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COMPARATIVE VINIFICATION ASSAYS WITH SELECTED PATAGONIAN STRAINS OF OENOCOCCUS OENI AND LACTOBACILLUS PLANTARUM

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Abstract

The performance of Patagonian *Lactobacillus plantarum* and *Oenococcus oeni* strains as malolactic starter cultures was compared. Two autochthonous strains of each species were selected, based on the presence of aroma-related genes, and inoculated in sterile wine of high ethanol content. The effects of initial inoculum size and pre-acclimation treatment on the efficiency of malolactic fermentation (MLF) were analyzed for each strain. *O. oeni* strains were able to successfully conduct the MLF only when the inoculum concentration was higher than 1.10^8 CFU/mL and cells were acclimated in sublethal ethanol concentrations. The increase of ethanol concentration in the acclimation medium also improved the kinetics of malic acid consumption. Successful MLF with *L. plantarum* strains required lower inocula and no pre-acclimation treatment. In addition, these strains showed a better profile of aroma-related genes than *O. oeni*. *L. plantarum* strains appeared to be more efficient than *O. oeni* strains as candidates for malolactic starter cultures to be used in Patagonian red wines.

Keywords: *Oenococcus oeni*, *Lactobacillus plantarum*, Patagonian Pinot noir wine, vinification assays, acclimation, inoculum size.

Highlights

► *L. plantarum* and *O. oeni* strains were isolated and fermentation examined ►*L. plantarum* was more efficient at MLF than *O. oeni* in Patagonian wines ► Standardization of acclimation and inoculum was necessary for successful MLF by *O. oeni* ► Acclimation in high ethanol content improved the malic acid consumption of *O. oeni* ► MLF with *L. plantarum* strains required lower inocula and no pre-acclimation treatment.

Abbreviations

LAB: Lactic Acid Bacteria

MLF: Malolactic Fermentation

PAP: Proline aminopeptidase

PAD: Phenolic acid decarboxylase

MAC: Malic acid consumption

AF: Alcoholic fermentations

16S-ARDRA: Amplified Ribosomal DNA Restriction Analysis

UPGMA: Unweighted Pair Group Method using Arithmetic Averages

hdc: histamine decarboxylase

tdc: tyramine decarboxylase

*ptc*A: putrescine carbamoyl transferase

PFGE: Pulsed-Field Gel Electrophoresis

1. Introduction

Oenococcus oeni and *Lactobacillus plantarum* have been described as the best adapted LAB species involved in malolactic fermentation (MLF) (Wibovo et al. 1985; Lonvaud-Funel 1999; Bartowsky and Henschke 1999; Pozo-Bayón et al. 2005, Valdés La Hens et al. 2015). MLF is a secondary fermentation that occurs during winemaking, and it is a crucial step that provides enhanced organoleptic properties and microbial stabilization of the wine (Lonvaud-Funel 1999; Liu 2002). MLF can occur spontaneously and randomly, but some winemakers suggest the use of MLF starter cultures to avoid delay and spoilage during this process (Capozzi et al. 2010, Ribereau-Gayon et al. 2006, Cecconi et al. 2009). Although, some strains of *O. oeni* and *L. plantarum* are available as commercial starter cultures, the use of indigenous strains is recommended to maintain the *terroir* characteristics (López et al. 2008; du Toit et al. 2011; Garofalo et al. 2015; Berbegal et al. 2016). The selection of autochthonous strains as potential candidates for MLF starter cultures requires the isolation, identification and study of their oenological properties, as well as their capacity to grow and consume malic acid under the harsh conditions that occur in wine, mainly high ethanol concentration and low pH (G-Alegría et al. 2003; Spano et al. 2006).

In Patagonian red wines MLF mainly occurs spontaneously and both species, *O. oeni* and *L. plantarum*, have been found in different vintages and in all stages of MLF of Pinot noir and Merlot wines (Valdés La Hens et al. 2015, Bravo-Ferrada et al. 2013). Several strains of these LAB species were previously isolated and studied in order to know their oenological and technological properties, particularly their capacity to grow and consume malic acid under the harsh wine environment (Bravo-Ferrada et al. 2013, 2014, 2015a, 2015b, 2016). Stress factors as high ethanol concentration, low pH, presence of sulfite are the main cause of death of inoculated cultures (Bravo-Ferrada et

al. 2016). However, it has been shown that bacteria are able to grow under these conditions if cultures are pre-adapted to wine environment (Maicas et al. 2000, Cecconi et al. 2009). In previous works we found that some *L. plantarum* and *O. oeni* strains can grow in wine-like media when they were previously acclimated in a rich medium containing a high fructose and glucose concentration and a low ethanol concentration (6 or 10% v/v) (Bravo-Ferrada et al. 2014, 2016). In addition, the acclimated cultures showed an improved capacity to consume malic acid. This adaptation was related to a change in the composition and properties of the bacterial membranes, such as a modification in fatty acid composition and protein profile (Bravo-Ferrada et al. 2014, 2015a, Chu-ky et al. 2005, da Silveira et al. 2004). However, the effect of acclimation of selected Patagonian *L. plantarum* and *O. oeni* strains on growing capacity and malolactic activity in sterile red wine with high ethanol concentration (≥ 14% v/v) have not been reported yet.

On the other hand, LAB strains can positively alter the chemical composition of wine through metabolism of flavor precursor compounds as a result of bacterial enzyme activities such as citrate lyase, beta-glucosidase, proline aminopeptidase (PAP) and phenolic acid decarboxylase (PAD) (Mtshali et al. 2010). The citrate lyase activity is related with the production of diacetyl; the enzyme cleaves citrate molecules into oxalacetate and acetate (Bekal et al. 1998). PAP is one of enzymes with protease activity that contributes to development of flavor by releasing free amino acids that are precursors of aroma compounds (Matos et al. 1998). PAD metabolize phenolic acids present in must and wine (Cavin et al. 1993, Swiegers et al. 2005) and beta-glucosidase releases different aroma-compounds by cleavage of glycosidic bonds, transforming terpenes, alcohols, fatty acids, etc. from bound to free forms (Grimaldi et al. 2000, Spano et al. 2005).

These enzyme activities could be explored by quantification of diverse metabolites *in vitro*. However, the screening of the genes coding these enzymes is an easier method to select strains that could have better oenological properties (Lerm et al. 2011, Olguin et al. 2010, Mtshali et al. 2010, Spano et al. 2005).

With this background, the aim of this work was to select and compare native Patagonian strains of *L. plantarum* and *O. oeni* by the presence of different aroma-related genes and studying the effect of pre-acclimation treatment, inoculum sizes, implantation capacity, and kinetics of malic acid consumption (MAC) in sterile Pinot noir wine, in order to understand its behavior as MLF starter cultures candidates for Patagonian red wines.

2. Materials and Methods

2.1. Bacterial isolates and growth conditions

O. oeni and *L. plantarum* isolates were obtained from Pinot noir wine samples from a 2012 vintage, in which alcoholic fermentation (AF) and MLF were spontaneous. At the end of MLF the wine had the following values: pH 3.75, 14.3% (v/v) ethanol, L-malic acid 0.5 g/L.

Samples were aseptically collected from a commercial cellar in General Roca, Argentinean North Patagonia, and inoculated in MLO (Maicas et al. 1999) and MRS (Biokar Diagnostic, Beauvais, France) (De Man et al. 1960) plates supplemented with cycloheximide 100 mg/L, under anaerobic conditions (AnaeroPack – Mitsubishi Gas Chemical America, Inc., New York, NY), at 28 °C, during 7 days or 48 h, respectively. Isolates identified as *O. oeni* or *L. plantarum* were grown in MLO or MRS broth, respectively. Cultures were kept frozen at -20 °C in the corresponding broth supplemented with glycerol (30% v/v).

2.2. Identification of isolates

DNA extraction from bacterial isolates was performed according to Bravo Ferrada et al. (2011). DNA samples were quantified using a Nanodrop spectrophotometer (Thermo Scientific, 1000) and visualized on a 1.0% (w/v) agarose gel.

Isolates were identified by 16S-ARDRA (Amplified Ribosomal DNA Restriction Analysis), using the primers pA and pH to amplify *16S rRNA* gene (Ulrike et al. 1989). Restrictions with *Mse*I enzyme were carried out according to Rodas et al. (2003). *L. plantarum* ATCC 14917 and *Oenococcus oeni* ATCC 27310 were used as reference strains. To confirm the identity of the selected *L. plantarum* and *O. oeni* strains, the *16S rRNA* gene was sequenced. Amplification of this gene was performed according to Delfederico et al. (2006). Sequences were obtained by using universal primers T7 and SP6 by means of DNA automatic sequencer (Macrogen Korea).

2.3. Typing of isolates by RAPD-PCR analysis

O. oeni and *L. plantarum* isolates were typed by RAPD-PCR analysis using primer Coc (Cocconcelli et al. 1995). Amplification reactions were performed as described Delfederico et al. (2006), and products were analyzed by electrophoresis in 1.5% (w/v) agarose gels. The evaluation of PCR profiles was made by calculation of genetic similarity index using a simple matching coefficient (Apostol et al. 1993). Unweighted Pair Group Method using Arithmetic Averages (UPGMA) cluster analysis was carried out by using PAUP* 4.0b10 (Sinauer Associates, MS, USA).

2.4. Screening of genes that encode for biogenic amines

The genes histamine decarboxylase (*hdc*), tyramine decarboxylase (*tdc*), and putrescine carbamoyl transferase (*ptc*A), implicated in the synthesis of histidine, tyrosine and

pustrescine, respectively, were screened in the potential MLF starter culture strains according to Bravo-Ferrada et al. (2013).

2.5. Detection of β-glucosidase, phenolic acid decarboxilase, proline aminopeptidase and β subunit-citrate lyase genes

LAB strains were analyzed for the presence of genes coding β-glucosidase, phenolic acid decarboxilase, proline aminopeptidase and β subunit-citrate lyase. The primers and reaction conditions used to amplify each gene are listed in **Supplementary 1.** PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gel. Estimation of fragment lengths was done by comparison to a 100-bp ladder marker as size standard (Productos Bio-Lógicos, Argentina).

2.6. Cell acclimation

Bacterial cells in the early stationary phase (approximately 10^9 CFU/ml) were harvested by centrifugation at 5000 x g for 10 min and suspended in the same volume of a modified acclimation medium (50 g/l MRS, 40 g/l D(-) fructose, 20 g/l D (-) glucose, 4 g/l L-malate, 1 g/l Tween 80, and 0.1mg/l pyridoxine, pH 4.6) (Lerm et al. 2011) supplemented with 6% or 10% (v/v) ethanol **(**Bravo-Ferrada et al. 2014). Culture incubations were carried out at 21 °C for 48 h.

2.7. Vinification assays

Two strains of each LAB species (UNQLp 11, UNQLp 22, UNQOe3 1 and UNQOe 6) were selected for carrying out vinification assays, at laboratory scale, in sterile Pinot noir wine at final stage of alcoholic fermentation (AF). A volume of 100 mL of wine (14.5% v/v ethanol, pH 3.82, < 2.00 g/L residual sugars, 2 g/L malic acid, 96 mg/L total

191 SO_2) was sterilized by filtration through 0.2 μm pore size (Sartorius Stedim Biotech
192 GmbH, Göttingen, Germany). Acclimated and non-acclimated cells were harvested by
193 centrifugation and inoculated (~5 x 10^7 CFU/mL) in 10 mL of wine. Incubation was
194 performed at 21 ºC during 20 days, without shaking.
195
196 ***2.8. Malic acid consumption and implantation capacity***
197 Malic acid consumption (MAC) and bacterial implantation capacity by acclimated (see
198 above) and non-acclimated cultures were evaluated by cell inoculation in sterile wine.
199 Cultivable cells were determined by plating on MRS or MLO agar, as appropriate,
200 sampled at days 0, 5, 10, 15 and 20. Remaining L-malic acid was measured with a malic
201 acid enzymatic kit (L-Malic Acid Enology enzymatic kit, BioSystems SA, Barcelona,
202 Spain).
203 An exponential one-phase decay equation model was used for fitting the performed
204 MAC kinetic by the different strains tested. The equation for this model was obtained
205 by the GraphPad Prism® software and it is:
206 $$[MA_t] = ([MA_0] - [MA_i])\ e^{-Kt} + [MA_i]$$ **(Equation 1)**
207 Where $[MA_t]$ is the malic acid concentration at time = t, $[MA_0]$ is the initial
208 concentration of malic acid (which was 2 g/L in the wine used), $[MA_i]$ is the malic acid
209 concentration at infinite time and K is the rate constant.
210 Also, the percentage of malic acid consumed (MAC %) after 20 days of incubation was
211 calculated following the equation:
212 $$MAC\ \% = 100 - ([MA_f]\ 100 / [MA_0])$$ **(Equation 2)**
213 Where $[MA_0]$ is the initial concentration of malic acid in the wine used and $[MA_f]$ is the
214 final concentration measured in the wine after 20 days of incubation.
215

2.9. Reproducibility of the results

All experiments were carried out on duplicate samples using three independent cultures of bacteria. The statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, 2007). Means were compared by one-way ANOVA, and if $P < 0.05$ the difference was considered statistically significant.

3. Results and discussion

In previous works we showed that *L. plantarum* and *O. oeni* are the main LAB species involved in conducting spontaneous MLF of Patagonian Pinot noir and Merlot wines (Valdés La Hens et al. 2015, Bravo-Ferrada et al. 2013). It is now widely accepted that use of autochthonous LAB strains as starter cultures, best adapted to the conditions of a specific wine-producing area, has the potential to retain the *terroir* characteristics of wine (Carreté et al. 2006; Ruiz et al. 2010, Bokulich et al. 2014; Garofalo et al. 2015). With the aim to enrich our collection of Patagonian oenological LAB strains, *L. plantarum* and *O. oeni* were surveyed from a Pinot noir wine, 2012 vintage, suffering spontaneous MLF, and a genetic screening of aroma-related enzymes was performed with the aim to select those with possible effect on sensorial quality of wine.

A total of sixty isolates were identified as LAB from MRS culture, and thirty from MLO culture, by morphology, Gram positive staining and catalase negative reaction. Twenty-seven isolates were presumptively identified as belonging to *L. plantarum* species and thirty isolates as *O. oeni* by 16S-ARDRA (Rodas et al. 2003). The other LAB species identified were *Lactobacillus brevis* and *Pediococcus acidilactici* (data not shown). After this presumptive identification, a clustering analysis was performed because a pool of different genotypes reduces the number of isolates to be studied and simplifies the evaluation of the implantation capacity and malic acid consumption. For this purpose, RAPD-PCR profiles with Coc primer were obtained from 30 *O. oeni* and

27 *L. plantarum* isolates. The clonal relationship between each single genomic fingerprinting of the *L. plantarum* and *O. oeni* isolates is shown in the UPGMA dendogram of **Fig 1**. Considering an arbitrary percentage similarity of 80.5% for *L. plantarum* isolates, they were grouped into 4 clusters (**Fig 1a**). Clusters 2 and 4 contain the largest number of members (6 and 8 strains, respectively), while clusters 1 and 3 contain 2 and 3 members respectively. All the clusters included members which showed a 100% of similarity among them.

For *O. oeni* isolates, and considering an arbitrary percentage similarity of 86.5%, they were grouped into 4 clusters **(Fig 1b)**. Cluster 2 has the largest number of members (7) and three pairs of them have a 100% of similarity. Clusters 1 and 3 have 4 members, while cluster 4 contains 3 members.

The 19 biotypes discriminated from *L. plantarum* isolates and the 18 from *O. oeni* isolates, contained in a single wine, suggest a notable intraspecific diversity for both LAB species. These results are in agreement with other reports which indicated a rich biodiversity of *L. plantarum* strains of oenological origin, even higher than *O. oeni* (López et al. 2008, Testa et al. 2014; Berbegal et al. 2016). On the other hand, different RAPD patterns of *O. oeni* isolates were often recovered in most of wine samples analyzed by Solieri et al. (2010). Similar results were reported using Pulsed-Field Gel Electrophoresis (PFGE), confirming that several strains can occur in a single spontaneous MLF (Ruiz et al. 2008, González-Arenzana et al. 2012, Solieri et al. 2010).

3.1. PCR detection of genes encoding enzymes of oenological interest

One strain of each cluster of *O. oeni* (UNQOe 6, UNQOe 17, UNQOe 31b, UNQOe 24b) and *L. plantarum* (UNQLp 11, UNQLp 12a, UNQLp 22, UNQLp 27) were chosen to investigate the presence of genes coding aroma-related enzymes and their ability to

conduct MLF in laboratory conditions. This selection was made according to the isolates ability to grow in MLO or MRS broth supplemented with 10% (v/v) ethanol (Bravo-Ferrada et al., 2013, 2016) in order to reduce the number of isolates used for vinification assays, considering that a best adaptation to ethanol is an important factor to survive in the wine harsh conditions. In order to confirm the presumptive identification of these strains, the *16S rRNA* gene was sequenced (GenBank Accession Numbers KU693340, KU693341, KU985242, KU985241, KU693338, KU693339, KU985239 and KU985240 for strains UNQLp 11, UNQLp 22, UNQLp 27, UNQLp 12a, UNQOe 6, UNQOe 31b, UNQOe 17 and UNQOe 24, respectively) confirming the previous identification. The absence of genes involved in biogenic amines synthesis was tested, and none of the eight strains showed the presence of these genes (data not shown). The inability to produce biogenic amines is an important characteristic for any strain to be used as starter culture, since these compounds have a negative impact on wine wholesomeness (Lerm et al. 2011, Mtshali et al. 2010).

The presence of genes coding β-glucosidase, phenolic acid decarboxylase (PAD), citrate lyase and proline aminopeptidase (PAP) enzymes were also screened. Although the presence of these genes does not guarantee its expression during a vinification process, the PCR screening allows knowing which strains have the potential to synthesizing enzymes related to aroma production in wine. The screening of aroma-related genes is showed in **Table 1**. *L. plantarum* strains showed the higher presence of these genes, being UNQLp 11 and UNQLp 22 positive for the four genes studied. In contrast, UNQOe 31b and UNQOe 6 were positive for two of them, citrate lyase and beta-glucosidase genes. These results are in agreement with the genetic screening of aroma-related enzymes accomplished from South African *O. oeni* and *L. plantarum* strains (Lerm et al. 2011), and with the enzyme activities detected in other Patagonian *O. oeni*

292 and *L. plantarum* strains (Bravo-Ferrada et al. 2013, 2016). Also, Mtshali et al. (2010)
293 reported that some South African *L. plantarum* strains were positive for β-glucosidase
294 (40.4%), citrate lyase (72.5%), and PAD (85.7%). Based on the results obtained, two
295 strains of *L. plantarum* (UNQLp 11 and UNQLp 22) and two of *O. oeni* (UNQOe 6 and
296 UNQOe 31b) were selected to screen them in sterile wine with a high ethanol content
297 (14.5% v/v).
298
299 ***3.2. Vinification assays in sterile Pinot noir wine***
300 The effect of pre-acclimation treatment on ethanol tolerance of MLF starter cultures is
301 widely reported (Cecconi et al. 2009; Solieri et al. 2010; Lerm et al. 2010). In addition,
302 in previous works we reported the positive effect of acclimation of Patagonian *L.*
303 *plantarum* and *O. oeni* strains exposed to wine-like medium (Bravo-Ferrada et al. 2014,
304 2015a, 2015b, 2015c, 2016). However, the effects of ethanol concentration in the
305 acclimation medium and inoculum size on the MLF of sterile Patagonian wine, has not
306 been reported yet.
307 Vinification assays, at laboratory scale, were performed in a sterile Pinot red wine
308 (14.5% v/v ethanol) in order to compare the implantation ability and the MAC kinetic of
309 *L. plantarum* and *O. oeni* strains. **Fig 2 and 3** shows the evolution of viable cell number
310 and MAC of *L. plantarum* (UNQLp 11 and UNQLp 22) and *O. oeni* (UNQOe 6 and
311 UNQOe 31b) cultures, respectively, inoculated at a concentration of ≅ 5.10^7 CFU/mL,
312 and incubated during 20 days at 21 ºC. Cultures were previously acclimated in the
313 presence of 6% (v/v) ethanol at 21°C for 48 h or non-acclimated. The number of viable
314 cells of *L. plantarum* strains (UNQLp 11 and UNQLp 22) remained relatively constant
315 along the MLF, except for the non-acclimated culture of UNQLp 22, which decay 3 log
316 after 20 days of incubation (**Fig 2A, 2 B**), indicating that acclimation improves the

317 survival of this strain in the wine environment. MLF was carried out successfully for
318 both strains and no significant differences were observed in MAC between acclimated
319 or non-acclimated cultures (Table 2). MAC in all conditions was higher than 85% after
320 20 days of incubation (Table 2).
321 In contrast, when the sterile wine was inoculated with *O. oeni* cultures, at the same
322 conditions than *L. plantarum* cultures, a drastic decrease of cell viability of both strains
323 was observed after 5 days (**Fig 3 A, 3 B)**. At this time, acclimated cultures started to
324 grow, being the MAC value lower than 40% after 20 days of incubation.
325 With the aim to improve the performance of *O. oeni* cultures and considering the
326 inoculum size reported by du Toit et al. (2011) for *O. oeni* strains ($\geq 1.10^8$ CFU/mL),
327 this bacterial concentration was proved (**Fig 4**). In this condition, an improvement in the
328 viability of both *O. oeni* strains was observed, being better the behavior of acclimated
329 cultures **(Fig 4 A, 4 B)**. In addition, the positive effect of acclimation became more
330 evident in the MAC values for both *O. oeni* strains (**Fig 4 A, 4 B**). When the UNQOe 6
331 culture was acclimated, an increase in the MAC value from 48 to 82.50% was observed
332 after 20 days of incubation. In the case of UNQOe 31b culture, this value increased
333 from 81.13 to 99.98% when cells were acclimated. Comparing both *O. oeni* strains,
334 UNQOe 31b seems more efficient as malolactic starter culture than UNQOe 6.
335 Furthermore, acclimated culture of UNQOe 31b (using an inoculum size $\cong 1.10^8$
336 CFU/mL) (**Fig 4**) showed a similar efficiency to consume malic acid than both *L.*
337 *plantarum* strains tested (**Fig 2, Fig 4, Table 2**).
338 Taking into account the drastic differences observed **(Fig 4)** between acclimated and
339 non-acclimated *O. oeni* cultures, and previous results regarding the ethanol
340 concentration during the acclimation treatment (Bravo-Ferrada et al. 2014, 2015a,
341 2015c, 2016), the effect of different sizes of inoculum (1.10^8, 1.10^9 and 1.10^{10} CFU/mL)

342 and ethanol concentrations (6% or 10% v/v) during acclimation, were also studied. **Fig**
343 **5 (A, B, C)** show the viability of UNQOe 31b cultures, acclimated at different ethanol
344 concentrations or non-acclimated. The viability of non-acclimated cells decreases after
345 inoculation in sterile wine and after 5 days remains relatively constant, except for the
346 smaller inoculum tested (Fig 5A), where the cell population falls up to 10 day. The
347 acclimated cultures reach higher cell populations than non acclimated ones after 20
348 days. The MAC kinetic of these cultures (**Fig 6 A, B, C**) was faster at higher inoculum
349 size, as expected. For the three bacterial concentrations inoculated, the MAC value was
350 higher than 95% after 20 days of incubation, only when cultures were acclimated. In
351 addition, the increase of ethanol concentration in the acclimation medium improved the
352 MAC values.
353 The four selected strains were able to tolerate the high ethanol concentration of the
354 Patagonian Pinot noir wine employed in vinification assays (14.5% v/v ethanol). In
355 addition, the survival exhibited by these strains was better than the previously reported
356 for other Patagonian *O. oeni* and *L. plantarum* strains inoculated in a synthetic wine
357 (Bravo-Ferrada et al. 2014, 2016). Although the strains previously analyzed were
358 different, and the phenotypic traits are strain-dependent, it is probable that some wine
359 compounds, such as phenolic acids, have a protective action on bacterial strains and
360 support MLF (Reguant et al. 2000).
361 Vinification assays showed that acclimation in low ethanol concentrations improves the
362 viability and the L-malic acid consumption of Patagonian *O. oeni* strains when the
363 inoculant size was higher than 1.10^8 CFU/mL. The data displayed in **Fig 6** showed that,
364 although the MAC was successful in all acclimation conditions tested, the MAC
365 kinetics were affected by the ethanol concentration in the acclimation medium, being
366 faster for higher ethanol content. In contrast, for *L. plantarum* strains assayed, the pre-

acclimation treatment was not relevant for the viability and MAC in the sterile Pinot noir wine. Furthermore, MLF was successfully with smaller inoculum sizes than *O. oeni* strains. The shortest incubation time and the better viability conditions make *L. plantarum* strains an economic alternative to produce malolactic starter cultures with potential application in Patagonian red wines.

4. Conclusion

In this study, *L. plantarum* and *O. oeni* strains obtained from Patagonian Pinot noir wine were selected, based on the presence of aroma-related genes, and successfully adapted to survive and conduct MLF in a sterile Pinot noir wine with high ethanol content (> 14% v/v). Although *O. oeni* has been reported as the main LAB starter culture for MLF, the results obtained in this work show that some *L. plantarum* strains have some potential advantages, such as the presence of more flavor-relative genes, a higher ability to consume malic acid with a smaller inoculum size and without pre-acclimation treatment, with the consequent economic advantages on the production of the indigenous starter cultures. More studies at medium and high scales and the effect of storage conditions are necessary to determine the effectiveness of these strains.

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558
559

560 ***Captions to Figures***

561 **Fig 1** Dendrogram based on the UPGMA clustering of Coc-RAPD patterns of *L.*
562 *plantarum*(a) and *O. oeni*(b) strains from spontaneous MLF of a Patagonian Pinot noir
563 wine

564

565 **Fig 2** Cultivability and malic acid consumption after inoculation of *L. plantarum*
566 UNQLp11 or UNQLp 22) in sterile Pinot noir wine with an inoculums ≅ 5.10^7
567 CFU/mL. Cultures were previously acclimated, or not, in the presence of ethanol 6%
568 (v/v) at 21ºC. Solid symbols denotes the Log CFU/mL for non-acclimated (▲) or
569 acclimated cultures (■). Open symbols represents the malic acid concentration for non-
570 acclimated (△) or acclimated cultures (□). Dashed line represents the fit according
571 exponential one-phase decay.

572

573 **Fig 3** Cultivability and malic acid consumption after inoculation of *O. oeni* strains
574 (UNQOe 6 or UNQOe 31), in sterile Pinot noir wine with an inoculum ≅ 5.10^7
575 CFU/mL. Cultures were previously acclimated, or not, in the presence of ethanol 6%
576 (v/v) at 21ºC. Solid symbols denotes the Log CFU/mL for non-acclimated (▲) or
577 acclimated cultures (■). Open symbols represents the malic acid concentration for non-
578 acclimated (△) or acclimated cultures (□). Dashed line represents the fit according
579 exponential one-phase decay.

580

581 **Fig 4** Cultivability and malic acid consumption after inoculation of *O. oeni* strains
582 (UNQOe 6 or UNQOe 31), in sterile Pinot noir wine with an inoculum ≅ 1.10^8
583 CFU/mL. Cultures were previously acclimated, or not, in the presence of ethanol 6%
584 (v/v) at 21ºC. Solid symbols denotes the Log CFU/mL for non-acclimated (▲) or
585 acclimated cultures (■). Open symbols represents the malic acid concentration for non-

586 acclimated (△) or acclimated cultures (□). Dashed line represents the fit according
587 exponential one-phase decay.
588
589 **Fig 5** Cultivability after inoculation of *O. oeni* strain UNQOe 31b in sterile Pinot noir
590 wine with different size inoculums (A) 1.10^8 CFU/mL, (B) 1.10^9 CFU/mL and (C)
591 1.10^{10} CFU/mL. Cultures were previously acclimated in the presence of ethanol 6 %
592 (v/v) at 21 °C (▼), ethanol 10 % (v/v) at 21 °C (●),or non-acclimated (■).
593
594 **Fig 6** Malic acid consumption after inoculation of *O. oeni* strain UNQOe 31b in sterile
595 Pinot noir wine with different size inoculums (A) 1.10^8 CFU/mL, (B) 1.10^9 CFU/mL
596 and (C) 1.10^{10} CFU/mL. Cultures were previously acclimated in the presence of ethanol
597 6 % (v/v) at 21 °C (▽), ethanol 10 % (v/v) at 21 °C (○),or non-acclimated (□). Dashed
598 line represents the fit according exponential one-phase decay.
599
600

Table 1. Presence (+) or absence (-) of genes coding enzymes of the *L. plantarum* and *O. oeni* strains.

Strain	PAD	β-glucosidase	Citrate-lyase	PAP
UNQLp 11	+	+	+	+
UNQLp 22	+	+	+	+
UNQLp 27	+	+	-	+
UNQLp 12a	+	-	+	+
UNQOe 24b	-	-	+	-
UNQOe 31b	-	+	+	-
UNQOe 6	-	+	+	-
UNQOe 17	-	-	+	-

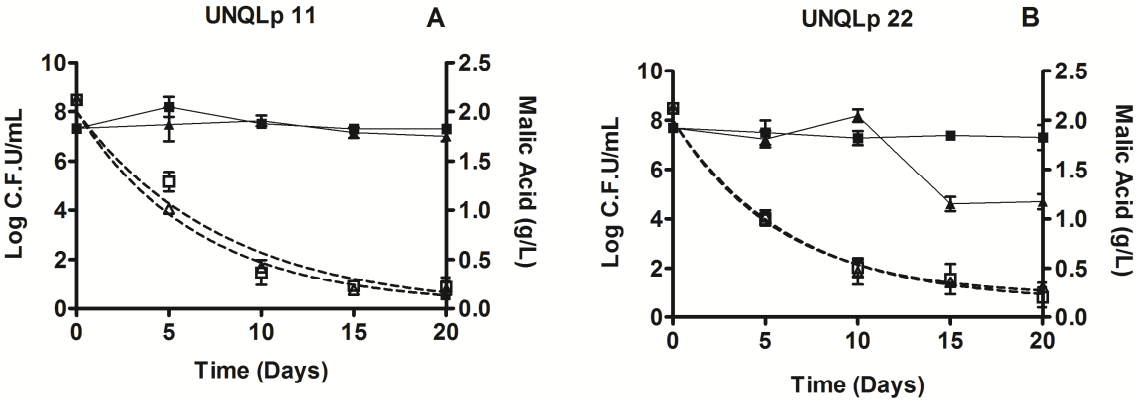
PAD: phenolicaciddecarboxylase

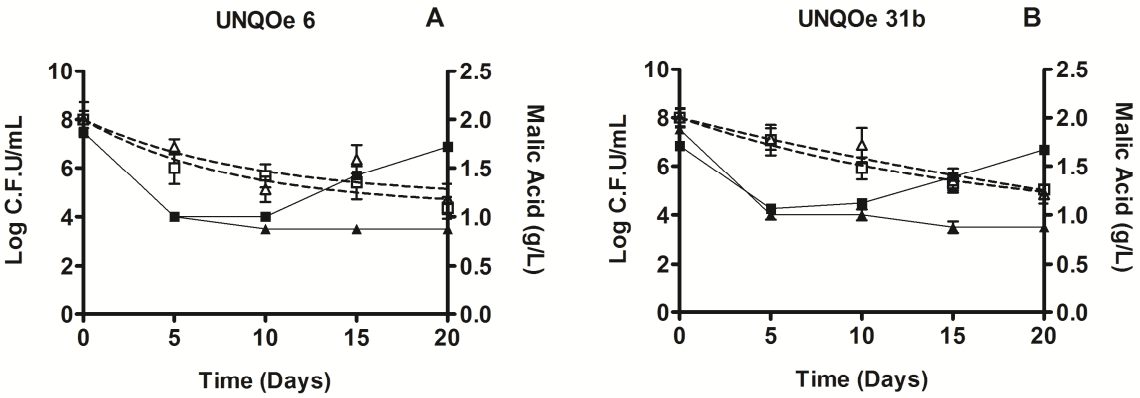
PAP: Prolineaminopeptidase

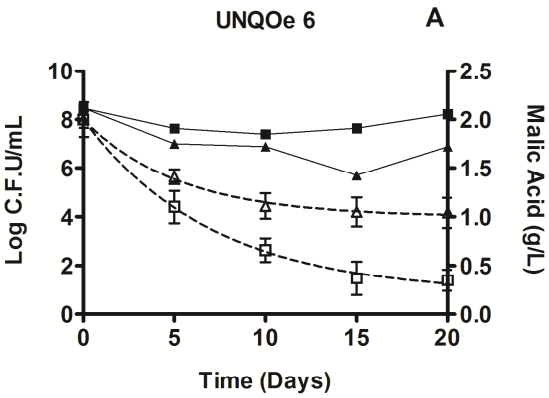
Table 2: Number of viable cells, of *L. plantarum* and *O. oeni* strains, before and after 20 days of inoculation in Pinot noir wine and kinetics parameter of malic acid consumption (equation 1) obtained from **Fig 2** and **Fig 4.**

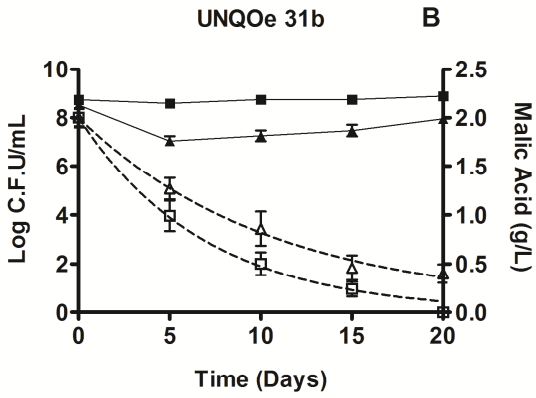
	UNQLp 11		**UNQLp 22**		**UNQOe 6**		**UNQOe 31b**	
	Non Accl	Accl	Non Accl	Accl	Non Accl	Accl	Non Accl	Accl
N_0 (CFU/mL)	2.00E+07	2.00E+07	5.01E+07	5.01E+07	3.16E+08	3.16E+08	3.16E+08	5.01E+08
Log N/N_0	-0.30	-0.02	-3.00	-0.30	-1.59	-0.24	-0.57	0.16
MAC (%)	91.04 ± 3.12^{ab}	89.62 ± 2.69^{ab}	85.84 ± 1.68^{ab}	90.57 ± 4.33^{ab}	48.00 ± 4.38^{c}	82.50 ± 3.50^{ab}	78.50 ± 3.75^{b}	99.98 ± 2.27^{a}
K	0.15 ± 0.03	0.13 ± 0.05	0.17 ± 0.03	0.16 ± 0.03	0.19 ± 0.02	0.14 ± 0.01	0.10 ± 0.01	0.14 ± 0.02
R^2	0.9905	0.9600	0.9896	0.9916	0.9963	0.9977	0.9948	0.9946
[MA*i*]	0.04	0.00	0.21	0.16	0.99	0.21	0.08	0.00

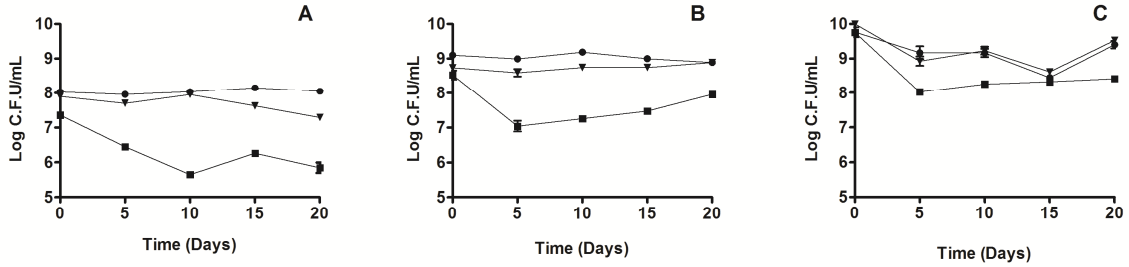
N_0: initial number of viable cells at time = 0
Log N/N_0: Change in the number of viable cells after 20 days of incubation
MAC (%): Percentage of malic acid consume after 20 days of incubation.
K: constant of first order exponential decay
R^2: coefficient of determination.
[MA*i*]: Minimum malic acid concentration (time = infinite)
Different letters (a, b and c) denote statistically significant difference ($P < 0.05$).

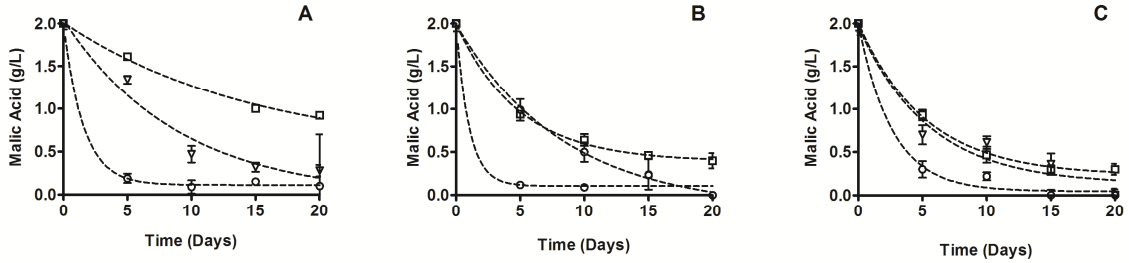


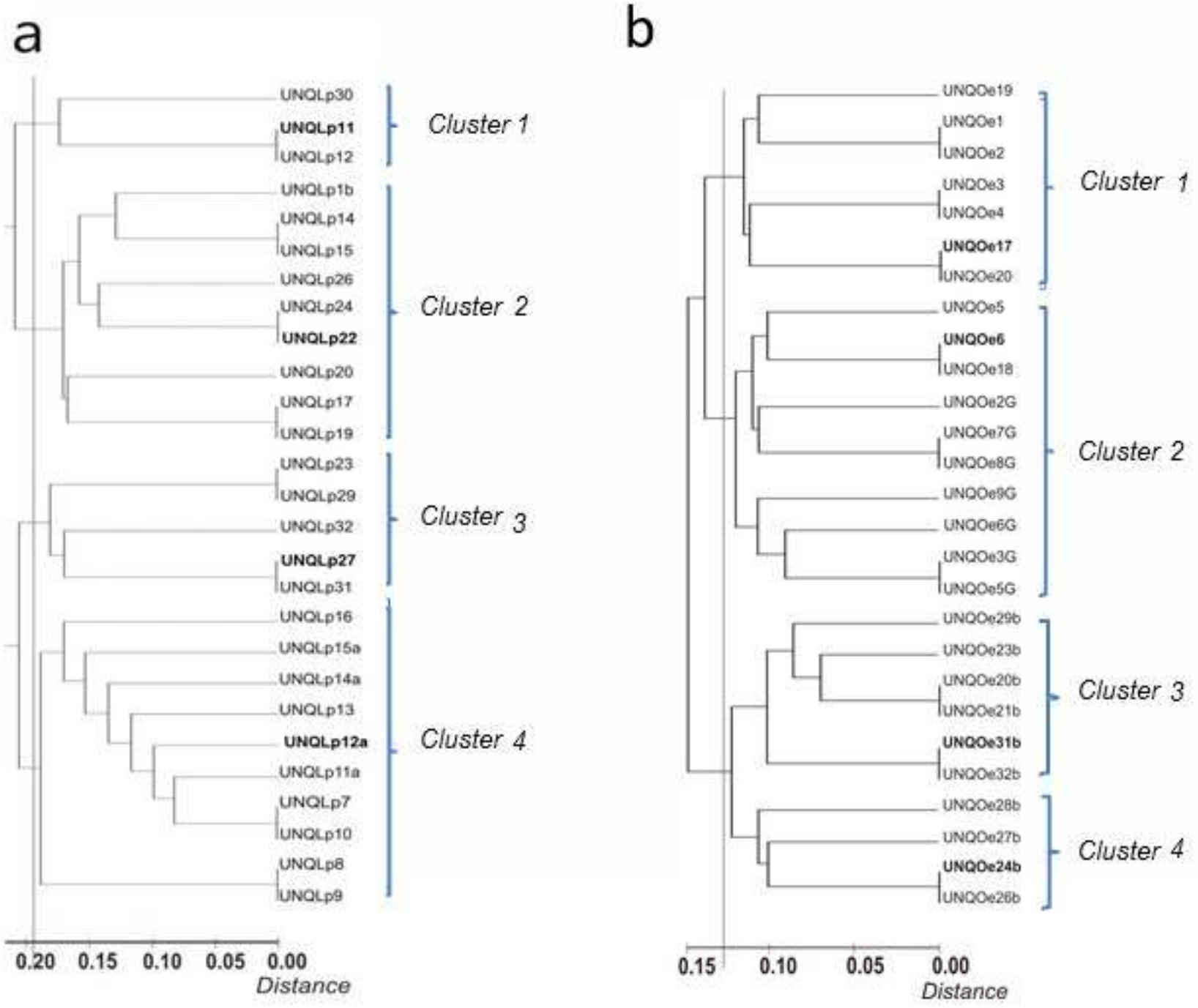











a
UNQLp30
UNQLp11
UNQLp12
UNQLp1b
UNQLp14
UNQLp15
UNQLp26
UNQLp24
UNQLp22
UNQLp20
UNQLp17
UNQLp19
UNQLp23
UNQLp29
UNQLp32
UNQLp27
UNQLp31
UNQLp16
UNQLp15a
UNQLp14a
UNQLp13
UNQLp12a
UNQLp11a
UNQLp7
UNQLp10
UNQLp8
UNQLp9
Cluster 1
Cluster 2
Cluster 3
Cluster 4
0.20 0.15 0.10 0.05 0.00
Distance
b
UNQOe19
UNQOe1
UNQOe2
UNQOe3
UNQOe4
UNQOe17
UNQOe20
UNQOe5
UNQOe6
UNQOe18
UNQOe2G
UNQOe7G
UNQOe8G
UNQOe9G
UNQOe6G
UNQOe3G
UNQOe5G
UNQOe29b
UNQOe23b
UNQOe20b
UNQOe21b
UNQOe31b
UNQOe32b
UNQOe28b
UNQOe27b
UNQOe24b
UNQOe26b
Cluster 1
Cluster 2
Cluster 3
Cluster 4
0.15 0.10 0.05 0.00
Distance

Highlights

► *L. plantarum* and *O. oeni* strains were isolated and fermentation examined ►*L. plantarum* was more efficient at MLF than *O. oeni* in Patagonian wines ► Standardization of acclimation and inoculum was necessary for successful MLF by *O. oeni* ► Acclimation in high ethanol content improved the malic acid consumption of *O. oeni* ► MLF with *L. plantarum* strains required lower inocula and no pre-acclimation treatment