, V. (2017). Microbial Resources and Innovation in the Wine Production Sector. *South African Journal of Enology and Viticulture*, *38*, 156–166.

Capozzi, V., Berbegal, C., Tufariello, M., Grieco, F., Spano, G., & Grieco, F. (2019). Impact of co-inoculation of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and

Oenococcus oeni autochthonous strains in controlled multi starter grape must fermentations. *LWT*, *109*, 241–249. https://doi.org/10.1016/j.lwt.2019.04.045

Ciani, M., & Comitini, F. (2011). Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. *Annals of Microbiology*, *61*(1), 25–32. https://doi.org/10.1007/s13213-010-0069-5

Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, J. A., Oro, L., Rodrigues, A. J., & Gonzalez, R. (2016). Non-conventional Yeast Species for Lowering Ethanol Content of Wines. *Frontiers in Microbiology*, *7*, 642. https://doi.org/10.3389/fmicb.2016.00642

Contreras, A., Hidalgo, C., Henschke, P. A., Chambers, P. J., Curtin, C., & Varela, C. (2014). Evaluation of Non-*Saccharomyces* Yeasts for the Reduction of Alcohol Content in Wine. *Applied and Environmental Microbiology*, *80*(5), 1670–1678. https://doi.org/10.1128/AEM.03780-13

Costantini, A., Rantsiou, K., Majumder, A., Jacobsen, S., Pessione, E., Svensson, B., Garcia-Moruno, E., & Cocolin, L. (2015). Complementing DIGE proteomics and DNA subarray analyses to shed light on *Oenococcus oeni* adaptation to ethanol in wine-simulated conditions. *Journal of Proteomics*, *123*, 114–127. https://doi.org/10.1016/j.jprot.2015.04.019

Costello, P. J., Siebert, T. E., Solomon, M. R., & Bartowsky, E. J. (2013). Synthesis of fruity ethyl esters by acyl coenzyme A: alcohol acyltransferase and reverse esterase activities in *Oenococcus oeni* and *Lactobacillus plantarum*. *Journal of Applied Microbiology*, *114*(3), 797–806. https://doi.org/10.1111/jam.12098

du Plessis, H., Du Toit, M., Nieuwoudt, H., Van der Rijst, M., Kidd, M., & Jolly, N. (2017). Effect of *Saccharomyces*, Non-*Saccharomyces* Yeasts and Malolactic Fermentation

Strategies on Fermentation Kinetics and Flavor of Shiraz Wines. *Fermentation*, *3*(4), 64. https://doi.org/10.3390/fermentation3040064

du Plessis, H. W., du Toit, M., Hoff, J. W., Hart, R. S., Ndimba, B. K., & Jolly, N. P. (2017). Characterisation of Non-*Saccharomyces* yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation. *South African Journal of Enology and Viticulture*, *38*, 46–63.

du Toit, M., Engelbrecht, L., Lerm, E., & Krieger-Weber, S. (2011). Lactobacillus: the Next Generation of Malolactic Fermentation Starter Cultures—an Overview. *Food and Bioprocess Technology*, *4*(6), 876–906. https://doi.org/10.1007/s11947-010-0448-8.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., & Cocolin, L. (2015). Exploitation of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: Physiological and molecular characterizations. *International Journal of Food Microbiology*, *199*, 33–40. https://doi.org/10.1016/j.ijfoodmicro.2015.01.009

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Ortiz-Julien, A., Gerbi, V., Rolle, L., & Cocolin, L. (2016). *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Applied Microbiology and Biotechnology*, *100*(12), 5515–5526. https://doi.org/10.1007/s00253-016-7413-z

Englezos, V., Giacosa, S., Rantsiou, K., Rolle, L., & Cocolin, L. (2017). *Starmerella bacillaris* in winemaking: opportunities and risks. *Current Opinion in Food Science*, *17*, 30–35. https://doi.org/10.1016/j.cofs.2017.08.007.

Englezos, V., Cocolin, L., Rantsiou, K., Ortiz-Julien, A., Bloem, A., Dequin, S., & Camarasa, C. (2018). Specific Phenotypic Traits of Starmerella bacillaris Related to Nitrogen Source Consumption and Central Carbon Metabolite Production during Wine

574 Fermentation. *Applied and Environmental Microbiology*, *84*(16), e00797-18.
575 https://doi.org/10.1128/AEM.00797-18
576 Englezos, V., Cravero, F., Torchio, F., Rantsiou, K., Ortiz-Julien, A., Lambri, M., Gerbi, V.,
577 Rolle, L., & Cocolin, L. (2018). Oxygen availability and strain combination modulate
578 yeast growth dynamics in mixed culture fermentations of grape must with *Starmerella*
579 *bacillaris* and *Saccharomyces cerevisiae*. *Food Microbiology*, *69*, 179–188.
580 https://doi.org/10.1016/j.fm.2017.08.007
581 Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Giacosa, S., Ortiz-Julien, A., Gerbi, V.,
582 Rolle, L., & Cocolin, L. (2018). Volatile profiles and chromatic characteristics of red
583 wines produced with *Starmerella bacillaris* and *Saccharomyces cerevisiae*. *Food*
584 *Research International*, *109*, 298–309. https://doi.org/10.1016/j.foodres.2018.04.027
585 Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Pollon, M., Fracassetti, D., Ortiz-Julien,
586 A., Gerbi, V., Rolle, L., & Cocolin, L. (2018). Volatile profile of white wines
587 fermented with sequential inoculation of *Starmerella bacillaris* and *Saccharomyces*
588 *cerevisiae*. *Food Chemistry*, *257*, 350–360.
589 https://doi.org/10.1016/j.foodchem.2018.03.018
590 Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Research*, *8*(7), 979–995.
591 https://doi.org/10.1111/j.1567-1364.2008.00427.x
592 Garofalo, C., Russo, P., Beneduce, L., Massa, S., Spano, G., & Capozzi, V. (2016). Non-
593 *Saccharomyces* biodiversity in wine and the '*microbial terroir*': a survey on Nero di
594 Troia wine from the Apulian region, Italy. *Annals of Microbiology*, *66*(1), 143–150.
595 https://doi.org/10.1007/s13213-015-1090-5
596 Grandvalet, C., Coucheney, F., Beltramo, C., & Guzzo, J. (2005). CtsR Is the Master
597 Regulator of Stress Response Gene Expression in *Oenococcus oeni*. *Journal of*

598 *Bacteriology*, *187*(16), 5614–5623. https://doi.org/10.1128/JB.187.16.5614-
599 5623.2005
600 Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2006). The Role and Use of Non-
601 *Saccharomyces* Yeasts in Wine Production. *South African Journal of Enology and*
602 *Viticulture*, *27*, 15–38.
603 Jolly, N. P., Varela, C., & Pretorius, I. S. (2014). Not your ordinary yeast: non-
604 *Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Research*, *14*(2),
605 215–237. https://doi.org/10.1111/1567-1364.12111
606 Knoll, C., Fritsch, S., Schnell, S., Grossmann, M., Krieger-Weber, S., du Toit, M., & Rauhut,
607 D. (2012). Impact of different malolactic fermentation inoculation scenarios on
608 Riesling wine aroma. *World Journal of Microbiology and Biotechnology*, *28*(3),
609 1143–1153. https://doi.org/10.1007/s11274-011-0917-x
610 Lee, J.-E., Hwang, G.-S., Lee, C.-H., & Hong, Y.-S. (2009). Metabolomics Reveals
611 Alterations in Both Primary and Secondary Metabolites by Wine Bacteria. *Journal of*
612 *Agricultural and Food Chemistry*, *57*(22), 10772–10783.
613 https://doi.org/10.1021/jf9028442
614 Lucio, O., Pardo, I., Heras, J. M., Krieger, S., & Ferrer, S. (2018). Influence of yeast strains
615 on managing wine acidity using *Lactobacillus plantarum*. *Food Control*, *92*, 471–
616 478. https://doi.org/10.1016/j.foodcont.2018.05.027
617 Magyar, I., & Tóth, T. (2011). Comparative evaluation of some oenological properties in
618 wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and
619 *Saccharomyces cerevisiae*. *Food Microbiology*, *28*(1), 94–100.
620 https://doi.org/10.1016/j.fm.2010.08.011

621 Maicas, S., Gil, J.-V., Pardo, I., & Ferrer, S. (1999). Improvement of volatile composition of
622 wines by controlled addition of malolactic bacteria. *Food Research International*,
623 *32*(7), 491–496. https://doi.org/10.1016/S0963-9969(99)00122-2
624 Maicas, S., Ferrer, S., & Pardo, I. (2002). NAD(P)H regeneration is the key for heterolactic
625 fermentation of hexoses in *Oenococcus oeni*. *Microbiology*, *148*, 325–332.
626 Margalef-Català, M., Araque, I., Bordons, A., Reguant, C., & Bautista-Gallego, J. (2016).
627 Transcriptomic and Proteomic Analysis of *Oenococcus oeni* Adaptation to Wine
628 Stress Conditions. *Frontiers in Microbiology*, *7*, 1554. PubMed.
629 https://doi.org/10.3389/fmicb.2016.01554
630 Masneuf-Pomarede, I., Juquin, E., Miot-Sertier, C., Renault, P., Laizet, Y., Salin, F.,
631 Alexandre, H., Capozzi, V., Cocolin, L., Colonna-Ceccaldi, B., Englezos, V., Girard,
632 P., Gonzalez, B., Lucas, P., Mas, A., Nisiotou, A., Sipiczki, M., Spano, G., Tassou,
633 C., Bely, M., & Albertin, W. (2015). The yeast *Starmerella bacillaris* (synonym
634 *Candida zemplinina*) shows high genetic diversity in winemaking environments.
635 *FEMS Yeast Research*, *15*(5), fov045. https://doi.org/10.1093/femsyr/fov045
636 Medina, K., Boido, E., Dellacassa, E., & Carrau, F. (2018). Effects of Non-*Saccharomyces*
637 Yeasts on Color, Anthocyanin and Anthocyanin-Derived Pigments of Tannat Grapes
638 during Fermentation. *American Journal of Enology and Viticulture*, *69*, 148–156.
639 https://doi.org/10.5344/ajev.2017.17055
640 Miller, B. J., Franz, C. M. A. P., Cho, G.-S., & du Toit, M. (2011). Expression of the
641 Malolactic Enzyme Gene (mle) from *Lactobacillus plantarum* Under Winemaking
642 Conditions. *Current Microbiology*, *62*(6), 1682. https://doi.org/10.1007/s00284-011-
643 9914-4

644 Nadai, C., Fernandes Lemos, W. J., Favaron, F., Giacomini, A., & Corich, V. (2018).
645 Biocontrol activity of *Starmerella bacillaris* yeast against blue mold disease on apple
646 fruit and its effect on cider fermentation. *Plos One*, *13*(9), e0204350.
647 Nardi, T., Panero, L., Petrozziello, M., Guaita, M., Tsolakis, C., Cassino, C., Vagnoli, P., &
648 Bosso, A. (2019). Managing wine quality using *Torulaspora delbrueckii* and
649 *Oenococcus oeni* starters in mixed fermentations of a red Barbera wine. *European*
650 *Food Research and Technology*, *245*(2), 293–307. https://doi.org/10.1007/s00217-
651 018-3161-x
652 OIV. (2015). *Recueil international des méthodes d'analyse des vins et des moûts. Paris,*
653 *France: Organisation Internationale de la Vigne et du Vin.*
654 Olguín, N., Bordons, A., & Reguant, C. (2010). Multigenic expression analysis as an
655 approach to understanding the behaviour of *Oenococcus oeni* in wine-like conditions.
656 *Int. J. Food Microbiol.*, *144*(1), 88–95.
657 https://doi.org/10.1016/j.ijfoodmicro.2010.08.032.
658 Padilla, B.,Gil, J. V., & Manzanares, P. (2016). Past and Future of Non-*Saccharomyces*
659 Yeasts: From Spoilage Microorganisms to Biotechnological Tools for Improving
660 Wine Aroma Complexity. *Frontiers in Microbiology*, *7*, 411. PubMed.
661 https://doi.org/10.3389/fmicb.2016.00411
662 Pozo-Bayón, M. A., G-Alegría, E., Polo, M. C., Tenorio, C., Martín-Álvarez, P. J., Calvo de
663 la Banda, M. T., Ruiz-Larrea, F., & Moreno-Arribas, M. V. (2005). Wine Volatile and
664 Amino Acid Composition after Malolactic Fermentation: Effect of *Oenococcus oeni*
665 and *Lactobacillus plantarum* Starter Cultures. *Journal of Agricultural and Food*
666 *Chemistry*, *53*(22), 8729–8735. https://doi.org/10.1021/jf050739y
667 Ramírez, M., Velázquez, R., Maqueda, M., Zamora, E., López-Piñeiro, A., & Hernández, L.
668 M. (2016). Influence of the dominance of must fermentation by *Torulaspora*

669 *delbrueckii* on the malolactic fermentation and organoleptic quality of red table wine.
670 *International Journal of Food Microbiology*, *238*, 311–319.
671 https://doi.org/10.1016/j.ijfoodmicro.2016.09.029
672 Rantsiou, K., Englezos, V., Torchio, F., Risse, P.-A., Cravero, F., Gerbi, V., Rolle, L., &
673 Cocolin, L. (2017). Modeling of the Fermentation Behavior of *Starmerella bacillaris*.
674 *American Journal of Enology and Viticulture*, *68*, 378–385.
675 https://doi.org/10.5344/ajev.2017.16108
676 Rizk, Z., El Rayess, Y., Ghanem, C., Mathieu, F., Taillandier, P., & Nehme, N. (2016).
677 Impact of inhibitory peptides released by *Saccharomyces cerevisiae* BDX on the
678 malolactic fermentation performed by *Oenococcus oeni* Vitilactic F. *International*
679 *Journal of Food Microbiology*, *233*, 90–96.
680 https://doi.org/10.1016/j.ijfoodmicro.2016.06.018
681 Roudil, L., Russo, P., Berbegal, C., Albertin, W., Spano G., & Capozzi, V. (2019). Non-
682 *Saccharomyces* Commercial Starter Cultures: Scientific Trends, Recent Patents and
683 Innovation in the Wine Sector. *Recent Patents on Food, Nutrition & Agriculture*, *10*,
684 1–13. https://doi.org/10.2174/2212798410666190131103713
685 Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., & Suzzi, G.
686 (2012). Diversity of *Candida zemplinina* strains from grapes and Italian wines. *Food*
687 *Microbiology*, *29*(1), 18–26. https://doi.org/10.1016/j.fm.2011.08.014
688 Tristezza, M., Vetrano, C., Bleve, G., Spano, G., Capozzi, V., Logrieco, A., Mita, G., &
689 Grieco, F. (2013). Biodiversity and safety aspects of yeast strains characterized from
690 vineyards and spontaneous fermentations in the Apulia Region, Italy. *Food*
691 *Microbiology*, *36*(2), 335–342. https://doi.org/10.1016/j.fm.2013.07.001
692 Tristezza, M., di Feo, L., Tufariello, M., Grieco, F., Capozzi, V., Spano, G., Mita, G., &
693 Grieco, F. (2016). Simultaneous inoculation of yeasts and lactic acid bacteria: Effects

on fermentation dynamics and chemical composition of Negroamaro wine. *LWT - Food Science and Technology*, *66*, 406–412. https://doi.org/10.1016/j.lwt.2015.10.064

Ugliano, M., & Moio, L. (2005). Changes in the Concentration of Yeast-Derived Volatile Compounds of Red Wine during Malolactic Fermentation with Four Commercial Starter Cultures of *Oenococcus oeni*. *Journal of Agricultural and Food Chemistry*, *53*(26), 10134–10139. https://doi.org/10.1021/jf0514672

Ugliano, M., & Moio, L. (2006). The influence of malolactic fermentation and *Oenococcus oeni* strain on glycosidic aroma precursors and related volatile compounds of red wine. *Journal of the Science of Food and Agriculture*, *86*(14), 2468–2476. https://doi.org/10.1002/jsfa.2650

Wang, C., Mas, A., & Esteve-Zarzoso, B. (2016). The Interaction between *Saccharomyces cerevisiae* and Non-*Saccharomyces* Yeast during Alcoholic Fermentation Is Species and Strain Specific. *Frontiers in Microbiology*, *7*, 502. https://doi.org/10.3389/fmicb.2016.00502

Zapparoli, G., Tosi, E., Azzolini, M., Vagnoli, P., & Krieger, S. (2009). Bacterial Inoculation Strategies for the Achievement of Malolactic Fermentation in High-alcohol Wines. *South African Journal of Enology and Viticulture*, *30*. https://doi.org/10.21548/30-1-1424

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

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

		Co-inoculation		**Sequential inoculation**	
	Inoculation protocol	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

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

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
Coinoculum	*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
Coinoculum	*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
Sequential	*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
Sequential	*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
Coinoculum	(*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
Coinoculum	(*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
Sequential	(*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
Sequential	(*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
Coinoculum	(*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
Coinoculum	(*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
Sequential	(*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
Sequential	(*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

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

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

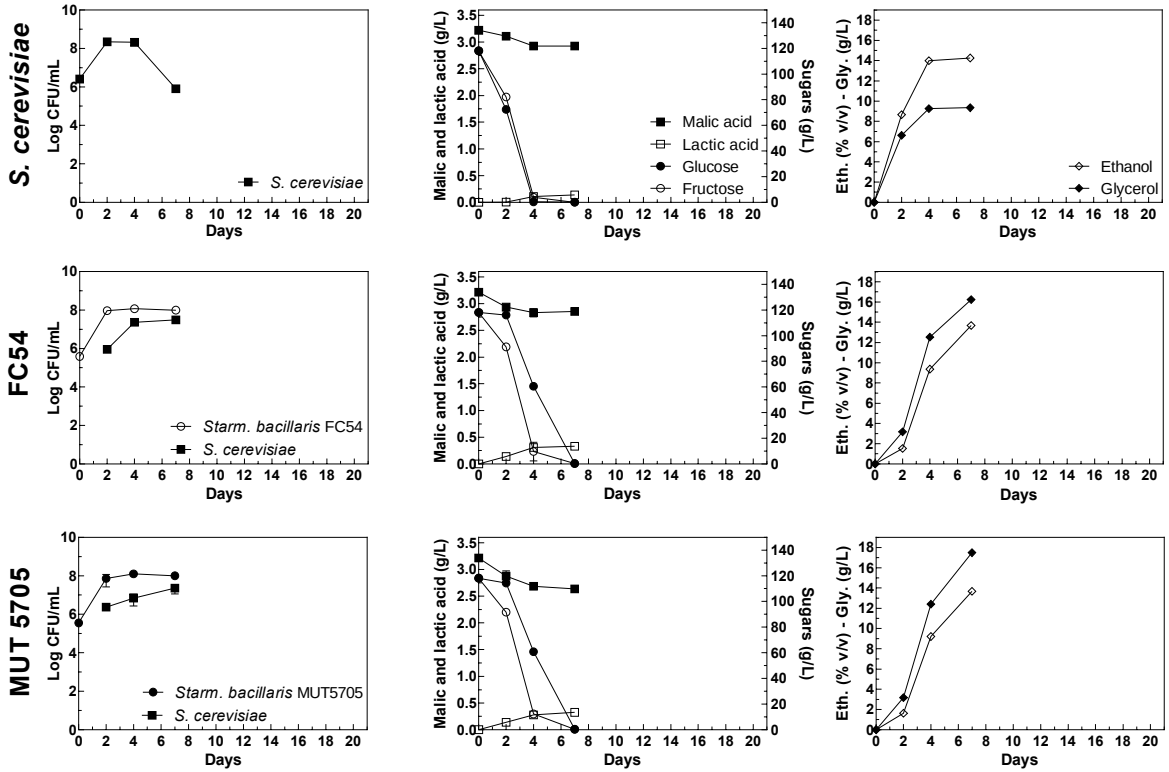
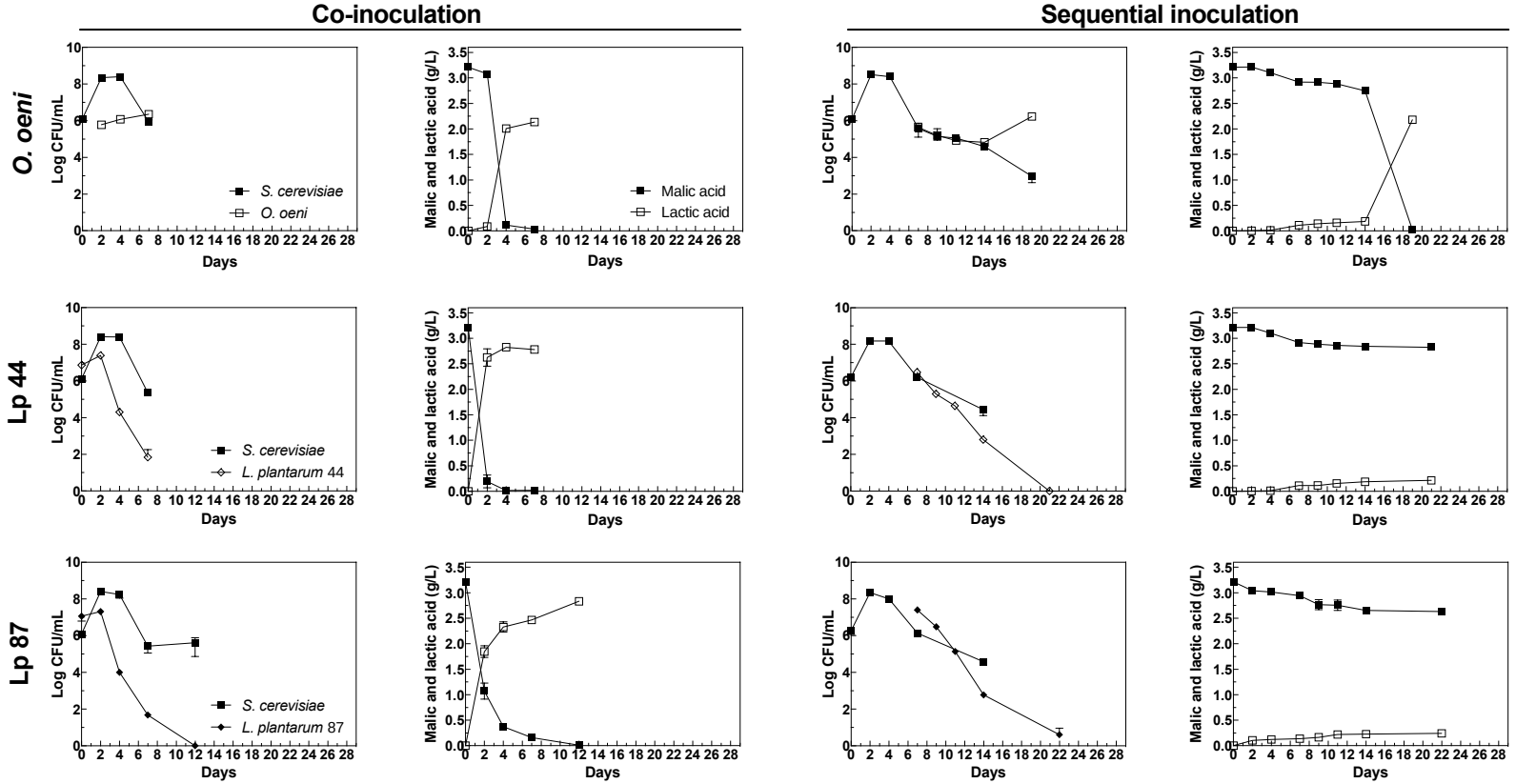
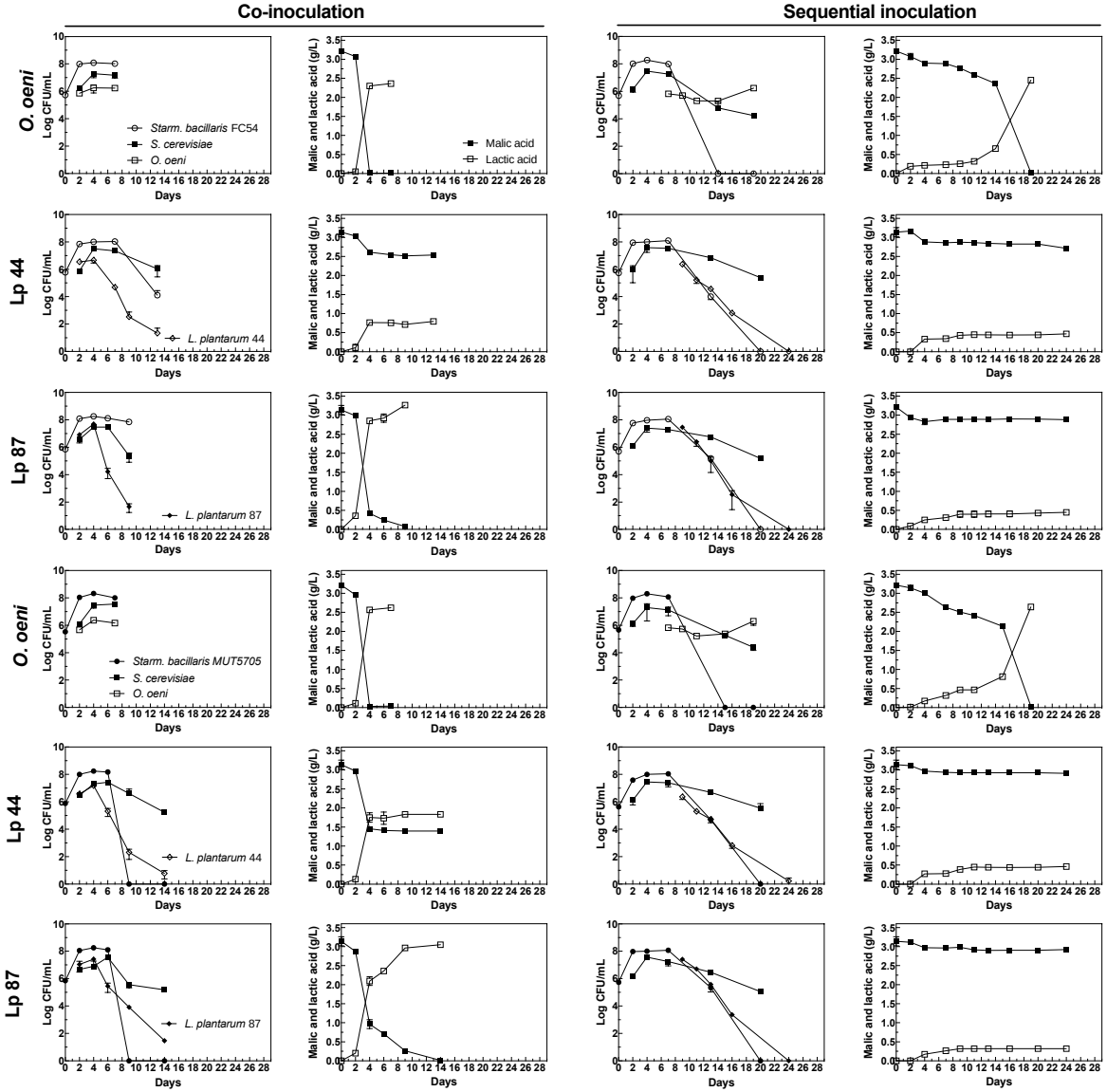


Fig. 2

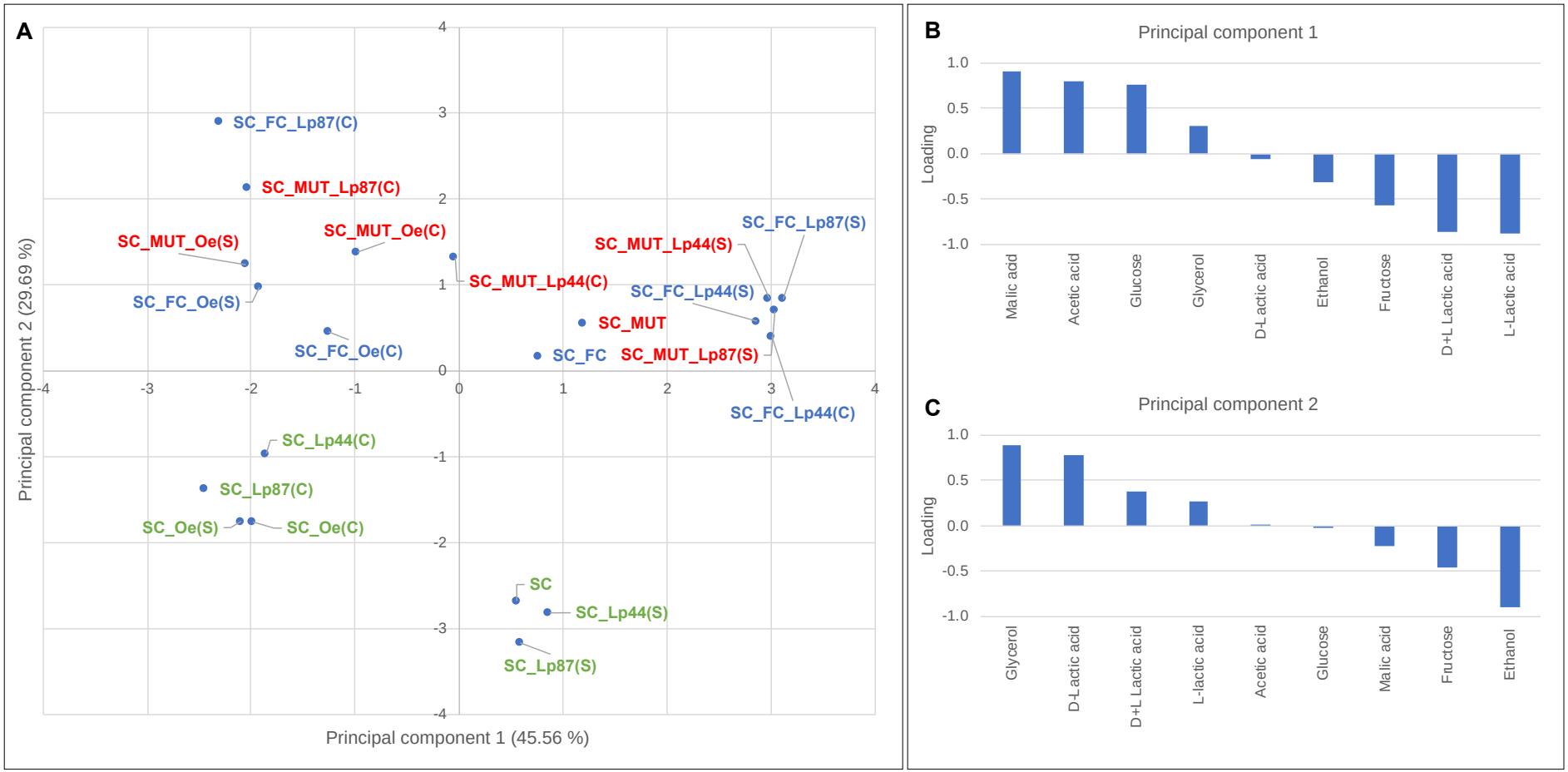

Co-inoculation
Sequential inoculation
O. oeni
Lp 44
Lp 87
Log CFU/mL
Malic and lactic acid (g/L)
Days
S. cerevisiae
O. oeni
L. plantarum 44
L. plantarum 87
Malic acid
Lactic acid

819 **Fig. 3**


Co-inoculation
Sequential inoculation
O. oeni
Lp 44
Lp 87
O. oeni
Lp 44
Lp 87
Starm. bacillaris FC54
S. cerevisiae
O. oeni
L. plantarum 44
L. plantarum 87
Starm. bacillaris MUT5705
Malic acid
Lactic acid
Log CFU/mL
Malic and lactic acid (g/L)
Days

820

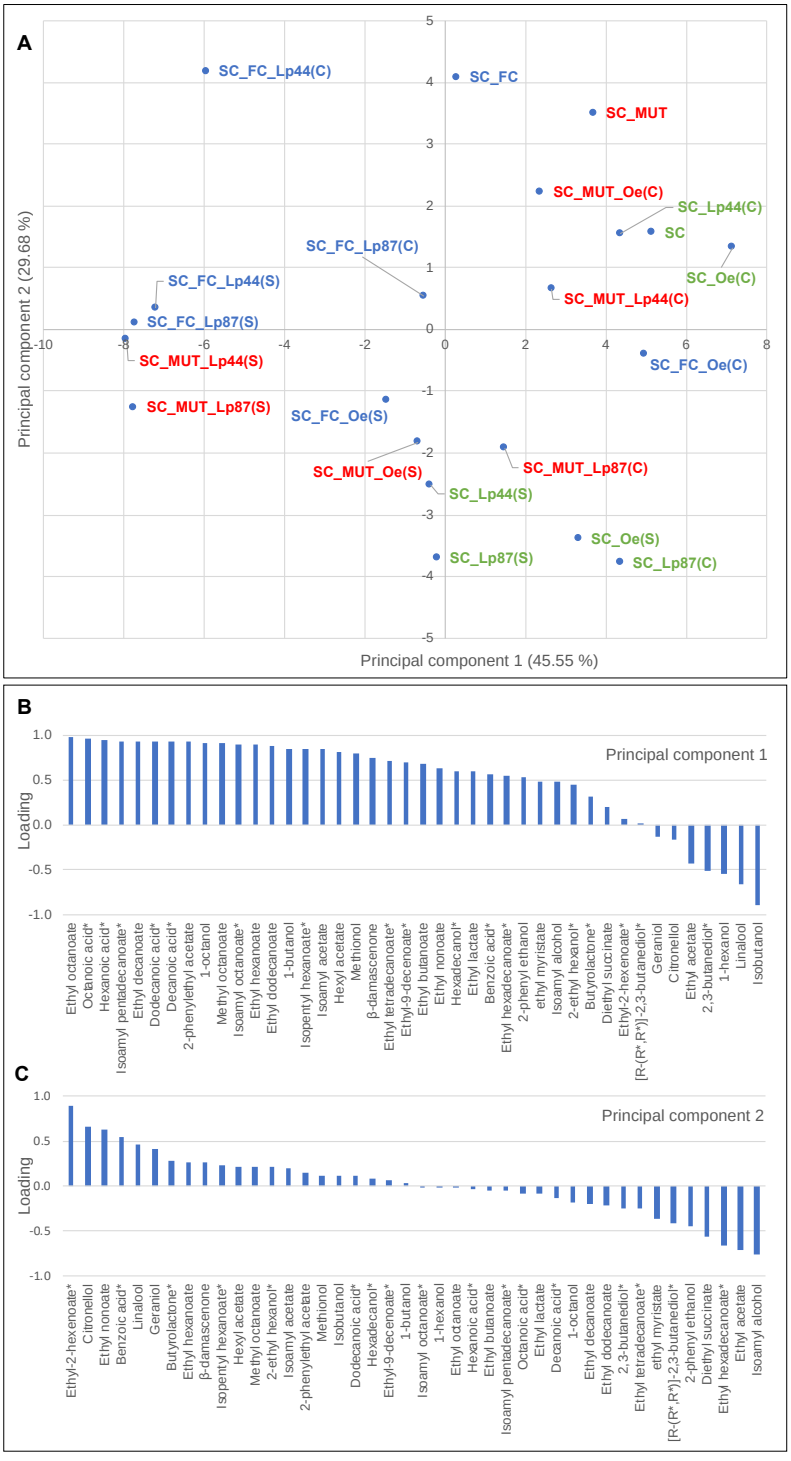
821 **Fig. 4**


A
SC_FC_Lp87(C)
SC_MUT_Lp87(C)
SC_MUT_Oe(S)
SC_MUT_Oe(C)
SC_FC_Lp87(S)
SC_MUT_Lp44(S)
SC_MUT_Lp44(C)
SC_FC_Lp44(S)
SC_FC_Oe(S)
SC_MUT
SC_FC_Oe(C)
SC_FC
SC_MUT_Lp87(S)
SC_FC_Lp44(C)
SC_Lp44(C)
SC_Lp87(C)
SC_Oe(S)
SC_Oe(C)
SC
SC_Lp44(S)
SC_Lp87(S)
Principal component 2 (29.69 %)
Principal component 1 (45.56 %)
B
Principal component 1
Loading
Malic acid
Acetic acid
Glucose
Glycerol
D-Lactic acid
Ethanol
Fructose
D+L Lactic acid
L-Lactic acid
C
Principal component 2
Loading
Glycerol
D-Lactic acid
D+L Lactic acid
L-lactic acid
Acetic acid
Glucose
Malic acid
Fructose
Ethanol

822

823 **Fig. 5**

824



825