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Characterization of malolactic fermentation by *Lactiplantibacillus plantarum* in red grape must

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ABSTRACT

Malolactic fermentation (MLF) is a desirable biotransformation in red and some high-acidity white wines. This process involves the decarboxylation of L-malic acid into L-lactic acid by lactic acid bacteria (LAB). Among them, *Oenococcus oeni* and *Lactiplantibacillus plantarum* are the most common, being the last usually used as starter to perform MLF in wine. Compared with *O. oeni*, *L. plantarum* is less tolerant to wine conditions such as pH and ethanol; thus, it is preferable to inoculate it in must. The current literature has not yet addressed the impact of some parameters on the inoculation of this bacterium in grape must. Therefore, the aim of this work was to characterize the effect of the inoculum concentration, pH and ethanol tolerance, as well as the inoculation strategy, on the MLF performance of *L. plantarum* in grape must. We observed that a population $< 10^8$ CFU/mL could compromise MLF performance. Additionally, *L. plantarum* was able to survive at low pH values, such as 2.50, but in the presence of ethanol or a fermenting yeast, it could tolerate pH values in the range of 3.00–3.25. Finally, coinoculation or sequential inoculation with *S. cerevisiae* allows for the completion of MLF before the end of alcoholic fermentation.

1. Introduction

Malolactic fermentation (MLF) is a biotransformation process performed by lactic acid bacteria (LAB) in fermented beverages (Liu, 2002). This process involves the decarboxylation of L-malic acid into L-lactic acid, which increases the pH value and is also related to microbial stabilization and the organoleptic modulation of wines (Davis, Wibowo, Eschenbruch, Lee, & Fleet, 1985). MLF usually occurs spontaneously in red wines after the completion of alcoholic fermentation (AF).

The LAB community produced from grape must is usually low, approximately 10^2–10^3 CFU/mL (Costello, Morrison, Lee, & Fleet, 1983), and is mainly composed of *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) and *Oenococcus oeni* (Franquès et al., 2018). During the first stages of AF, this population begins to lose diversity. Nevertheless, some species with high ethanol tolerance can survive in significant populations. Under spontaneous fermentation conditions, after AF, the LAB population begins to increase, and once it reaches a population of 10^6 CFU/mL, the consumption of L-malic acid begins (Balmaseda et al., 2023; Lonvaud-Funel, 1999). *L. plantarum* and *O. oeni* are the LAB species to best tolerante wine environments. However, *O. oeni* strains are more likely to survive and develop because of their notable pH and ethanol resistance (Bech-Terkilsen, Westman, Swiegers, & Siegumfeldt, 2020).

To better control the fermentation process, starter cultures of *O. oeni* are usually inoculated in wine to ensure the completion of MLF. Unfortunately, the use of *L. plantarum* starter cultures has yet to be explored. In general, when comparing the MLF performance of these two bacteria in the literature, *L. plantarum* is inoculated directly into wine, as is done for *O. oeni* (Brizuela et al., 2017, 2021). However, the more restricted tolerance of *L. plantarum* to pH or ethanol usually results in stuck fermentation (Russo, Englezos, Capozzi, Pollon, Río, et al., 2020). *O. oeni* has a heterofermentative hexose metabolism pathway, which limits its suitability for inoculation into grape must. In contrast, *L. plantarum* has a facultative heterofermentative pathway that does not compromise volatile acidity under wine conditions due to the low pentose and oxygen concentrations (Quatravaux, Remize, Bryckaert, Colavizza, & Guzzo, 2006). This feature increases the possibility of using *L. plantarum* in grape must to initiate MLF simultaneously with AF (Berbegal et al., 2016; Lombardi et al., 2020; Urbina, Calderón, & Benito, 2021). In addition, the short duration of MLF with this bacterium

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enables the completion of MLF before the production of a significant amount of ethanol by fermenting yeasts, which may compromise bacterial survival (Berbegal et al., 2016; Englezos et al., 2020). In addition, in the current climate emergency situation, the pH is significantly greater than it has been in the past (Ubeda, Hornedo-ortega, Cerezo, Garcia-parrilla, & Troncoso, 2020), allowing the growth of *L. plantarum*, which appears to be more affected by lower pH values than *O. oeni* (Bravo-Ferrada et al., 2013).

This issue has been recurrently addressed in the literature, but a complete characterization of the MLF performance of *L. plantarum* in grape must is necessary. Overall, the aim of this work was to characterize the MLF of *L. plantarum* in red grape must to fully address the oenological potential of this bacterium as a starter culture.

2. Materials and methods

2.1. Microbial strains

Two commercial *L. plantarum* strains were used in this study: Lp1 (ML Prime™, Lallemand, Inc., Montreal, Canada) and Lp2 (Viniflora® NOVA™, Chr. Hansen Holding AS, Hoersholm, Denmark). The strains were maintained in grape juice medium consisting of 250 mL/L commercial red grape must (Carrefour Bio, Carrefour S.A., Madrid, Spain), 5 g/L yeast extract (Panreac Química, Castellar del Vallès, Spain), and 1 mL/L Tween 80 (Panreac Química), adjusted to pH 4.80. In addition, *O. oeni* PSU-1 (ATCC BAA-331) was used. LAB were maintained in MRSmf medium (55 g/L MRS broth (BD™ Difco™, Fisher Scientific, Madrid, Spain), 4 g/L DL-malic acid, 5 g/L fructose, 100 mg/L nystatin (Panreac Química), pH 5.00). 20 g/L of agar (Panreac Química) was added for preparing plates of each described medium.

For AF, a commercial *S. cerevisiae* strain was used (Lalvin CLOS, Lallemand, Inc.). This strain was used as an active dry yeast, prepared following the manufacturer's instructions, and maintained at 4 °C.

2.2. Malolactic fermentation in commercial red grape must

The commercial red grape must (163 g/L glucose + fructose, 2.67 g/L L-malic acid, 88 mg/L primary amino nitrogen (PAN), 48 mg/L NH_4, pH 3.25) was adjusted to 200 g/L glucose + fructose and 200 mg/L total assimilable nitrogen by adding equal concentrations of glucose and fructose and Nutrient VIT (Lallemand, Inc.). This control must was modified when required to different pH values with 2 M HCl — pH 3.00, 2.75, 2.50 —, 2 M NaOH — pH 3.50 — or to higher sugar concentrations— 220, 240, 260, 280 g/L — with glucose and fructose. The resulting musts were pasteurized (85 °C, 30 min) before use. Sterility was confirmed by plating in YPD (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 100 mg/L chloramphenicol, and 20 g/L agar) or MRSmf plates.

2.2.1. Effect of inoculum size

Tubes containing 12 mL of the standard red must were inoculated with each *L. plantarum* strain for an initial population of 10^8, 10^7, or 10^6 CFU/mL. The tubes were statically incubated at 25 °C. L-malic acid and bacterial viability were monitored periodically. L-malic acid was determined by enzymatic methods using an ANALYZER Y15 (Biosystems, Barcelona, Spain). Viability was assessed by plating appropriate dilutions of fermentation must on MRSmf plates. Fermentations were performed in duplicate.

2.2.2. Effect of pH

Tubes containing 12 mL of grape must at different pH values — 3.25, 3.00, 2.75, 2.50 — were inoculated with each *L. plantarum* strain to obtain a population of 10^8 CFU/mL. The tubes were incubated statically at 25 °C. Similarly, the L-malic acid concentration and bacterial population were monitored periodically. Fermentations were performed in duplicate.

2.2.3. Combined effect of pH in the presence of ethanol

Tubes containing 12 mL of grape must at pH 3.25, 3.00, and 2.75 were supplemented with 6 or 8% (vol/vol) absolute ethanol. Then, each *L. plantarum* strain was inoculated for an initial population of 10^8 CFU/mL. The tubes were incubated statically at 25 °C. Similarly, the L-malic acid concentration and bacterial population were monitored periodically. Fermentations were performed in duplicate.

2.2.4. Effect of sulfur dioxide (SO_2)

A stock solution of $K_2S_2O_5$ (Fisher Scientific, Madrid, Spain) was used to supplement tubes containing 12 mL of standard grape must — pH 3.25 — with different initial concentrations of total SO_2 —20, 40, and 60 mg/L —. A must without $K_2S_2O_5$ used as the control. Subsequently, each *L. plantarum* strain was inoculated for an initial population of 10^8 CFU/mL. The tubes were incubated at 25 °C statically. Similarly, the L-malic acid concentration and bacterial population were monitored periodically. Fermentations were performed in duplicate.

2.3. Simultaneous alcoholic and malolactic fermentation in red grape must

Twenty millilitre-screwed vials (Thermo Fisher Scientific, Madrid, Spain) filled with 14 mL of red grape must at the different above mentioned pH values were inoculated with 2×10^6 cell/mL *S. cerevisiae*. *L. plantarum* strains were inoculated for a population of 10^8 CFU/mL in the must at the beginning of fermentation together with *S. cerevisiae* (coinoculation) or 24 h later (sequential inoculation). In addition, a double set of grape musts was inoculated with only *S. cerevisiae*. One grape must was used as a control for AF, and the other was inoculated with *O. oeni* after AF completion. For each condition, 50 mL of grape must were inoculated with the appropriate yeast and *L. plantarum* combination, and then the volume was split in 14 mL into the vials. In the case of the sequential inoculation of *L. plantarum*, the corresponding cell pellet was introduced into the vial by resuspending with the fermenting must. Caps were perforated by two hypodermic needles — one for allowing CO_2 release (0.8 × 40 mm; B. Braun, Melsungen, Germany) and the other (0.6 × 80 mm; B. Braun) coupled with a 1 mL syringe (Luer-Lok™, BD, Madrid, Spain) for sampling —. The fermentations were incubated with agitation (120 rpm) at 25 °C (New Brunswick Innova® 42, Eppendorf Corporate, Hamburg, Germany). Each fermentation was performed in triplicate. All the combinations described are summarized in Table 1.

Table 1
Summary of the fermenting combinations performed in this study. AF: alcoholic fermentation.

Code	*S. cerevisiae*	Lactic acid bacteria (LAB)	Inoculation timing of the LAB
Sc	Lalvin-CLOS	None	–
Lp1Co	Lalvin-CLOS	*L. plantarum* ML Prime™	Coinculation with *S. cerevisiae*
Lp1Se	Lalvin-CLOS	*L. plantarum* ML Prime™	24 h after *S. cerevisiae*
Lp2Co	Lalvin-CLOS	*L. plantarum* Viniflora® NOVA™	Coinculation with *S. cerevisiae*
Lp2Se	Lalvin-CLOS	*L. plantarum* Viniflora® NOVA™	24 h after *S. cerevisiae*
Oo	Lalvin-CLOS	*O. oeni* PSU-1	After AF

In the case of the wines inoculated with *O. oeni*, after AF, the resulting wine was inoculated with a population of approximately 10^7 CFU/mL *O. oeni* and subsequently transferred to a 10 mL syringe (Luer-Lok™, BD, Madrid, Spain) coupled to a needle (0.8 × 40 mm, B. Braun). The fermentations were incubated statically at 25 °C.

AF progression was monitored by weight loss at least once per day with a precision balance (Entris® II Essential, Sartorius, Goettingen, Germany). AF was considered to be finished when the weight was constant for two consecutive days and confirmed by a residual sugar concentration <1 g/L glucose + fructose measured via enzymatic method by an ANALYZER Y15 system (Biosystems). During the fermentation process, bacterial viability and L-malic acid content were monitored.

Once fermentation was complete, after AF, all the wines except for the *O. oeni*-inoculated wines that were analysed after MLF were chemically characterized. The pH was determined using a Crison micro pH 2002 pH meter (Barcelona, Spain). D-lactic acid, L-lactic acid, acetic acid, citric acid, glycerol, ammonium, and PAN (primary amino nitrogen) were determined with an ANALYZER Y15 system (Biosystems). Acetaldehyde concentrations were determined using a K-ACHYD (Megazyme, Wicklow, Ireland) assay kit.

The phenolic composition of the obtained wines was analysed in terms of the total polyphenol index (TPI) as described previously Balmaseda et al., 2021b.

2.4. Statistical analysis

ANOVA and the Tukey test were used for statistical analysis of the obtained data. These analyses were performed using XLSTAT 2020.2.3 software (Addinsoft, Paris, France). Principal component analysis (PCA) was also performed using the same software.

Finally, analysis of variance (ANOVA)-simultaneous component analysis (ASCA) was used to determine and assess the sources of variability affecting the chemical properties of the wines fermented with *L. plantarum* and *S. cerevisiae,* as already described in (Schorn-García et al., 2023). The considered factors were the initial pH, inoculation strategy and the *L. plantarum* strain, which were used to decompose the variance in the original matrix (**X**) as

$$\mathbf{X} = \mathbf{X}_{\text{initial pH}} + \mathbf{X}_{\text{inoculation strategy}} + \mathbf{X}_{\text{Lp strain}} + \mathbf{X}_{\text{initial pH x inoculation strategy}} + \mathbf{X}_{\text{initial pH x Lp strain}} + \mathbf{X}_{\text{inoculation strategy x Lp strain}} + \mathbf{E} \quad (1)$$

where $\mathbf{X}_{\text{factor}}$ are the decomposed matrices for the main factors, $\mathbf{X}_{\text{factor 1 x factor 2}}$ are the decomposed matrices for factor interactions and **E** is the matrix of residuals.

3. Results and discussion

3.1. L-malic acid consumption in red grape must

The consumption of L-malic acid in wine by *O. oeni* has been extensively studied (Balmaseda et al., 2023; Bartowsky, 2005; Betteridge, Sumby, Sundstrom, Grbin, & Jiranek, 2018; Ferrando et al., 2020). However, the effect of the MLF performance of *L. plantarum* in grape must still has not been fully characterized. Several studies have evaluated the presence of *L. plantarum* in wine, but its low ethanol tolerance and facultative heterofermentative character make *L. plantarum* suitable for inoculation into grape must during the first fermentative stages. Therefore, in this work, we aimed to study the effect of different factors potentially affecting the MLF of *L. plantarum*. In this sense, two commercial strains of the species were used.

3.1.1. Evaluation of the effect of inoculum size on the MLF

First, the inoculum size was evaluated. There has been no consensus about the optimal population of *L. plantarum* for inoculation in grape must or in wine to perform MLF. Apart from the heterogeneity of inoculation strategies — coinoculation with *S. cerevisiae* in grape must, 24 h after *S. cerevisiae* inoculation in must (Berbegal et al., 2016; Lerena et al., 2016), after 7 days of AF (Lucio, Pardo, Heras, Krieger-Weber, & Ferrer, 2017), or even after AF (Brizuela et al., 2017; Cinquanta, De Stefano, Formato, Niro, & Panfili, 2018) — the inoculated population in grape must can range from 10^6 to 10^8 (Brizuela et al., 2021; Lombardi et al., 2020; Lucio et al., 2017; Wang et al., 2018), even 5×10^8 CFU/mL (Mesas, Rodríguez, & Alegre, 2011). Thus, evaluating different inoculum sizes under the same fermentative conditions is highly important for evaluating the optimal and minimum populations of *L. plantarum* in grape must.

Under the studied conditions, both *L. plantarum* strains at 10^8 CFU/mL could consume almost all the L-malic acid in 24 h (Fig. 1), leaving a residual concentration of 0.28 g/L on average — from an initial concentration of approximately 2.64 g/L — in the case of Lp1. After 24 h, cell viability was maintained, and Lp1 was able to deplete L-malic acid during the following hours.

When inoculating less than 10^8 CFU/mL, the MLF was compromised due to a rapid loss of viability during the first days. In the case of Lp2, the inoculation of 10^7 CFU/mL was enough to ensure that the MLF was successful, which was completed after five days. Nevertheless, extrapolation to real wine fermentation with fermenting yeasts that are metabolically active could compromise this less effective MLF, especially because of a gradual increase in the ethanol content (Lombardi et al., 2020). Lp1 at 10^7 CFU/mL had a dramatic decrease in viability;

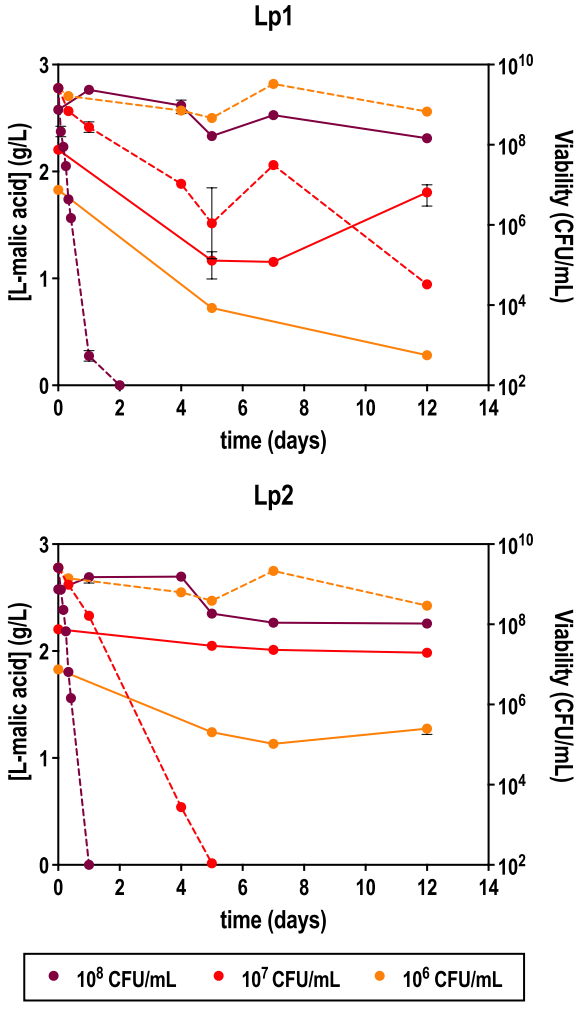

Fig. 1. L-malic acid consumption (dotted lines) and viability (continuous lines) of the two *L. plantarum* strains in red grape must with different inoculum sizes.

the bacterial count decreased to 10^5 CFU/mL on day four, which resulted in poor L-malic acid consumption. Nevertheless, after 12 days, an increase in viability was observed, and L-malic acid consumption increased. As noted before, under real fermentative conditions, the loss of viability could have been more dramatic, resulting in a stuck MLF. 10^6 CFU/mL was not enough sufficient to perform MLF under any conditions (Fig. 1).

Overall, the optimal inoculation ratio of *L. plantarum* was observed to be 10^8 CFU/mL, which achieved optimal MLF performance, whereas 10^7 CFU/mL was observed to be the minimal *L. plantarum* population needed to initiate MLF. Inoculation with 10^6 CFU/mL can also perform MLF in some wines, depending on the medium or the inoculated strain, according to other studies (Berbegal et al., 2016; Lucio et al., 2017; Russo, Englezos, Capozzi, Pollon, Río; Segade et al., 2020; Urbina et al., 2021). However, this inoculum can fail, even at pH > 3.60 (Lerena et al., 2016).

Finally, the optimal inoculation ratio—10^8 CFU/mL—was used for further experiments.

3.1.2. Evaluation of the effect of pH on the MLF performance

The MLF performance of the two selected *L. plantarum* strains was evaluated in the same red grape must at different pH values: 3.25, 3.00, 2.75, and 2.50. This is the first study in which MLF was tested in grape musts at these low pH values; this finding is interesting since pH is considered the most inhibitory stressor among ethanol and SO_2 (Sun et al., 2022). Other studies have rarely evaluated the pH tolerance of *L. plantarum* under 3.50 (Bravo-Ferrada et al., 2013; Sun et al., 2022). All the strains in all the conditions resulted in MLF in approximately one or two days (Fig. 2), with an optimal inoculation rate of 10^8 CFU/mL, as described in the previous section. This result confirmed the ability of *L. plantarum* to perform MLF at very low pH values with no other stressors, such as ethanol.

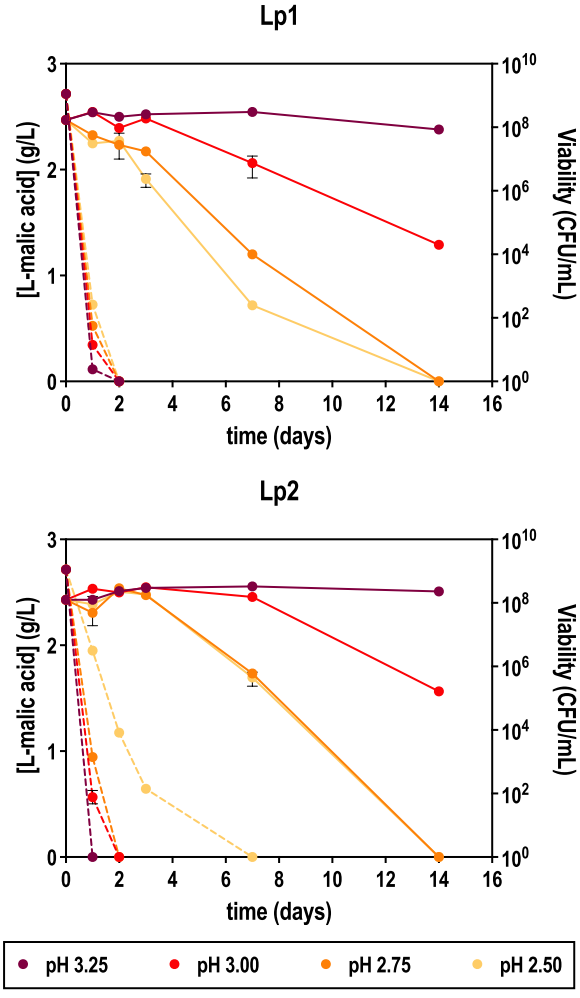

Fig. 2. L-malic acid consumption (dotted lines) and viability (continuous lines) of the two *L. plantarum* strains in red grape must at different pHs. The initial L. *plantarum* population was 10^8 CFU/mL.

Even if a decrease in viability was observed under all conditions except for the musts at pH 3.25 and 3.00, *L. plantarum* could complete the MLF. The only difference among the conditions was Lp2 at pH 2.50, which took seven days to finish fermentation (Fig. 2). This finding confirms the suitability of *L. plantarum* strains for performing MLF in grape musts at pH values typically found in oenology. Nevertheless, the studied conditions did not consider the synergistic effect of pH and ethanol during fermentation or competition with fermenting yeasts.

The combined effect of low pH and ethanol was tested in the same musts with different pH values (Fig. 3). In this case, the pH 2.50 fermentation was discarded because it showed poor MLF performance for Lp2, which needed one week to deplete L-malic acid. The pH 3.50 fermentation was also discarded due to the complete maintenance of viability. These musts were supplemented with 6 or 8% (vol/vol) ethanol to mimic an intermediate ethanol concentration of a wine with a 12–16% (vol/vol) potential degree of alcohol.

No effect was observed at increasing sugar concentrations — 220, 240, 260, or 280 g/L glucose + fructose — due to osmotic pressure (Suppl. Fig. 1).

3.1.3. Evaluation of the effect of sulfur dioxide (SO_2) on the MLF

The resistance of the commercial *L. plantarum* strains to SO_2 was evaluated in standard musts — pH 3.25 — with different initial SO_2 concentrations — 20, 40, and 60 mg/L — which are in the range of the current oenological practices. The two commercial strains displayed different levels of SO_2 resistance in red grape must (Fig. 4). Lp2 was more strongly affected by the addition of this compound, as it was associated with a dramatic decrease in viability in all the cases. The only SO_2 concentration that was compatible with MLF was 20 mg/L, which showed an initial decrease in viability comparable to that observed with higher SO_2 concentrations. Nevertheless, after 4 days, the viability increased from 10^6 to 10^8 CFU/mL, allowing the completion of MLF in 14 days. Higher concentrations did not allow for MLF due to a quick decrease in viability during the first week.

Other works have evaluated the SO_2 tolerance of *L. plantarum* strains in culture media at relatively high pH values. These authors have reported increased tolerances up to 40 mg/L in MRS supplemented with apple juice (20 % vol/vol) (Sun et al., 2022) or even 70 mg/L in MRS supplemented with tomato juice (15 % vol/vol) at pH 4.00 (Filimon et al., 2022), both with natural *L. plantarum* isolates. However, the initial population was approximately 10^6 CFU/mL, which, when combined with less stressful culture conditions — MRS medium and higher pH values — could explain the observed results.

Lp1 showed enhanced SO_2 resistance since the only tested SO_2 concentration of 60 mg/L inhibited the bacterium (Fig. 4). This concentration had the greatest effect on decreasing viability, but the effect was still more gradual than that observed for Lp2. Nevertheless, the amount of L-malic acid consumed in Lp1 musts was always greater than that consumed in Lp2 wines.

The gradual increase in viability after a quick initial loss of viability could be related to the evaporation or combination of molecular SO_2 in the must. In addition, the adaptation of the bacterial cells to the medium could also be related to the observed increase in viability.

Notably, a SO_2 concentration of approximately 20 mg/L is very common in winemaking, and together with other potential inhibitors, such as fermenting yeasts, the MLF in must by *L. plantarum* could be compromised.

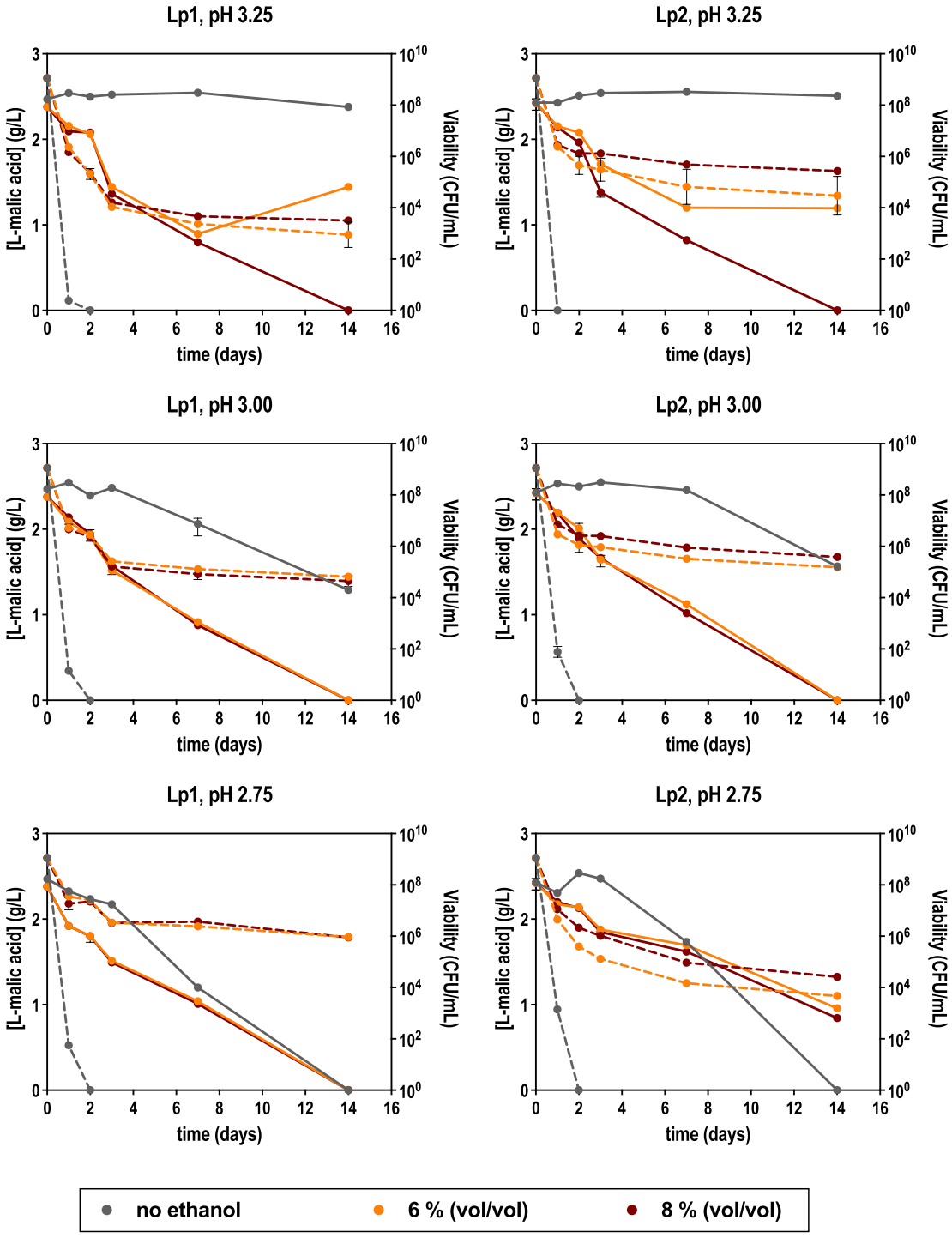

Fig. 3. L-malic acid consumption (dotted lines) and viability (continuous lines) of the two *L. plantarum* strains in red grape must with different pHs and ethanol concentrations at 10^8 CFU/mL.

3.2. Simultaneous alcoholic and malolactic fermentation

Once the behaviour of *L. plantarum* in red grape must was assessed, its MLF performance was evaluated in the same must but inoculated with *S. cerevisiae* (Fig. 5). In this sense, the red must was adjusted to different pH values — 3.50, 3.25, 3.00, and 2.75 — to evaluate whether pH, together with the gradual increase in ethanol concentration, could alter *L. plantarum* performance.

In regard to AF, the different initial pH values did not have any negative effect on fermentation kinetics (data not shown). However, AF in all samples finished in 14 days except in the must at pH 3.50, which finished after 13 days, one day before. During this time, MLF-related parameters (bacterial viability and L-malic acid concentration) were monitored. The bacterial viability decreased gradually in all the wines. This difference should be related mainly to the production of ethanol during AF, apart from microbial competition with *S. cerevisiae*. In addition, this decrease in viability was more dramatic in wines with lower pH values. The synergistic effect of ethanol on membrane function is enhanced at lower pH values (Bech-Terkilsen et al., 2020; Sun et al., 2022). This fact explains the obtained results at pH 3.00 and pH 2.75,

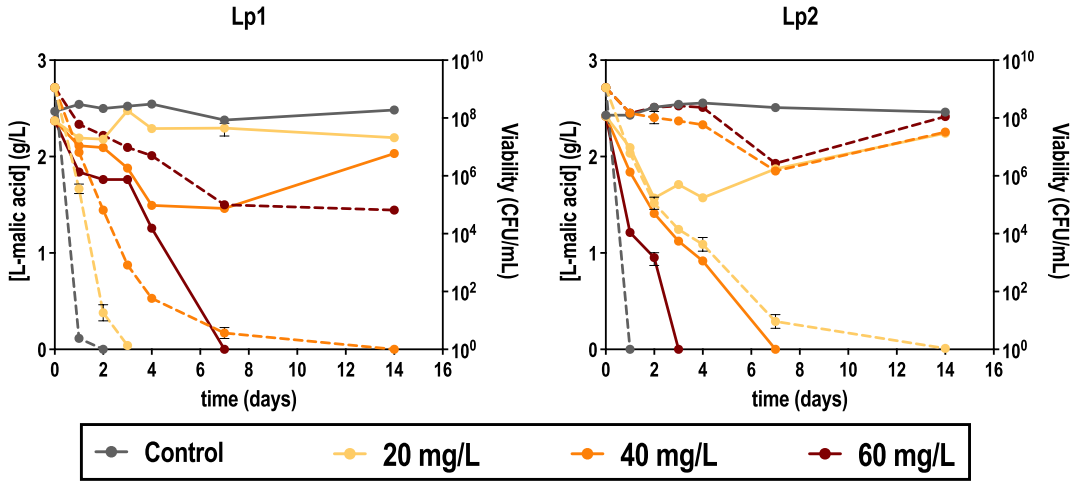

Fig. 4. L-malic acid consumption (dotted lines) and viability (continuous lines) of the two *L. plantarum* strains in red grape must with different sulfur dioxide concentrations at 10^8 CFU/mL.

where bacterial viability was completely lost before the end of AF (Fig. 5). The observed loss of viability at lower pH values also corresponded to stuck MLF. Wines produced with musts adjusted to pH 3.00 and 2.75 did not finish MLF because of the loss of viability.

In general, the coinoculation strategy showed a quick L-malic acid consumption at relatively high pH values (3.50, 3.25) (Fig. 5). It was as fast as finishing MLF in 1 day at pH 3.50. At pH 3.25, the duration was increased, and at pH 3.00, only Lp2 could finish MLF (Fig. 5). The results obtained for sequential inoculation 24 h after *S. cerevisiae* inoculation were extended to the MLFs. In this case, the decrease in viability followed the same trend as that observed for the coinoculated wines.

For comparison, wine after AF, without *L. plantarum*, was inoculated with *O. oeni*. The only pH condition that could enable MLF by *O. oeni* in this grape must was 3.50. A lower pH resulted in a dramatic decrease in viability after 2 days (data not shown). At pH 3.50, the duration of MLF was 22 days. At pH 3.25, only an initial L-malic acid consumption of approximately 0.20 g/L was observed, after which the consumption stopped as a result of the loss of viability.

The use of *L. plantarum* in the initial must guaranteed lower failure of MLF regarding to the inoculation of *O. oeni* in wine. It is worth noting that the evaluated musts with pH values of 3.00 or 2.75 represent very extreme oenological conditions for MLF. However, the inoculation of Lp2 enhanced the MLF performance, and sequential inoculation was the best inoculation strategy, occurring mainly at pH 3.00. Overall, the use of *L. plantarum* as an MLF starter culture not only enables simultaneous AF and MLF, reducing the fermentation duration but also the consumption of L-malic acid at lower pH values than *O. oeni* when inoculated in wine.

The wines obtained with the tested inoculation strategies exhibited chemical differences (Table 2). Wines after MLF or partial MLF — when AF finished — presented similar pH values to those obtained with *S. cerevisiae* as the sole starter (Table 2). This is true for *L. plantarum*-inoculated wines but not for *O. oeni*-inoculated wines. In these wines, the pH increase was much greater (3.74 on average) than that in Sc wine (3.57 on average) or Lp wine (from 3.50 to 3.68 on average) at an initial pH of 3.50. Other authors have described an acidification phenomenon when *L. plantarum* is inoculated in wine (Lucio, Pardo, Heras, Krieger, & Ferrer, 2018; Onetto & Bordeu, 2015). Even if this species decarboxylates L-malic acid, it can also produce lactic acid from sugars (Lucio et al., 2018). The obtained results for pH suggest that the final values, where some *L. plantarum* wines present a lower pH than Sc wines, are related to an accumulation of lactic acid in the medium (Table 2).

The residual L-malic acid correspond to the final concentration of this compound when wines finished AF. As expected, Sc wine not inoculated with LAB maintained a high L-malic acid concentration. From the initial L-malic acid concentration of 2.67 g/L, a significant decrease in this compound was observed after AF (Table 2). This consumption of L-malic acid during AF is a common behaviour of fermenting yeast that appears to be strain dependent (Ferrando et al., 2020; Martín-García, Balmaseda, Bordons, & Reguant, 2020; Ruiz-de-Villa, Poblet, et al., 2023). In addition, a strong effect of pH was observed on the L-malic acid consumption pattern of the yeast.

The production of D- and L-lactic acid was quantified. Typically, L-lactic acid in wine is generated from L-malic acid, mainly when LAB are inoculated after AF, where no sugars are found. D-lactic acid comes from the lactic fermentation of sugars via both heterofermentative and homofermentative pathways (Lonvaud-Funel, 1999). The L-lactic acid produced in Oo wine at pH 3.50 corresponds to the theoretical stoichiometrically produced concentration of approximately 1.72 g/L L-lactic acid from the initial L-malic acid of Sc wine (2.57 g/L), with some losses. However, in Lp wines, the L-lactic acid concentration was greater than possible for MLF (Table 2). It is known that some LAB have D- or L-lactate dehydrogenases and racemases that can transform one isomer to the other under different biological conditions (Liu, 2003); these enzymes exhibit complex regulation (Desguin, Soumillion, Hausinger, & Hols, 2017). However, the general transformation occurs from the L to D isomer since cell wall-related processes can occur (Goffin et al., 2005). Under our conditions, we found a generally higher concentration of the L isomer, which could have resulted from sugar metabolism.

The consumption of citric acid is another typical characteristic of LAB in wine. Low citric acid consumption was observed at pH 3.50 for both LAB. This difference could be related to an adaptation to low pH, as reported by Julliat et al. (2023), who reported a decrease in citrate consumption in *O. oeni* strains that evolved in acidic environments.

The concentration of nitrogen-related compounds was generally low and increased as the pH decreased. In addition, the Oo wine at an initial pH of 3.50 had the highest concentration, probably due to its peptidase activity, which occurs under wet conditions (Balmaseda, Rozès, Bordons, & Reguant, 2022; Margalef-Català, Araque, Bordons, Reguant, & Bautista-gallego, 2016; Remize et al., 2006; Ritt et al., 2009).

Residual nitrogen after AF can be used by spoiling microorganisms, causing undesirable microbial growth. Thus, low concentrations or complete depletion of nitrogen sources can be interesting in wines that have already finished both AF and MLF. The obtained results showed that when fermenting at low pH values, the residual nitrogen was greater in terms of primary amino nitrogen and ammonium (Table 2). Nevertheless, the risk of spoilage at lower pH values is considered low. In addition, in Oo wine, an enhanced nitrogen composition was

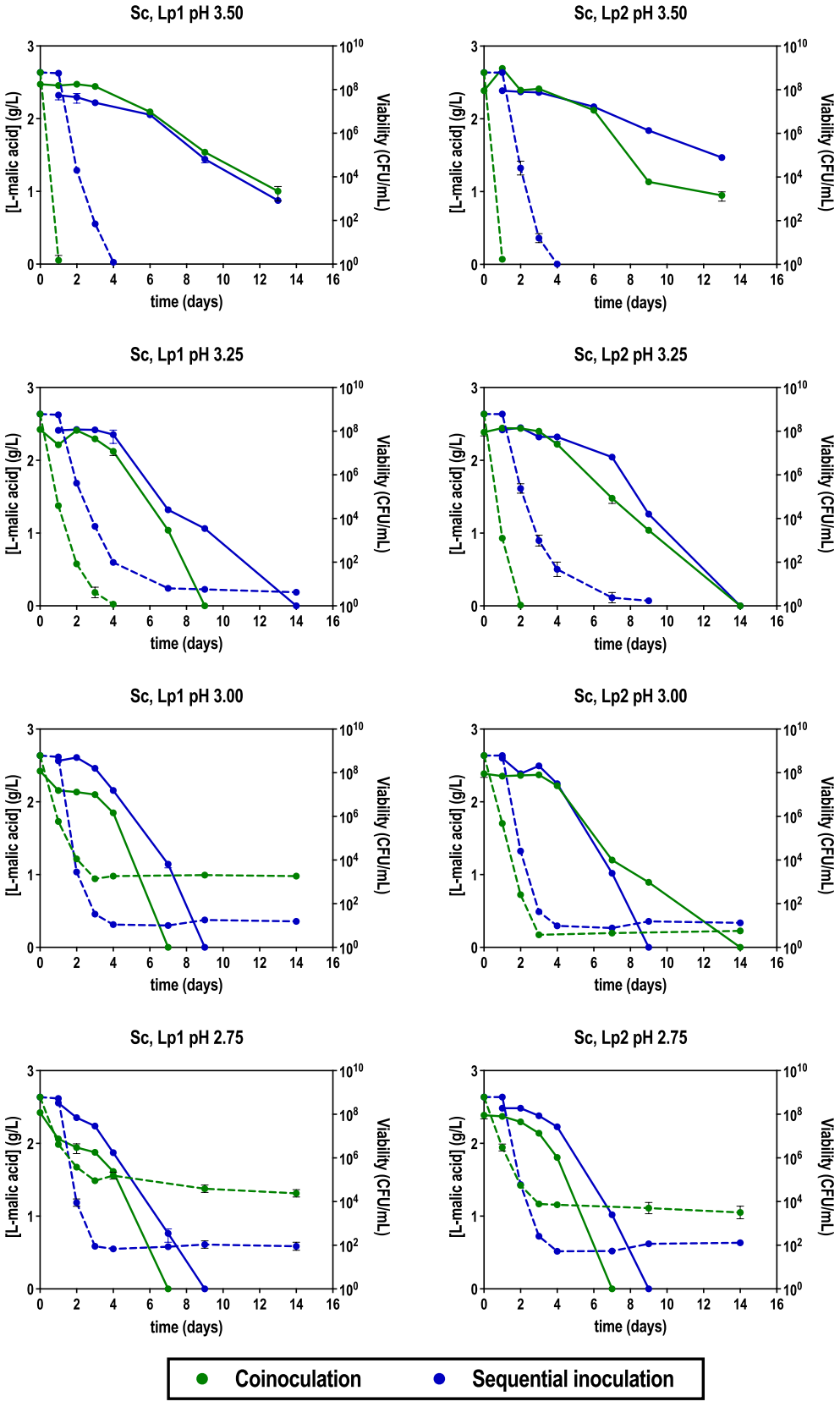

Fig. 5. L-malic acid consumption (dotted lines) and bacterial viability (continuous lines) in fermenting red grape must with *S. cerevisiae* at 10^8 CFU/mL. Coinoculation: *L. plantarum* coinoculated with *S. cerevisiae* in must. Sequential inoculation: *L. plantarum* was sequentially inoculated with *S. cerevisiae* after 24 h in must.

Table 2
General oenological parameters of the wines obtained at different initial pHs. PAN: primary amino nitrogen, TPI: total polyphenolic index, pH_f: final pH after fermentation.

	pH_f	L-malic acid (g/L)	L-lactic acid (g/L)	D-lactic acid (g/L)	Citric acid (mg/L)	Acetic acid (g/L)	Ammonium (mg/L)	PAN (mg N/L)	Acetaldehyde (mg/L)	Glycerol (g/L)	TPI
						pH 3.50					
Sc	3.57 ± 0.02^{b}	2.57 ± 0.04^{c}	0.26 ± 0.02^{a}	0.21 ± 0.01^{a}	306 ± 4^{e}	0.13 ± 0.01^{a}	4.67 ± 2.31^{a}	10.36 ± 3.06^{b}	21.5 ± 1.6^{a}	6.97 ± 0.12^{b}	9.3 ± 0.4^{a}
Lp1Co	3.58 ± 0.02^{b}	n.d.	2.49 ± 0.06^{e}	1.13 ± 0.08^{d}	248 ± 14^{d}	0.14 ± 0.02^{a}	1.00 ± 1.00a	8.33 ± 1.53^{b}	16.9 ± 0.5^{a}	6.30 ± 0.20^{a}	9.5 ± 0.2^{ab}
Lp1Se	3.65 ± 0.02^{c}	n.d.	2.15 ± 0.04^{c}	0.40 ± 0.02^{b}	300 ± 8^{e}	0.15 ± 0.02^{a}	4.00 ± 5.20^{a}	2.67 ± 2.52^{a}	20.7 ± 2.2^{a}	6.57 ± 0.32^{ab}	10.0 ± 0.6^{abc}
Lp2Co	3.50 ± 0.01^{a}	n.d.	2.33 ± 0.03^{d}	2.51 ± 0.08^{e}	92 ± 11^{a}	0.22 ± 0.01^{b}	2.67 ± 2.08^{a}	7.67 ± 0.58^{ab}	14.1 ± 0.7^{a}	6.50 ± 0.20^{ab}	10.7 ± 0.4^{bc}
Lp2Se	3.68 ± 0.02^{c}	n.d.	2.11 ± 0.08^{c}	0.62 ± 0.06^{c}	197 ± 15^{c}	0.20 ± 0.01^{b}	3.67 ± 2.08^{a}	7.00 ± 1.00^{ab}	43.7 ± 13.7^{b}	6.43 ± 0.21^{ab}	10.7 ± 0.7^{bc}
Oo*	3.74 ± 0.01^{d}	0.18 ± 0.03^{b}	1.33 ± 0.01^{b}	0.17 ± 0.01^{a}	148 ± 4^{b}	0.27 ± 0.02^{c}	14.33 ± 1.15^{b}	20.00 ± 1.00^{c}	56.9 ± 10.9^{b}	6.60 ± 0.01^{ab}	11.0 ± 0.3^{c}
						pH 3.25					
Sc	3.36 ± 0.03^{a}	2.22 ± 0.10^{c}	0.20 ± 0.01^{a}	0.10 ± 0.01^{a}	303 ± 6^{c}	0.22 ± 0.01^{a}	4.67 ± 2.52	16.00 ± 1.00^{c}	27.3 ± 2.7^{a}	6.30 ± 0.20	9.5 ± 0.4^{a}
Lp1Co	3.41 ± 0.02^{ab}	0.10 ± 0.01^{a}	1.83 ± 0.01^{b}	0.32 ± 0.01^{b}	307 ± 6^{c}	0.22 ± 0.02^{a}	4.33 ± 3.21	9.00 ± 1.00^{ab}	74.8 ± 3.4^{b}	6.33 ± 0.25	10.3 ± 0.2^{ab}
Lp1Se	3.43 ± 0.01^{b}	0.23 ± 0.02^{b}	1.67 ± 0.02^{b}	0.20 ± 0.01^{a}	310 ± 4^{c}	0.29 ± 0.02^{b}	n.d.	5.00 ± 1.00^{a}	93.8 ± 20.3^{b}	6.33 ± 0.15	11.0 ± 0.7^{b}
Lp2Co	3.37 ± 0.01^{ab}	n.d.	2.15 ± 0.13^{c}	1.20 ± 0.01^{c}	195 ± 11^{a}	0.30 ± 0.02^{b}	n.d.	12.67 ± 2.08^{bc}	84.8 ± 4.3^{b}	6.57 ± 0.35	9.5 ± 0.3^{a}
Lp2Se	3.41 ± 0.01^{ab}	n.d.	1.80 ± 0.04^{b}	0.35 ± 0.04^{b}	273 ± 2^{b}	0.29 ± 0.02^{b}	n.d.	8.33 ± 2.03^{a}	98.6 ± 18.4^{b}	6.30 ± 0.50	14.8 ± 0.2^{c}
						pH 3.00					
Sc	3.03 ± 0.03^{a}	1.97 ± 0.15^{d}	0.19 ± 0.01^{a}	0.09 ± 0.01^{a}	295 ± 7^{c}	0.19 ± 0.01^{a}	n.d.	19.67 ± 2.52^{c}	77.4 ± 3.7^{a}	5.57 ± 0.15^{a}	11.3 ± 0.3
Lp1Co	3.13 ± 0.01^{c}	0.99 ± 0.02^{c}	1.21 ± 0.04^{b}	0.26 ± 0.03^{c}	301 ± 4^{c}	0.24 ± 0.01^{b}	8.67 ± 2.08^{b}	15.33 ± 2.08^{ab}	82.8 ± 10.1^{a}	6.47 ± 0.21^{b}	12.1 ± 0.1
Lp1Se	3.07 ± 0.02^{ab}	0.38 ± 0.02^{b}	1.46 ± 0.08^{c}	0.16 ± 0.02^{ab}	305 ± 5^{c}	0.23 ± 0.02^{ab}	9.67 ± 1.53^{b}	12.67 ± 3.51^{a}	171.1 ± 10.8^{b}	6.67 ± 0.06^{b}	12.0 ± 0.8
Lp2Co	3.17 ± 0.02^{c}	0.20 ± 0.02^{a}	1.88 ± 0.02^{d}	0.36 ± 0.05^{d}	246 ± 16^{a}	0.29 ± 0.01^{c}	11.67 ± 1.53^{b}	17.33 ± 1.15^{ab}	56.1 ± 13.1^{a}	5.77 ± 0.06^{a}	12.1 ± 0.4
Lp2Se	3.08 ± 0.01^{b}	0.34 ± 0.02^{ab}	1.35 ± 0.10^{bc}	0.22 ± 0.02^{bc}	270 ± 3^{b}	0.21 ± 0.03^{ab}	8.67 ± 1.53^{b}	12.33 ± 2.31^{a}	145.0 ± 13.6^{b}	6.87 ± 0.25^{b}	11.5 ± 0.4
						pH 2.75					
Sc	2.71 ± 0.02^{ab}	1.96 ± 0.11^{d}	0.18 ± 0.04^{a}	0.13 ± 0.01^{a}	294 ± 3^{bc}	0.27 ± 0.03^{a}	6.00 ± 2.00	22.33 ± 2.08^{b}	11.9 ± 5.8^{a}	5.97 ± 0.15^{a}	11.8 ± 0.6
Lp1Co	2.88 ± 0.01^{c}	1.38 ± 0.05^{c}	0.78 ± 0.01^{b}	0.21 ± 0.01^{b}	295 ± 3^{bc}	0.26 ± 0.02^{a}	9.67 ± 1.15	14.00 ± 1.00^{a}	66.6 ± 6.3^{bc}	6.30 ± 0.17^{ab}	11.6 ± 0.2
Lp1Se	2.68 ± 0.01^{a}	0.61 ± 0.05^{a}	1.14 ± 0.07^{d}	0.12 ± 0.07^{a}	300 ± 5^{c}	0.33 ± 0.01^{b}	6.33 ± 3.21	18.00 ± 1.15^{ab}	77.2 ± 13.3^{c}	6.40 ± 0.26^{ab}	11.3 ± 0.1
Lp2Co	2.86 ± 0.01^{c}	1.11 ± 0.08^{b}	1.02 ± 0.02^{c}	0.29 ± 0.02^{c}	282 ± 9^{ab}	0.26 ± 0.01^{a}	10.67 ± 3.06	18.33 ± 1.15^{ab}	90.5 ± 5.3^{c}	6.43 ± 0.15^{ab}	11.2 ± 0.1
Lp2Se	2.74 ± 0.02^{b}	0.63 ± 0.03^{a}	1.03 ± 0.05^{cd}	0.19 ± 0.02^{b}	273 ± 3^{a}	0.33 ± 0.03^{b}	11.33 ± 0.58	19.67 ± 0.58^{b}	48.0 ± 11.4^{b}	6.70 ± 0.35^{b}	11.5 ± 0.2

Superscripts mean that values are significantly different at $p \leq 0.05$, according to a Tukey post-hoc comparison test. Statistics were done independently for each pH. No superscript means no significant difference between values. *Oo wine is only addressed at pH 3.50 as it was the only pH condition where *O. oeni* could finish. n.d.: not detected.

observed, which can be related to the protease and peptidase activities of *O. oeni* (Balmaseda et al., 2021a; Ritt et al., 2009) or even to the autolytic process of yeast cells due to a prolonged fermentation time.

Acetaldehyde is an intermediary compound produced by the sugar fermentative metabolism of yeasts. Acetaldehyde, together with pyruvic acid, accumulates as a result of the carbon flux from hexoses to ethanol during fermentation. In addition, ethanol can be oxidized to acetaldehyde over time as a consequence of chemical oxidation. In general, *O. oeni* is responsible for the degradation of acetaldehyde during MLF, even if some strains do not seem to degrade it (Wells & Osborne, 2012). In contrast, *L. plantarum* is related with increases in its concentration (Wang et al., 2018). Here, we observed that the inoculation of *L. plantarum*, mainly in sequential inoculation in must, enhanced the accumulation of acetaldehyde at all the pH conditions tested (Table 2). The use of *L. plantarum* can increase the accumulation of acetaldehyde, which can increase the stability of coloured compounds (Schwarz, Wabnitz, & Winterhalter, 2003), such as vitisin B, a pyranoanthocyanin formed by cycloaddition of acetaldehyde with anthocyanin (Ruiz-de-Villa, Gombau, et al., 2023). The Oo wine had a higher acetaldehyde concentration than the control Sc wine (Table 2). Even if *O. oeni* is usually related to the degradation of this compound, this increase could be related to wine oxidation due to a more prolonged fermentation time, 13 days of AF, followed by 22 days of MLF.

Wines were also analysed in terms of the total polyphenolic index (TPI). No great differences were observed at low pH values (Table 2). In contrast, at pH 3.50 and 3.25, an increase in this index was observed in those wines where MLF was completed. The main effect was observed in wines sequentially inoculated with *L. plantarum* at pH 3.25.

Other parameters, such as acetic acid or glycerol, did not significantly differ according to the inoculation strategy (Table 2).

Principal component analysis (PCA) was applied to the wine analysis data (Table 2), from which the scores and loadings were plotted (Fig. 6). With the aim of studying the variability of the produced wines and focusing on the effect of *L. plantarum* on the studied variables, the Sc and Oo wines were discarded from this analysis. The obtained PCA results (Fig. 6) revealed the strong effect of pH on the obtained wines along

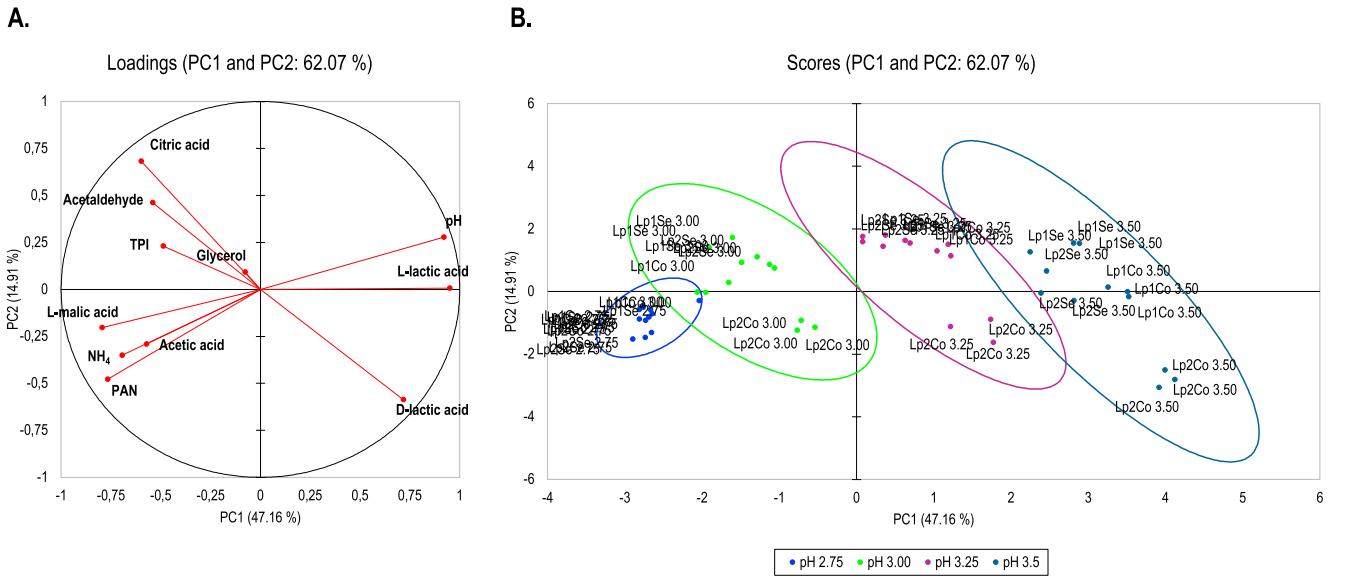

Fig. 6. Principal component analysis (PCA) with 95% confidence ellipses. The PCA shows the loadings (A) and scores (B) of measured variables of the wines obtained with *L. plantarum*. The triplicates of each wine are plotted individually. The color of the ellipses and scores represent the initial pH of the grape must. Wines are named based on Table 1 followed by the value of the initial pH of the must. PAN: primary amino nitrogen, TPI: total polyphenolic index.

PC1. Indeed, this was confirmed by an ANOVA-simultaneous component analysis (ASCA), in which the pH was determined to be a significantly affecting parameter — explaining 55.65% of the variance with a *p*-value of 0.0001 —. This indicates that even if the inoculation strategy and *L. plantarum* strain were significant parameters (*p*-value of 0.0001), their contributions to the observed variance were an order of magnitude smaller; 6.46% and 6.06%, respectively.

A higher initial pH in grape must was related to higher final pH values, together with higher L-lactic acid and D-lactic acid concentrations (Fig. 6). Interestingly, at pH 3.50, 3.25 and 3.00, the Lp2Co wines had negative scores on PC2, contrary to all the other wines, which was related to higher D-lactic acid concentrations and lower citric acid, acetaldehyde, TPI and glycerol contents.

4. Conclusion

We evaluated the technological implications of several relevant parameters for the use of *L. plantarum* as a starter culture in red grape musts with two commercial strains. First, it was determined that an inoculation concentration of 10^8 CFU/mL was the optimal inoculation ratio. Second, it was demonstrated that *L. plantarum* can perform MLF at low pH values, as low as 2.50. However, when a stressor such as ethanol is added to the medium, at the concentrations found during the intermediate AF stages, the bacterial viability is strongly affected, and MLF is stuck. Third, the SO_2 tolerance appears to be strain dependent, and concentrations greater than 20 mg/L can cause MLF failure in some *L. plantarum* strains. Finally, the inoculation strategy was evaluated with *S. cerevisiae*. The suitability of the use of *L. plantarum* as an MLF starter in grape must was confirmed, either by coinoculation or sequential inoculation, at pH 3.50 and 3.25. A strong pH effect was observed during fermentation with *S. cerevisiae*, with a pH of 3.00 being the threshold at which one strain could finish the fermentation process and the other could almost finish. Interestingly, potential acidification by *L. plantarum* in wine was observed under some conditions, mainly at higher initial pH values, due to an accumulation of lactic acid. Overall, we have better characterized the MLF performance of *L. plantarum* in grape must. Further research should be conducted to address the effect of MLF on the organoleptic profile of wines, mainly by comparing the MLF performed with *O. oeni* and *L. plantarum*.

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CRediT authorship contribution statement

Aitor Balmaseda: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Nicolas Rozès:** Writing – review & editing, Supervision, Funding acquisition. **Albert Bordons:** Supervision, Writing – review & editing. **Cristina Reguant:** Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116070.

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