Selection and technological potential of *Lactobacillus plantarum* bacteria suitable for wine malolactic fermentation and grape aroma release

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Running title: Selection and technological potential of wine *L. plantarum* bacteria

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Abstract

Lactobacillus plantarum strains have resistance mechanisms that enable them to survive and proliferate in wine, which makes them potential malolactic fermentation (MLF) starter cultures. This work focused on the technological characterization of 11 *L.plantarum* strains isolated from Southern Italian wines that undergo spontaneous MLF, and proposes a selection of new *L.plantarum* malolactic starters. These strains were characterized according to their oenological characteristics, their ability to produce biogenic amines and bacteriocins, their response to the presence of phenolic compounds, their enzymatic activities and their ability to produce wine odorant aglycones from odourless grape glycosidic aroma precursors. Finally, the malolactic activity of one selected strain was assessed in Cabernet Sauvignon wine, using two inoculation methods. *L. plantarum* strains tested were not producers of biogenic amines. In particular, the M10 strain showed a good resistance to high levels of ethanol and low pH, it has a good malolactic performance and β-glucosidase activity, this last one demonstrated both directly through the measurement of this enzymatic activity and indirectly by following the release of volatile aglycones from commercial and natural grape glycosidic odourless precursors. These results demonstrated the potential applicability of M10 as a new MLF starter culture, especially for high-ethanol wines.

Keywords: *Lactobacillus plantarum*, wine malolactic fermentation, functional starter culture, co-inoculation, grape aroma hydrolytic activity

1. Introduction

Malolactic fermentation (MLF) plays an important role in the production of wine, especially red wines, resulting in microbial stability, biological deacidification, as well as contributing to the aroma profile (Moreno-Arribas and Polo, 2005; Bartowsky *et al*., 2008). Nowadays, the use of lactic acid bacteria (LAB) strains as malolactic starter cultures to improve wine quality is a common winemaking practice.

Spontaneous MLF is often unpredictable. It may occur during, or many months after the completion of alcoholic fermentation (Wibowo *et al*., 1985; Henschke, 1993), and it may also fail because of very harsh environmental conditions in the wine, impeding bacterial survival and growth, such as low pH, high alcohol content, high SO_2 concentrations and low temperatures (Lafon-Lafourcade *et al*., 1983; Wibowo *et al*., 1988). Moreover, some LAB have also undesirable effects on wine quality, because they produce off-flavours, a reduction in colour (Liu and Pilone, 2000) and the formation of biogenic amines (Moreno-Arribas *et al*., 2003). The overall effects of MLF are largely dependent on the strains that carry out the process and on the type of wine being manufactured. *Oenococcus oeni* is the major bacterial species found in wines during spontaneous MLF, as it is well adapted to the low pH and high ethanol concentration of wine. However, *O. oeni* can also be detected with other LAB, mainly *Lactobacillus* spp., and in particular *L. plantarum* species (Lonvaud-Funel, 2001; Lerm *et al*., 2011; Bravo-Ferrada *et al*., 2013). In 1988 the potential of *L. plantarum* as a malolactic starter culture was realised by Prahl (1988) with the first freeze-dried culture being released. Today there are a few *L. plantarum* strains commercially available as MLF starter cultures (Fumi *et al*., 2010; Lerm *et al*., 2011). Some relevant characteristics of *L. plantarum*, such as the ability to function well at low pH conditions, the tolerance of ethanol up to 14%, has a similar SO_2 tolerance to *O. oeni*, and it has a more diverse array of enzymes that could lead to more aroma compounds being produced, all contribute to making *L. plantarum* as the up-to-date generation wine MLF starter cultures (Spano *et al*., 2002; Du Toit *et al*., 2011; Lerm *et al*., 2011). The selection criteria for enological malolactic starters should include: (i) technological challenges

(resistance to the main wine parameters and withstanding the production processes); (ii) malolactic performance and flavour production (malic acid degradation; impact on wine aroma); (iii) production of ensured enhancement of the wholesomeness of wine (no production of biogenic amines) (Du Toit, 2012). A minor but also important aspect to be considered is the susceptibility of LAB to polyphenols, which are one of the most abundant groups of chemical compounds in wine (and in red wines in particular) and can have an extremely important impact on wine sensorial characteristics. Several studies have shown different effects of wine polyphenols on the growth and metabolism of enological LAB (García-Ruiz *et al*., 2008; García-Ruiz *et al*., 2013a; Campos *et al*., 2016). Particularly *O. oeni* and *L. plantarum* may be inhibited by tannins and phenolic acids, and so they have a negative impact on the development of malolactic fermentation, while anthocyanins and gallic acid seem to have a stimulatory effect (Reguant *et al*. 2000; Alberto *et al*., 2001; Campos *et al*., 2009).

Recently, some authors have evidenced that the *L. plantarum* species shows a different enzymatic profile to other LAB species, which could play an important role in the wine aroma profile (Swiegers *et al*., 2005; Lerm *et al*., 2011). The use of malolactic starter cultures has become widespread to control the MLF process and to prevent the production of off-flavours. However, the induction of malolactic fermentation by use of commercially available strains is not always successful. Several reports have shown that the success of MLF starters depends of the strain and is influenced by several factors, including geographical origin and adaptation to the winemaking conditions of each wine (Ruiz *et al*., 2010; Testa *et al*., 2014; Valdés la Hens *et al*., 2015). Because the resistance to wine conditions is strictly strain-dependent, the development of new malolactic starters is a multiphasic approach, whose identification and oenological characterization of *L. plantarum* strains naturally occurring in wines that have undergone spontaneous MLF are relevant steps.

With the final aim of proposing a selection of potential *L. plantarum* malolactic fermentation starter cultures, this study was focused on the oenological characterization of 11 *L. plantarum* strains

previously isolated from Southern Italian red wines. The first objective was to characterize the isolates by assessing their capacity to survive at low pH and high alcohol content, and their malic acid degradation performance in synthetic wine. Also, the production of bacteriocins and biogenic amines was examined, as well as the production of enzymatic activities that play a role in wine production; furthermore, the transformation of odourless glycosidic aroma precursors into odorant aglycones was investigated. The second objective was to evaluate the malolactic activity of one selected strain in a Cabernet Sauvignon wine using two inoculation methods: co-inoculation with yeast and sequential inoculum at the end of alcoholic fermentation.

2. Materials and Methods

2.1 Microorganisms and starters preparation

L. plantarum V22 (Lallemand Inc., Montreal, Canada) and 11 strains of *L. plantarum*, selected from southern Italian wines (Testa *et al*., 2014), were used in the characterization tests of MLF, after a first screening including 58 *L. plantarum* strains isolated from these wines. A commercial strain of *Saccharomyces cerevisiae* AM37 (Enobiotech, Novara Italy) was used to carry out the alcoholic fermentation. The AM37 and V22 strains were rehydrated according to the manufacturer's specifications before use.

At time of use, the strains of *L. plantarum*, were propagated overnight in Man, Rogosa and Sharpe (MRS) medium (Oxoid Ltd., UK) at 30 °C, reinoculated into a new MRS medium and incubated until the exponential phase growth was reached. The cells were pelleted by centrifugation at 10,000 rpm for 15 minutes at 4 °C, washed twice with sterile water and resuspended in must at a concentration of 10^8 CFU/mL (colony-forming units per millilitre).

2.2 Characterization of the L. plantarum *strains in synthetic wine medium*

In the first test, 58 *L. plantarum* strains were screened in synthetic wine (SW) media [4 g/L yeast extract, 2 g/L glycerol, 6 g/L D,L-malic acid] (Carreté *et al*., 2002). The pH was adjusted to 3.5

with 4N NaOH and the ethanol concentration to 14% (v/v). Cells grown at exponential phase on MRS (Oxoid Ltd., UK) for 48 h at 28 °C were washed with physiological solution and resuspended in SW at a final concentration of 10^8 CFU/ml. The viable cell number was measured by plating diluted SW aliquots on MRS agar at different times on days 5, 10, 15 of incubation at 30 °C under anaerobic conditions.

The second screening panel was performed on the selection of strains (11 *L. plantarum*). Their capacity to grow in SW with the following combination of pH and ethanol concentrations was evaluated: a) pH 3.5 and 11% (v/v) ethanol; b) pH 3.5 and 13% ethanol; c) pH 3.2 and 11% ethanol; d) pH 3.2 and 13% ethanol; e) pH 3.0 and 10% ethanol; each medium was incubated at 24 °C for 15 days. The cell counts were monitored at four different stages during MLF (0, 10, 15 days) by conducting plate counts on MRS agar plates incubated at 30 °C in anaerobic conditions. The L-malic acid concentration was determined with a malic acid enzymatic assay (Steroglass, San Martino in Campo, Italy) at different times (0, 5, 15 days).

2.3 Multi-enzymatic activities

The strains used in this study were assayed for their enzymatic activities using the Api-Zym galleries (BioMérieux, Montalieu-Vercieu, France) as described by the manufacturer. Rapid semi-quantitative evaluation of 19 hydrolytic enzymes was carried out. The colour that developed in each enzymatic reaction was graded from (+) positive to (-) negative and (W) weakly positive by the API-ZYM colour reaction chart.

2.4 Odourless glycosidic aroma precursor transformation by L. plantarum strains

As an indirect measurement of β-D-glucosidase activity in *L. plantarum*, each of the strains tested in this study was first incubated with a commercial glucoside (Octyl-β-D-glucopyranoside) (Sigma-Aldrich, St. Louis, MO, USA) and then with a natural odourless glycosidic aroma precursor extract, which can better represent the ability of these microorganisms to release positive aromatic notes in

wines. The natural aroma precursor extract was obtained from white grapes using methodologies based on the protocol already published by Rodríguez-Bencomo *et al*. (2013). The incubation procedure was one described elsewhere (Muñoz-González *et al*., 2015) with slight modifications. Briefly, strains were inoculated in 10 mL of MRS broth (Oxoid Ltd., UK) and incubated in the presence of each of the glycosidic aroma precursors at 30°C. In addition, a control without bacteria was prepared, confirming that the release of volatile compounds was due to the presence of *L. plantarum*. The analysis of free volatile compounds released from the glycosides was carried out by headspace solid phase microextraction coupled to gas chromatography mass spectrometry (HS-SPME-GC-MS) at 0 h, 2 h and 24 h of incubation. Preliminary tests were performed in order to establish whether the glycoside concentration employed in these experiments might inhibit the bacterial growth, concluding that none of the bacteria assayed were inhibited by the glycosidic extract at the assayed concentration (data not shown).

For this study, two wine LAB strains, *L. paracasei* CIAL-94 and the *Pediococcus pentosaceus* CIAL-85, exhibiting weak enzymatic activity (unpublished results), were used as reference strains. All the experiments were performed in duplicate.

2.5 Bacteriocins production

The production of bacteriocins by *L. plantarum* strains was investigated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF) using a Bruker Daltoniks instrument provided by a Bruker MALDI Biotyper 3.0 system. The strains were inoculated in MRS agar and incubated overnight at 30 °C in anaerobic conditions. A colony, for each strain, was spotted onto the MALDI-TOF/TOF target. The spectra obtained by the strains tested were compared with the spectral fingerprints of *Lactococcus lactis* CECT (producer of Lacticin 3147) and IFPL 105-3 (not a producer of Lacticin 3147) (Martínez-Cuesta *et al*., 2000).

2.6 Determination of biogenic amine-forming capacity

Qualitative detection of amine formation in decarboxylase assay medium was tested by inoculating each strain in the decarboxylase medium described by Bover-Cid and Holzapfel (1999). The medium contained the corresponding precursor amino acid at 0.5% final concentration (L-histidine monohydrochloride, tyrosine di-sodium salt, L-ornithine monohydrochloride and L-arginine monohydrochloride), pyridoxal-5-phosphate, growth factors, buffer compounds and purple bromocresol as pH indicator. The pH was adjusted to 5.3 and the medium was autoclaved. The precursor amino acids were purchased from Sigma (St. Louis, MO, USA). A bacterial suspension (10^9 CFU/mL) was made from a plate culture in decarboxylase medium without amino acids. An aliquot of the suspension (0.2 mL) was inoculated into 2 mL of the same medium with and without amino acids (as control). After 7 days incubation at 30 ºC under anaerobic conditions, the medium was centrifuged and the supernatant was kept at -20 ºC until biogenic amines analysis.

For quantitative determination of biogenic amine producers, cells grown at exponential phase on MRS broth overnight at 30 °C were suspended in MRS broth, containing 0.1% of the corresponding amino acid precursor (L-histidine monohydrochloride, tyrosine di-sodium salt and L-ornithine monohydrochloride), pyridoxal-5'-phosphate (Sigma) and growing factors, previously described in Moreno-Arribas *et al*., (2003). The pH was adjusted to 5.3 and the medium was autoclaved. The precursor amino acids were purchased from Sigma (St. Louis, MO, USA). Samples were incubated at 30 °C for 7 days with stirring at 80 rpm. Two mL of culture were taken and centrifuged at 4000 rpm for 10 minutes at 5 °C; 1mL of supernatant was filtered through a 0.22 μm filter and placed in vials for HPLC. Biogenic amines were analysed by RP-HPLC according to the method described by Marcobal *et al*. (2005), using a liquid chromatograph consisting of a Waters 600 controller programmable solvent module (Waters, Milford, MA, USA), a WISP 710B autosampler (Waters, Milford, MA, USA) and an HP 1046-A fluorescence detector (Hewlett-Packard). Chromatographic data were collected and analysed with a Millenium 32 system (Waters, Milford, MA, USA). The separations were performed on a Waters Nova-Pak C18 (150 x 3.9 mm i.d., 60 Å, 4 μm) column, with a matching guard cartridge of the same type. Samples were submitted to an automatic pre-

column derivatization reaction with *o*-phthaldialdehyde (OPA) prior to injection. Derivatized amines were detected using the fluorescence detector (excitation wavelength of 340 nm, and emission wavelength of 425 nm).

2.7 Effects of malvidin 3,5-diglucoside on the growth of L. plantarum

The *L. plantarum* strains were cultured at 37 ºC in MRS broth (pH 3.5) to obtain overnight cultures. The effect of malvidin 3,5-diglucoside on the growth of these strains was evaluated following the protocol described by Tabasco *et al.* (2011). Growth was performed in triplicate in sterile 96-well microplates with lid (Sarstedt Inc., Newton, USA). Wells containing 300 mL of ZMB1 medium in the absence (control) and in the presence of malvidin 3,5-diglucoside (0.500, 0.250 and 0.125 mg/mL), were inoculated (1%) with an overnight culture of each strain. Bacteria growth for 48 h at 37 ºC under aerobic conditions was monitored at 60 min intervals (preceded by 15 s of shaking at variable speed) by assessing optical density (OD) at 600 nm (OD600) using an automated microplate reader (Varioskan Flash, Thermo Electron Corporation, Vantaa, Finland). The effect (inhibition/stimulation) of malvidin 3,5-diglucoside on the bacteria growth was calculated from the data at 48 h as: % Inhibition = $(Abs_{sample} - Abs_{control})/ Abs_{control}$.

2.8 Malolactic fermentation (co-inoculation and sequential inoculum) in small-scale vinification procedures

Vinifications were conducted at the Giagnacovo winery (San Biase of Molise, Italy) using red grapes of the Cabernet Sauvignon variety. The must showed the following chemical composition: pH 3.37, titratable acidity 8.61 g/L tartaric acid, L-malic acid 5.0 g/L, L-lactic acid 0.05 g/L, D-lactic acid 0.01 g/L, acetic acid 0.01 g/L and sugar content 21.7 °Brix. The chemical–physical analyses were performed according to EC Official Methods (1999).

Fermentations were carried out in five stainless steel tanks of 10 hL each with the addition of 50 mg/L of $K_2S_2O_5$. The alcoholic fermentation was conducted at 22 °C in the presence of grape skins,

seeds and stalks, until the residual reducing sugar content was less than 2 g/L, with an inoculum of a commercial *S. cerevisiae* strain AM37. For each experiment, the wine samples were collected at different times and subjected to microbiological analysis.

Malolactic fermentation was performed using two different procedures, co-inoculation and sequential inoculum, using the *L. plantarum* M10 strain (Testa *et al*. 2014) and *L. plantarum* V22 commercial strain (Lallemand).

The alcoholic fermentation was carried out in 25 hL of Cabernet Sauvignon grape juice by the addition of *S. cerevisiae* AM37 and divided equally into five stainless steel tanks (A, B, C, D, E). The tanks A and B, after 12 h, were inoculated, respectively, with *L. plantarum* M10 and *L. plantarum* V22 (co-inoculum). The tanks C and D, after the alcoholic fermentation, were inoculated, respectively, with *L. plantarum* M10 and *L. plantarum* V22 (sequential inoculum). Tank E represents a control, inoculated only with the *S. cerevisiae* AM37 strain.

The alcoholic fermentation was considered concluded when the reducing sugars level was below 2 g/L. Malolactic fermentation was monitored up to 30 days of incubation at a temperature of 22 °C. The L-malic acid degradation and the DL-lactic acid formation in all tanks were determined using enzymatic kits (Steroglass, Italy) from 0 days to up to 30 days of incubation.

The chemical–physical analyses were performed according to EC Official Methods (1999).

2.9 Identification of L. plantarum strains during the malolactic fermentation

Wine samples were serially diluted in sterile saline solution (9 g/L NaCl) and then plated in triplicate on MRS agar supplemented with 0.2 g/L sodium azide, as the selective medium for LAB medium. Plates were incubated at 30 °C under anaerobic conditions (GasPak, Oxoid Ltd., UK) at 48 h, five colonies were randomly picked from plates at highest dilutions and identified by their morphology, Gram staining, catalase test, and PCR-DGGE and RAPD-PCR analysis (Testa *et al*., 2014).

2.10 Statistical analysis

All analytical assays were carried out in three replicates by determining the mean and standard deviation. Statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Significant difference was evaluated using ANOVA LSD test at $p < 0.01$.

3. Results

3.1 Characterization of L. plantarum strains in synthetic wine

Fifty-eight *L. plantarum* strains were submitted at screening to evaluate the growth in synthetic wine (SW), with pH 3.5 and an ethanol content of 14% (v/v) (data not shown). Eleven *L. plantarum* strains were selected, and then submitted to a comparative assay of malolactic performance at laboratory scale, using SW medium at different pH and ethanol concentrations. Figure 1 shows the L-malic acid evolution in SW medium with different combinations of pH and ethanol content for the isolated strains tested and the commercial *L. plantarum* V22.

All 11 strains were able to consume the L-malic acid completely (respectively at pH 3.5 with 11% v/v ethanol, Fig. 1**a**, and pH 3.5 with 13% v/v ethanol, Fig. 1**b**) after five days, with the P5 strain being the exception. Three strains, P5, M26 and V22 were not able to consume L-malic acid in the SW medium at pH 3.2 with 11% v/v of ethanol (Fig. 1**c**). The SW medium pH 3.2 with 13% v/v of ethanol (Fig. 1**d)**, was more selective for the majority of strains. Eight strains (A1, M17, M26, P9, P5, M22, M24, T13) and the commercial strain V22 were unable to deplete the L-malic acid during the 15 days of microvinification, as can be seen in figure 1**d**, whereas three strains (M10, R1 and P1) had degraded all L-malic acid in the medium within ten days. Moreover, only the A1, R1, P1, M10 and M26 strains consumed L-malic acid in SW medium at pH 3.0 with 10% v/v of ethanol, as shown in Fig. 1**e**.

The results demonstrate that a pH value of 3.5 provides the best conditions for survival for the tested strains, permitting total L-malic acid consumption, independently from an ethanol content of either 11% or 13% v/v.

3.2. Production of enzymes of oenological interest

Enzymatic activities correlated with carbohydrate catabolism, α-galactosidase, β-glucoronidase, α-mannosidase and α-fucosidase were not observed in any of the strains tested (Table 1). However all the *L. plantarum* strains exhibited strong β-glucosidase and α-glucosidase activities. For the β-galactosidase and N-acetyl-β-glucosaminidase, strains showed weak activity, except the commercial V22 strain in which these enzymatic activities were not expressed.

3.3. Ability of L.plantarum strains to release free volatiles from odourless glycosidic aroma precursors

The ability of *L. plantarum* strains to release odorant aglycones from Octyl-β-D-glucopyranoside is shown in Figure 2. As can be seen, all the strains assayed were able to hydrolyse the glycoside and to release different amounts of the aglycone 1-octanol (Figure 2). However, this ability was bacteria-dependent, and therefore different depending on the type of bacteria assayed. Interestingly, all the *L. plantarum* strains studied produced significantly higher amounts of the aglycone than the strains *L. paracasei* CIAL-94 and *P. pentosaceus* CIAL-85, which were the lowest producers. In particular the strains M17 and M10 were the major producers of 1-octanol, suggesting that these strains could be potentially responsible for the generation of a greater amount of free aroma compounds in wines.

Grape glycosides represent a natural reservoir of odorant molecules in wines that can be naturally and slowly released during wine aging, or intentionally released by using oenological enzymes during winemaking. In order to take a step forward in the ability of these bacteria to release aroma compounds in wines, a natural precursor extract obtained from white grapes was incubated in the presence of each of the strains. Table 2 shows these results. As can be seen in the table, all the strains were able to generate odorant aglycones belonging to different chemical families (terpenes, benzenic derivatives and C6-alcohols). But what it is more interesting is that *L. plantarum* M10

296 released a considerable amount of important odorant compounds with low odour thresholds and
297 flowery-citric aroma nuances in wines, such as the terpenes limonene and linalool, among others
298 (see Table 2). However, these results need to be validated by additional experiments carried out
299 with real wines.
300
301 *3.4. Bacteriocins production*
302 The inhibitory activity of *L. plantarum* strains which was not detected in peptide MALDI-
303 TOF/TOF spectra obtained from *L. plantarum* strains, and the isogenic strains of *Lactococcus*
304 *lactis*, which differ in the ability to produce lacticin 3147, was compared. The appearance of two
305 peaks of molecular mass 2850 Da and 3300 Da in the spectra allowed us to detect this bacteriocin
306 production by *L. lactis* strains (García-Cayuela, unpublished results), but not in any of the wine *L.*
307 *plantarum* strains studied, suggesting that none of them were bacteriocin producers.
308
309 *3.5. Biogenic amines production*
310 None of the strains tested produced biogenic amines, histamine, tyramine, cadaverine or putrescine
311 when the modified decarboxylase screening media developed by Bover-Cid and Holzapfel (1999)
312 was used. This medium was shown in a previous work to be suitable for the screening of wine lactic
313 acid bacteria (Moreno-Arribas *et al*., 2003). When these strains were analysed by HPLC, it was
314 confirmed that these amines were not found to be produced by any of the bacterial strains studied
315 under the conditions applied. Furthermore, the wines obtained after the inoculation experiments
316 with the selected strain M10 were also analysed by RP-HPLC for the presence of the biogenic
317 amines histamine, methylamine, ethylamine, tyramine, phenylethylamine, putrescine and
318 cadaverine, concluding that none of these amines was detected in the final wines.
319
320 *3.6. Influence of malvidin 3,5-diglucoside on the growth of L. plantarum strains*

321 The *L. plantarum* strains tested showed a different response to the presence of malvidin 3,5-
322 diglucoside in the culture medium (Table 3). The growth of the strains R1 and M24 was clearly
323 stimulated by the presence of the anthocyanin (% growth >58, compared to the control: ZMB1
324 medium in the absence of malvidin 3,5-diglucoside) in a dose-dependent manner. Conversely,
325 inhibition of bacteria growth was markedly observed for strain P5 (% growth < -40), and for the
326 strains A1 and M17 to a lower extent (% growth < -10) for all the anthocyanin concentrations
327 tested. The strains P9 and V22 showed certain stimulation on their growth (% growth ~ 20) only at
328 the anthocyanin concentration of 0.5 mg/mL, but inhibitory effects (% growth ≤ -20) at the lower
329 concentration tested (0.125 mg/mL). Finally, the presence of malvidin 3,5-diglucoside at the
330 concentrations tested seemed not to affect the growth of the rest of the bacteria (M10, M22, M26,
331 P1 and T13).
332
333 *3.7. Malolactic fermentation in wine*
334 The Cabernet Sauvignon must, used in the study, had a high malic acid content, and in addition, in
335 order to help the development of MLF, a low SO_2 concentration (50 mg/L of $K_2S_2O_5$) was added
336 during alcoholic fermentation. The malolactic bacteria, *L. plantarum* M10, which showed the best
337 characteristics in the previous assays, was selected for small-scale malolactic fermentation
338 procedures in wine. The commercial culture V22 (Lallemand) was inoculated to compare the
339 fermentation performance of the selected strain with that of a commercial product. As reported in
340 Figure 3, with the co-inoculation method (tanks A and B), L-malic acid was degraded completely in
341 12 days. Moreover, there were no substantial differences among the inoculated wines with M10
342 (tank A) and V22 (tank B) strains.
343 On the other hand, in the control tank (E), inoculated only with the yeast strain (AM37) (i.e. under
344 spontaneous malolactic fermentation), the L-malic acid content did not change during this period,
345 so the malolactic fermentation was never completed. In the sequential inoculation wines (tanks C
346 and D), the complete degradation of L-malic acid had occurred after 30 days, as reported in Figure

347 3. The chemical characterization of the different wines is shown in Table 4. None of the inoculated
348 wines resulted in volatile acidity concentrations exceeding the sensory threshold value of 0.7 g/L
349 (Guth, 1997). The M10 *L. plantarum* strain shows similar final volatile acidity values to the
350 commercial starter.
351 Microbiological control of the LAB population in the inoculated tanks (A, B, C and D) was
352 performed by PCR-DGGE analysis and RAPD typing on five randomly selected colonies from
353 plates at the highest dilution collected in the middle phase of MLF (data not shown). According to
354 the DGGE profiles all the isolates belonged to *L. plantarum* species. Strain typing using the primer
355 M13 showed that all the isolates had the same M13-RAPD profile as either strain M10 or V22,
356 suggesting a good implantation of these two starters on the indigenous LAB population.
357

358 **4. Discussion**

359 Given the economic importance of MLF, the development of new starter cultures is an interesting
360 aim in oenology. In particular, the development of alternative malolactic starter cultures using
361 species other than *O. oeni* has become one of the main challenges for oenological research in recent
362 years (Lerm *et al*., 2011; Bravo-Ferrada *et al*., 2013). In this work, we proposed a selection plan for
363 a new *L. plantarum* malolactic starter culture, through the technological/functional characterization
364 of different native *L. plantarum* strains and an MLF test in synthetic wine and in a winery
365 environment (microvinification), by testing two different inoculation scenarios (co-inoculation with
366 yeast and sequential inoculum at the end of alcoholic fermentation).
367 The first assay of L-malic acid degradation in synthetic wine medium showed that low pH (3.0 and
368 3.2) is a crucial parameter to limit *L. plantarum* growth in wine. In synthetic wine, the study of the
369 combinations of pH and ethanol revealed that low pH values are the limiting feature of malolactic
370 activity. Independently from the ethanol percentage, the condition of pH 3.5 allowed the
371 accomplishment of MLF by ten out of the eleven *L. plantarum* strains analysed. However, pH
372 values of 3.0 and 3.2 (combined with an ethanol content of 10% and 13% (v⁄v) respectively) were

prohibitive conditions for most strains. Previous results have proved the resilience of *L.plantarum*, especially for high-pH wines (Du Toit *et al*., 2011; Lerm *et al*., 2011); in our experiment it was established that wild *L. plantarum* strains can tolerate a combination of acid pH (pH 3.5) and ethanol concentration up to 13%, which are normal values in wines, and proliferate under conditions that are normally lethal for LAB.

Bacteriocins are antimicrobial peptides produced by certain bacteria with inhibitory activity against related species, including organisms involved in food-borne disease and food spoilage. Since bacteriocin-producing bacteria have a high technological potential, we tested if this characteristic was present in the strains studied as potential starter cultures. Taxonomic bacterial identification based on the peptide profile obtained by MALDI-TOF/TOF is an extremely fast, simple and reliable approach compared to other microbiological methods used (Ferreira *et al*., 2010). Also, this technique can differentiate beyond the species level, characterizing strains in terms of functionality. Thus, MALDI-TOF/TOF has been used to detect the feature of bacteriocin production. As an example, production of bacteriocins in the *Lactococcus lactis* strain producer of Lacticin 3147 was confirmed by MALDI-TOF/TOF (García-Cayuela, unpublished results), demonstrating the utility of this technique not only for taxonomic identification but to facilitate the evaluation of the potential technological/functional application of LAB. Although previous studies showed that wine isolates from *L. paracasei*, *L.higardii* and *L. plantarum* produced bacteriocins (Rojo-Bezares *et al*., 2007; Knoll *et al*., 2008), MALDI-TOF/TOF data obtained in our study suggested that none of the wine *L plantarum* strains selected was associated to bacteriocin production, probably because, as demonstrated, this property is strain-dependent, and the encoding-bacteriocin structural and transporter genes are expressed to varying degrees, depending on the wine media and fermentation conditions (Knoll *et al*., 2008).

Another important characteristic for the oenological strain, used as starter culture is the inability to produce biogenic amines. These not only have an impact on wine wholesomeness, since they have

several health and commercial implications in wine, but some biogenic amines (i.e. putrescine) also impact on wine aroma (Shalaby, 1996; Álvarez and Moreno-Arribas, 2014). The major amines found in wine are histamine, tyramine, putrescine and cadaverine, and it is well known that the capability to produce amines might be strain-dependent rather than being related to specific species (Lonvaud-Funel, 2001; Moreno-Arribas *et al*., 2000, 2003; Landete *et al*., 2007). In our screening, none of the *L. plantarum* strains was identified as producing biogenic amines, either in an easy decarboxylase synthetic broth or in a quantitative method such as reversed-phase high performance liquid chromatography, used to ensure this. It is important to select strains that do not have this characteristic to minimize the risk of spoilage of wine. According to our results, the selected bacteria *L. plantarum* M10 did not produce biogenic amines, as demonstrated by the absence of these compounds in the malolactic fermentation wines obtained after inoculation with this strain.

Wine polyphenols are known to influence the growth of LAB and MLF performance. The study of malvidin 3,5-diglucoside, as representative of anthocyanins (considering the main phenolic compounds in red wines), was of interest because a potential limitation of the synthetic wine media is the lack of these wine components as well as other wine nutrients (aminoacids, vitamins, etc). Although malvidin 3,5-diglucoside was able to interact with the growth of the *L. plantarum* strains tested, the effect was variable, depending on the strain and the concentration, suggesting a high microbial diversity to wine phenolics, in agreement with previous studies (García-Ruiz et al., 2008; Campos et al., 2016).

In the selection of the strains of *L. plantarum* able to perform malolactic fermentation, the characteristic of the strain to supply β-glucosidase enzymes capable of influencing the flavours and of operating under the physicochemical conditions of wine is very important. All strains tested showed β-glucosidase activity, important because having the potential to release glycosidically bound flavour compounds influences the wine aroma profile (Boido *et al*., 2002; D'Incecco *et al*., 2004; Matthews *et al*., 2004; Spano *et al*., 2005). Furthermore, the ability of the strains to hydrolyse

423 grape glycosides, releasing different types of aglycones belonging to different chemical families
424 (terpenes, benzenic derivatives and C6-alcohols), was evaluated by using a commercial glycoside,
425 and then confirmed using a precursor glycoside extract obtained from white grapes. On the basis of
426 their aroma characteristics, some of the compounds generated by the strains studied might be
427 relevant to aroma perception. For instance, terpenes are important odorant compounds that exhibit a
428 very low odour threshold and flowery-citric aroma nuances in wines (Baumes, 2009). Linalool is
429 one of the most common odorant aglycones released from some floral grape varieties, and it was
430 found in all the strain cultures assayed. In addition, two benzenoid compounds (benzyl alcohol and
431 *β*-phenylethyl alcohol) were also identified. Among them, *β*-phenylethanol has been related to a
432 rose-like odour (Botelho *et al*., 2008). Furthermore, some lipid derivatives, such as C6-alcohols (1-
433 hexanol), were identified in the strains cultures. Nonetheless, other typical wine aroma compounds
434 from grape glycosidic aroma precursors, such as C-13 norisoprenoides, vanillins or volatile phenols
435 (Baumes, 2009), were not detected in the cultures.
436 As can be seen in Table 2, the ability to hydrolyse and release the corresponding odorant aglycones
437 was different depending on the type of bacteria assayed (bacteria-dependent). For example, the *L.*
438 *plantarum* strain M10 was one of the major producers of limonene, linalool and its corresponding
439 oxides, suggesting that this strain could be responsible for the generation of floral and flowery notes
440 from grape glycosides.
441 Finally, the malolactic performance of a selected strain *L. plantarum* M10, which demonstrated the
442 best activities, was then determined in small-scale winery conditions. Our work highlights that
443 degradation of L-malic acid was successfully completed in wines inoculated with *L.plantarum* M10
444 (in both the co-inoculation and the sequential inoculum fermentation procedures) but not in the non-
445 inoculated wine. The evaluation of the LAB population inoculated in the two MLF inoculation
446 experiments (tanks A, B, C and D), confirmed the dominance of the population of the *L. plantarum*
447 species, ensuring the dominance of the selected strain M10 in tanks A and C, and of the commercial
448 starter V22 in tanks B and D. In both MLF inoculation scenarios, the *L. plantarum* strain M10

selected and employed during the course of the industrial-scale fermentation seems to perform best and when wine was inoculated with the yeast strain *S. cerevisiae* AM37 was able to successfully complete MLF; in fact, no decrease in cell counts was observed after inoculation. Regarding the capacity of a selected strain to take over spontaneous LAB population, several works have evidenced that the dominance of the starter is not always guaranteed (Maicas *et al*., 2000; Arnink and Henick-Kling, 2005). The growth of indigenous LAB and many technological factors can significantly affect the implantation capacity of the starter (Wibowo *et al*., 1985). In our study, however, there was a good implantation of the selected strain and therefore a quick malolactic fermentation, confirmed by genetic analysis using PCR-DGGE and RAPD typing, in agreement with previous studies on the potential selection of *O. oeni* and *L. plantarum* South African wine isolates as malolactic starters (Lerm *et al*., 2011).

5. Conclusions

In conclusion, a good understanding of MLF offers great potential in the manufacture of wine quality. In this study a new *L. plantarum* M10 strain was selected, able to degrade L-malic acid in synthetic media with a low pH and high alcohol content, and furthermore was also able to complete the MLF for co-inoculation in must in a short time without producing biogenic amines. *L. plantarum* M10 strain could be used as a starter for MLF co-inoculation in the must, at pH 3.5 and alcohol content of 12% v/v, enhancing the wine flavour by releasing different types of wine odorants. Further studies will be carried out to assess the influence of *L. plantarum* M10 strain on the aroma and sensorial characteristics of wines.

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599

600 **Figure Captions**

601 **Figure 1.** Evolution of L-malic acid concentration in synthetic wine inoculated with *L. plantarum*
602 isolated strains (M10, A1, M17, M26, P9, P5, M24, M10, R1, P1, M22, T13) and commercial V22
603 strain at: **a)** pH 3.5 and 11% (v/v) ethanol content; **b)** pH 3.5 and 13% (v/v) ethanol content; **c)** pH
604 3.2 and 11% (v/v) ethanol content; **d)** pH 3.2 and 13% (v/v) ethanol content; **e)** pH 3.0 and 10%
605 (v/v) ethanol content.

606

607 **Figure 2.** 1-octanol production (absolute peak areas: apa) from Octyl-β-D-glucopyranoside by the
608 strains assayed in this study**.**

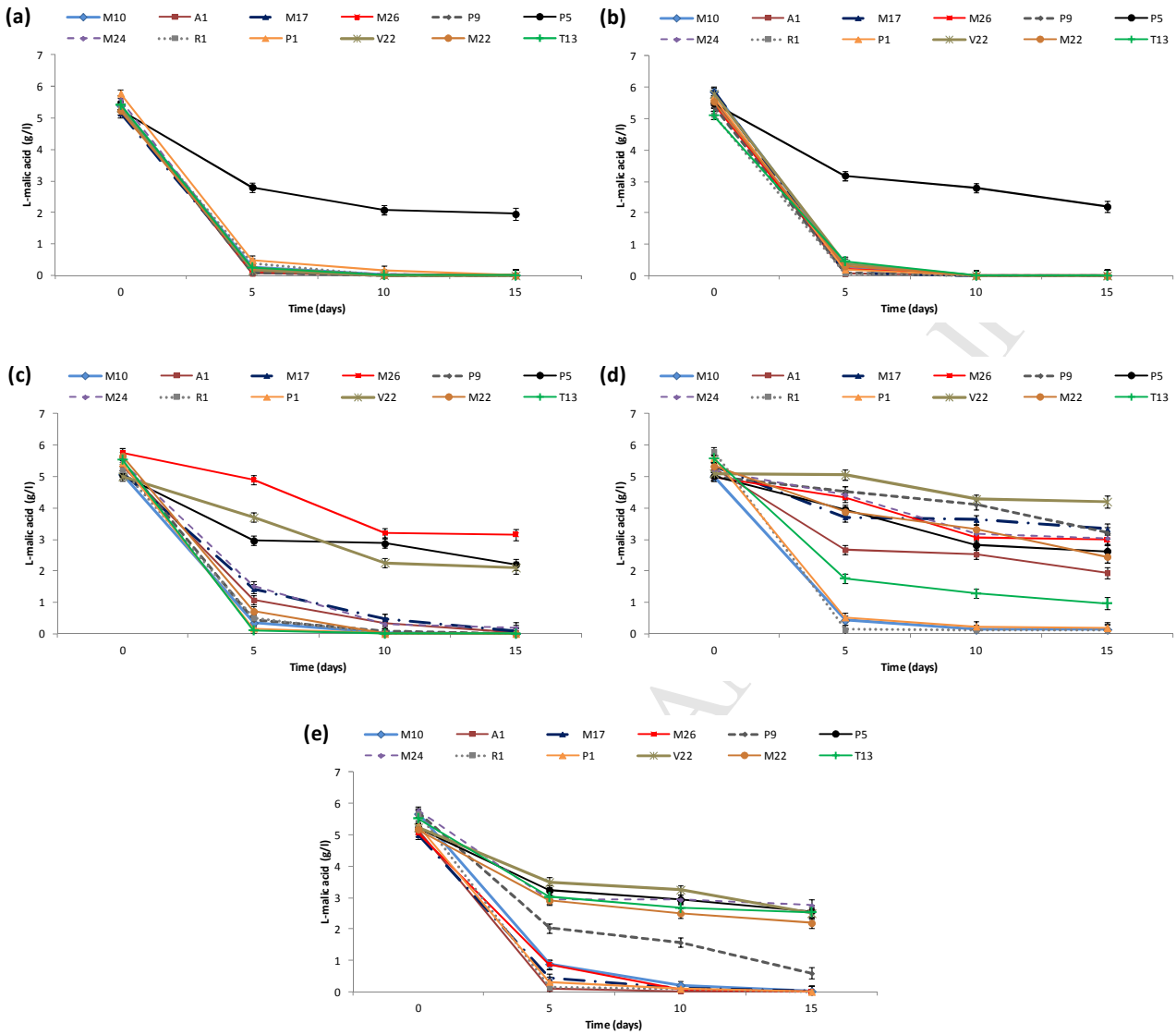
609

610 **Figure 3.** Evolution of L-malic acid concentration in Cabernet Sauvignon. Co-inoculum: A (*L.*
611 *plantarum* M10 + *S. cerevisiae* AM37) and B (*L. plantarum* V22 + *S. cerevisiae* AM37). Sequential
612 inoculum: C (*L. plantarum* M10) and D (*L. plantarum* V22). Control: E (*S. cerevisiae* AM37)

613

614

615



616

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618

619 **Figure 1:**

620

621

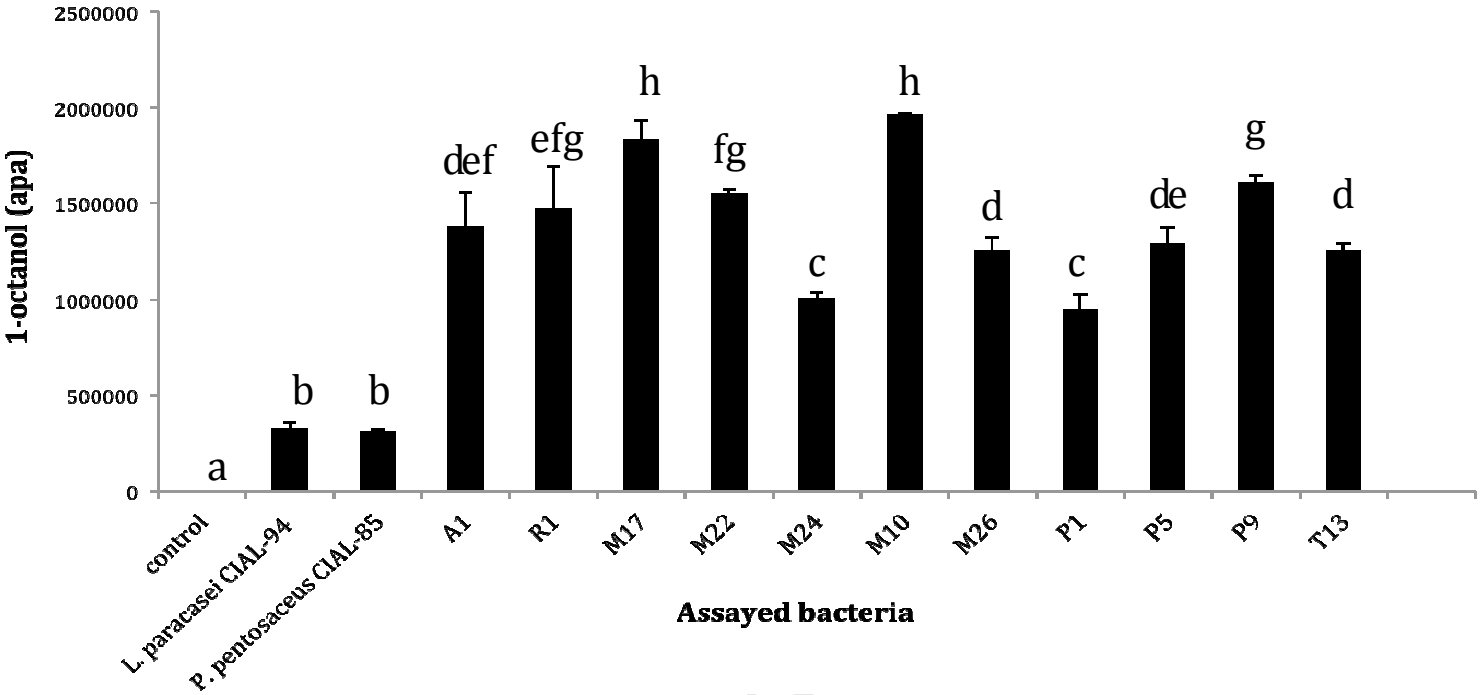

Figure 2:

Each value represents the mean ± standard deviation of two independent replicates. Different letters within columns indicate significant differences ($P < 0.05$).

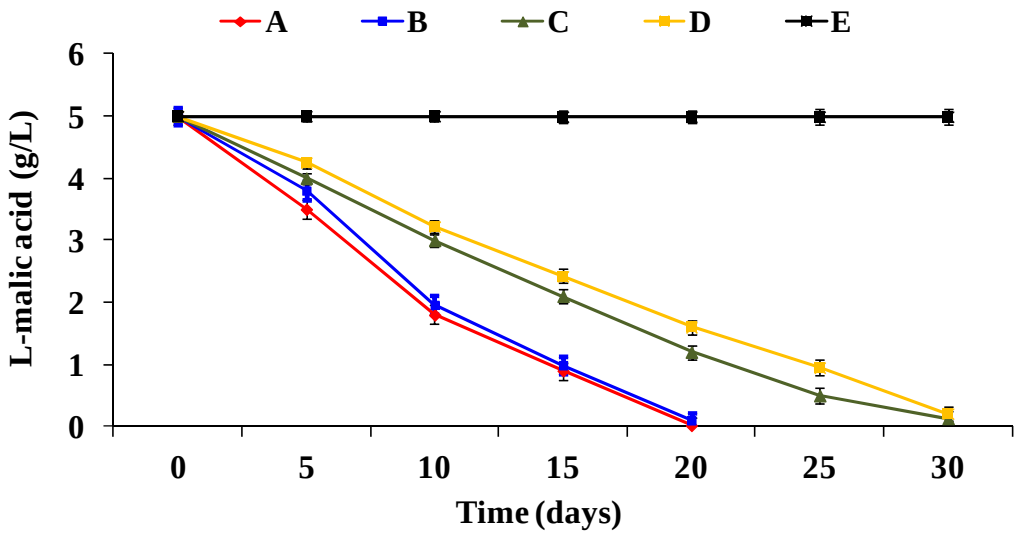

Figure 3:

668 **Table 1.** API-ZYM galleries of mean enzymatic activities corresponding to 12 *L. plantarum* strains

	Strains											
Enzymatic activities	**M10**	**M17**	**M26**	**M22**	**V22**	**M24**	**T13**	**P1**	**P9**	**P5**	**A1**	**R1**
Alkaline phosphatase	-	-	-	-	-	-	-	-	-	-	-	-
Esterase (C4)	-	-	-	-	-	-	-	-	-	-	-	-
Esterase lipase (C8)	-	-	-	-	-	-	-	-	-	-	-	-
Lipase (C14)	-	-	-	-	-	-	-	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
Valine arylamidase	W	W	W	W	W	W	W	W	W	W	W	W
Cystine arylamidase	-	-	-	-	-	-	-	-	-	-	-	-
Trypsin	-	-	-	-	-	-	-	-	-	-	-	-
α-hymotrypsin	-	-	-	-	-	-	-	-	-	-	-	-
Acid phosphatase	W	W	W	+	W	+	+	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	W	+	W	W	W	W	W	W	W
α-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-
β-galactosidase	W	W	W	W	-	W	W	W	W	W	W	W
β-glucoronidase		-	-	-	-	-	-	-	-	-	-	-
α-glucosidase	+	+	+	+	+	+	+	+	+	+	+	+
β-glucosidase	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-β-glucosaminidase	W	W	W	W	-	W	W	W	W	W	W	W
α-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-	-	-	-	-	-

669 Biochemical tests were performed using API-ZYM systems (BioMérieux). + Positive ; W weakly positive ; - negative.

670
671

672 **Table 2.** Ability of *L. plantarum* strains to release aromatic aglycones from odourless grape glycosidic precursors. Data represent the comparison of
673 the same aroma compound determined in different microbial cultures and are expressed in percentage (considering the highest value of a specific
674 compound as 100% and comparing this value with the amounts of the same compound determined in the other microbial cultures).
675

	control	**A1**	**R1**	**M17**	**M22**	**M24**	**M10**	**M26**	**P1**	**P5**	**P9**	**T13**	**V22**
Terpenes													
Limonene	0	46.46	84.43	42.32	74.36	40.91	**100**	59.37	44.28	37.08	39.95	40.90	60.77
Linalool	0	98.94	81.06	86.22	88.09	91.54	99.10	92.15	94.11	70.26	83.05	83.78	**100**
Linalool oxide 1	0	88.91	74.25	76.22	84.53	71.51	**100**	84.02	78.33	69.27	62.12	90.07	98.48
beta-myrcene	0	85.02	85.44	88.15	**100**	86.65	87.32	85.02	90.40	66.22	80.31	84.13	99.02
Benzenic derivatives													
Benzyl alcohol	0	73.03	81.24	77.41	66.84	65.39	93.88	61.61	58.21	58.56	47.77	70.50	**100**
beta-phenylethyl alcohol	0	55.99	81.44	87.07	51.69	49.21	98.96	45.81	45.38	34.92	34.12	48.96	**100**
C6 Alcohols													
1-Hexanol	0	**100**	81.89	70.68	63.40	63.13	96.61	64.14	68.10	51.02	52.41	59.29	90.15
trans-2-hexen-1-ol	0	67.63	46.23	**100**	47.68	49.12	58.42	53.82	45.91	45.70	32.91	44.10	49.04

676 **Table 3.** Turbidity difference at 600 nm between cultures with or without malvidin 3,5-diglucoside

Strain	mg/mL malvidin 3,5-diglucoside		
	0.500	**0.250**	**0.125**
A1	-18.4 ± 6.0	-18.2 ± 8.4	-10.8 ± 8.4
R1	94.5a ± 11.2	75.6b ± 6.1	69.9b ± 1.1
M17	-25.3c ± 2.88	-21.4b ± 2.7	-17.3a ± 2.5
M10	-5.91 ± 1.36	-5.85 ± 1.59	-6.73 ± 1.13
M22	-6.48 ± 0.17	-7.78 ± 1.57	-6.94 ± 3.21
M24	95.1 ± 21.2	72.6 ± 11.7	58.2 ± 14.4
M26	-14.0b ± 1.6	-9.16ab ± 1.90	-4.13a ± 3.84
P1	-3.98 ± 2.49	-1.49 ± 0.96	-1.61 ± 0.79
P5	-55.5 ± 2.02	-50.2 ± 0.5	-42.5 ± 8.6
P9	19.9a ± 14.2	6.01a ± 10.90	-19.5b ± 4.0
T13	-9.52 ± 3.79	-15.6 ± 0.6	-10.9 ± 2.4
V22	20.0a ± 1.9	-10.7b ± 5.8	-28.9c ± 4.6

677 Each value represents the mean ± standard deviation standards of three independent replicates.
678 Different letters within columns indicate significant differences ($P < 0.01$).
679 The effect of malvidin 3,5-diglucoside on the bacteria growth was expressed as % inhibition/stimulation.
680 Positive value: increased growth; Negative value: decreased growth.
681 Control: ZMB1 medium in the absence of malvidin 3,5-diglucoside.
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683 **Table 4.** Physical-chemical analysis of wines obtained with co-inoculation (A, B) and sequential
684 inoculum (C, D) at the end of the malolactic fermentation. Tank E represents a control, wine
685 inoculated with the *S. cerevisiae* AM37 strain

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	A	**B**	**C**	**D**	**E**
pH	3.54 ± 0.01	3.48 ± 0.04	3.38 ± 0.05	3.37 ± 0.06	3.38 ± 0.05
tritrable acidity (g/L)	8.52 ± 0.05	8.31 ± 0.06	8.60 ± 0.04	8.62 ± 0.03	8.67 ± 0.04
SO_2 total (mg/L)	22.5 ± 0.01	12.5 ± 0.50	22.5 ± 0.01	19.5 ± 0.02	17.5 ± 0.05
L-malic acid (g/L)	0.02 ± 0.01	0.05 ± 0.01	0.05 ± 0.06	0.06 ± 0.08	4.99 ± 0.20
L-lactic acid (g/L)	3.11 ± 0.01	2.72 ± 0.05	3.11 ± 0.06	3.12 ± 0.08	0.09 ± 0.34
D-lactic acid (g/L)	3.17 ± 0.01	2.68 ± 0.03	2.23 ± 0.01	2.18 ± 0.04	0.41 ± 0.05
acetic acid (g/L)	0.11 ± 0.01	0.15 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.10 ± 0.01
residual sugar (g/L)	0.66 ± 0.01	0.63 ± 0.01	0.65 ± 0.01	0.80 ± 0.04	1.66 ± 0.30
alcohol (% v/v)	12.4 ± 0.01	12.2 ± 0.01	12.2 ± 0.01	12.1 ± 0.01	12.0 ± 0.01

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Manuscript title: '**Selection and technological/functional potential of *Lactobacillus plantarum* bacteria suitable for wine malolactic fermentation and grape aroma release'**

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Highlights

- Selection of new *L. plantarum* malolactic starters
- None of the strains tested produced biogenic amines
- First application of MALDI-TOF/TOF in the study of bacteriocin-producing wine lactic acid bacteria
- First evidence about the ability of wine *L. plantarum* to hydrolyse grape glycosides, releasing different types of odorant aglycones
- *L. plantarum* M10 strain was proposed for co-inoculation in must to complete the MLF