Accepted Manuscript

Comparative vinification assays with selected Patagonian strains of *Oenococcus oeni* and *Lactobacillus plantarum*

Natalia S. Brizuela, Bárbara M. Bravo-Ferrada, Danay Valdés La Hens, Axel Hollmann, Lucrecia Delfederico, Adriana Caballero, E. Elizabeth Tymczyszyn, Liliana Semorile

PII: S0023-6438(16)30694-6

DOI: 10.1016/j.lwt.2016.11.023

Reference: YFSTL 5832

To appear in: *LWT - Food Science and Technology*

Received Date: 25 August 2016

Revised Date: 24 October 2016

Accepted Date: 10 November 2016

Please cite this article as: Brizuela, N.S., Bravo-Ferrada, B.M., La Hens, D.V., Hollmann, A., Delfederico, L., Caballero, A., Tymczyszyn, E.E., Semorile, L., Comparative vinification assays with selected Patagonian strains of *Oenococcus oeni* and *Lactobacillus plantarum*, *LWT - Food Science and Technology* (2016), doi: 10.1016/j.lwt.2016.11.023.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

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

Natalia S. Brizuela[1], Bárbara M. Bravo-Ferrada[1], Danay Valdés La Hens[1], Axel Hollmann[3], Lucrecia Delfederico[1], Adriana Caballero[2], E. Elizabeth Tymczyszyn[1]*, Liliana Semorile[1]

[1] Laboratorio de Microbiología Molecular, Instituto de Microbiología Básica y Aplicada (IMBA), Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Bernal, Argentina

[2] Facultad de Ciencia y Tecnología de los Alimentos, Universidad Nacional del Comahue and PROBIEN CONICET-Universidad Nacional del Comahue, Neuquén, Argentina.

[3] Laboratorio de Biointerfaces y Sistemas Biomiméticos – Universidad Nacional de Santiago del Estero (UNSE) y Centro de Investigaciones y Transferencia de Santiago del Estero (CITSE) – CONICET - UNSE, Santiago del Estero, Argentina

* Corresponding author: Elizabeth Tymczyszyn

Address: Roque Sáenz Peña Nº 352, (B1876BXD) Bernal, Buenos Aires, Argentina

Phone: +54- 11- 4365 7100 ext. 5636

Fax: +54- 11- 4365 7100

E-mail: elitym@yahoo.com.ar

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

25 **Abstract**

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

39

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

42

43 ***Highlights***

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

50

51

ACCEPTED MANUSCRIPT

52 ***Abbreviations***

53 LAB: Lactic Acid Bacteria

54 MLF: Malolactic Fermentation

55 PAP: Proline aminopeptidase

56 PAD: Phenolic acid decarboxylase

57 MAC: Malic acid consumption

58 AF: Alcoholic fermentations

59 16S-ARDRA: Amplified Ribosomal DNA Restriction Analysis

60 UPGMA: Unweighted Pair Group Method using Arithmetic Averages

61 *hdc*: histamine decarboxylase

62 *tdc*: tyramine decarboxylase

63 *ptc*A: putrescine carbamoyl transferase

64 PFGE: Pulsed-Field Gel Electrophoresis

65

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

66 **1. Introduction**

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

83 In Patagonian red wines MLF mainly occurs spontaneously and both species, *O. oeni*
84 and *L. plantarum*, have been found in different vintages and in all stages of MLF of
85 Pinot noir and Merlot wines (Valdés La Hens et al. 2015, Bravo-Ferrada et al. 2013).
86 Several strains of these LAB species were previously isolated and studied in order to
87 know their oenological and technological properties, particularly their capacity to grow
88 and consume malic acid under the harsh wine environment (Bravo-Ferrada et al. 2013,
89 2014, 2015a, 2015b, 2016). Stress factors as high ethanol concentration, low pH,
90 presence of sulfite are the main cause of death of inoculated cultures (Bravo-Ferrada et

91 al. 2016). However, it has been shown that bacteria are able to grow under these
92 conditions if cultures are pre-adapted to wine environment (Maicas et al. 2000, Cecconi
93 et al. 2009). In previous works we found that some *L. plantarum* and *O. oeni* strains can
94 grow in wine-like media when they were previously acclimated in a rich medium
95 containing a high fructose and glucose concentration and a low ethanol concentration (6
96 or 10% v/v) (Bravo-Ferrada et al. 2014, 2016). In addition, the acclimated cultures
97 showed an improved capacity to consume malic acid. This adaptation was related to a
98 change in the composition and properties of the bacterial membranes, such as a
99 modification in fatty acid composition and protein profile (Bravo-Ferrada et al. 2014,
100 2015a, Chu-ky et al. 2005, da Silveira et al. 2004). However, the effect of acclimation
101 of selected Patagonian *L. plantarum* and *O. oeni* strains on growing capacity and
102 malolactic activity in sterile red wine with high ethanol concentration (≥ 14% v/v) have
103 not been reported yet.
104 On the other hand, LAB strains can positively alter the chemical composition of wine
105 through metabolism of flavor precursor compounds as a result of bacterial enzyme
106 activities such as citrate lyase, beta-glucosidase, proline aminopeptidase (PAP) and
107 phenolic acid decarboxylase (PAD) (Mtshali et al. 2010). The citrate lyase activity is
108 related with the production of diacetyl; the enzyme cleaves citrate molecules into
109 oxalacetate and acetate (Bekal et al. 1998). PAP is one of enzymes with protease
110 activity that contributes to development of flavor by releasing free amino acids that are
111 precursors of aroma compounds (Matos et al. 1998). PAD metabolize phenolic acids
112 present in must and wine (Cavin et al. 1993, Swiegers et al. 2005) and beta-glucosidase
113 releases different aroma-compounds by cleavage of glycosidic bonds, transforming
114 terpenes, alcohols, fatty acids, etc. from bound to free forms (Grimaldi et al. 2000,
115 Spano et al. 2005).

116 These enzyme activities could be explored by quantification of diverse metabolites *in*
117 *vitro*. However, the screening of the genes coding these enzymes is an easier method to
118 select strains that could have better oenological properties (Lerm et al. 2011, Olguin et
119 al. 2010, Mtshali et al. 2010, Spano et al. 2005).
120 With this background, the aim of this work was to select and compare native Patagonian
121 strains of *L. plantarum* and *O. oeni* by the presence of different aroma-related genes and
122 studying the effect of pre-acclimation treatment, inoculum sizes, implantation capacity,
123 and kinetics of malic acid consumption (MAC) in sterile Pinot noir wine, in order to
124 understand its behavior as MLF starter cultures candidates for Patagonian red wines.
125

126 **2. Materials and Methods**

127 ***2.1. Bacterial isolates and growth conditions***

128 *O. oeni* and *L. plantarum* isolates were obtained from Pinot noir wine samples from a
129 2012 vintage, in which alcoholic fermentation (AF) and MLF were spontaneous. At the
130 end of MLF the wine had the following values: pH 3.75, 14.3% (v/v) ethanol, L-malic
131 acid 0.5 g/L.
132 Samples were aseptically collected from a commercial cellar in General Roca,
133 Argentinean North Patagonia, and inoculated in MLO (Maicas et al. 1999) and MRS
134 (Biokar Diagnostic, Beauvais, France) (De Man et al. 1960) plates supplemented with
135 cycloheximide 100 mg/L, under anaerobic conditions (AnaeroPack – Mitsubishi Gas
136 Chemical America, Inc., New York, NY), at 28 °C, during 7 days or 48 h, respectively.
137 Isolates identified as *O. oeni* or *L. plantarum* were grown in MLO or MRS broth,
138 respectively. Cultures were kept frozen at -20 °C in the corresponding broth
139 supplemented with glycerol (30% v/v).
140

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

141 ***2.2. Identification of isolates***

142 DNA extraction from bacterial isolates was performed according to Bravo Ferrada et al.
143 (2011). DNA samples were quantified using a Nanodrop spectrophotometer (Thermo
144 Scientific, 1000) and visualized on a 1.0% (w/v) agarose gel.
145 Isolates were identified by 16S-ARDRA (Amplified Ribosomal DNA Restriction
146 Analysis), using the primers pA and pH to amplify *16S rRNA* gene (Ulrike et al. 1989).
147 Restrictions with *Mse*I enzyme were carried out according to Rodas et al. (2003). *L.*
148 *plantarum* ATCC 14917 and *Oenococcus oeni* ATCC 27310 were used as reference
149 strains. To confirm the identity of the selected *L. plantarum* and *O. oeni* strains, the *16S*
150 *rRNA* gene was sequenced. Amplification of this gene was performed according to
151 Delfederico et al. (2006). Sequences were obtained by using universal primers T7 and
152 SP6 by means of DNA automatic sequencer (Macrogen Korea).
153

154 ***2.3. Typing of isolates by RAPD-PCR analysis***

155 *O. oeni* and *L. plantarum* isolates were typed by RAPD-PCR analysis using primer Coc
156 (Cocconcelli et al. 1995). Amplification reactions were performed as described
157 Delfederico et al. (2006), and products were analyzed by electrophoresis in 1.5% (w/v)
158 agarose gels. The evaluation of PCR profiles was made by calculation of genetic
159 similarity index using a simple matching coefficient (Apostol et al. 1993). Unweighted
160 Pair Group Method using Arithmetic Averages (UPGMA) cluster analysis was carried
161 out by using PAUP* 4.0b10 (Sinauer Associates, MS, USA).
162

163 ***2.4. Screening of genes that encode for biogenic amines***

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

ACCEPTED MANUSCRIPT

166 pustrescine, respectively, were screened in the potential MLF starter culture strains
167 according to Bravo-Ferrada et al. (2013).
168

169 ***2.5. Detection of β-glucosidase, phenolic acid decarboxilase, proline aminopeptidase***
170 ***and β subunit-citrate lyase genes***

171 LAB strains were analyzed for the presence of genes coding β-glucosidase, phenolic
172 acid decarboxilase, proline aminopeptidase and β subunit-citrate lyase. The primers and
173 reaction conditions used to amplify each gene are listed in **Supplementary 1.** PCR
174 products were resolved by electrophoresis in 1.5% (w/v) agarose gel. Estimation of
175 fragment lengths was done by comparison to a 100-bp ladder marker as size standard
176 (Productos Bio-Lógicos, Argentina).
177

178 ***2.6. Cell acclimation***

179 Bacterial cells in the early stationary phase (approximately 10^9 CFU/ml) were harvested
180 by centrifugation at 5000 x g for 10 min and suspended in the same volume of a
181 modified acclimation medium (50 g/l MRS, 40 g/l D(-) fructose, 20 g/l D (-) glucose, 4
182 g/l L-malate, 1 g/l Tween 80, and 0.1mg/l pyridoxine, pH 4.6) (Lerm et al. 2011)
183 supplemented with 6% or 10% (v/v) ethanol **(**Bravo-Ferrada et al. 2014). Culture
184 incubations were carried out at 21 °C for 48 h.
185

186 ***2.7. Vinification assays***

187 Two strains of each LAB species (UNQLp 11, UNQLp 22, UNQOe3 1 and UNQOe 6)
188 were selected for carrying out vinification assays, at laboratory scale, in sterile Pinot
189 noir wine at final stage of alcoholic fermentation (AF). A volume of 100 mL of wine
190 (14.5% v/v ethanol, pH 3.82, < 2.00 g/L residual sugars, 2 g/L malic acid, 96 mg/L total

ACCEPTED MANUSCRIPT

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 $$[MA_t] = ([MA_0] - [MA_i])\, e^{-Kt} + [MA_i] \qquad \textbf{(Equation 1)}$$

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 $$MAC\ \% = 100 - ([MA_f]\ 100 / [MA_0]) \qquad \textbf{(Equation 2)}$$

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

ACCEPTED MANUSCRIPT

216 ***2.9. Reproducibility of the results***

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

221

222 **3. Results and discussion**

223 In previous works we showed that *L. plantarum* and *O. oeni* are the main LAB species
224 involved in conducting spontaneous MLF of Patagonian Pinot noir and Merlot wines
225 (Valdés La Hens et al. 2015, Bravo-Ferrada et al. 2013). It is now widely accepted that
226 use of autochthonous LAB strains as starter cultures, best adapted to the conditions of a
227 specific wine-producing area, has the potential to retain the *terroir* characteristics of
228 wine (Carreté et al. 2006; Ruiz et al. 2010, Bokulich et al. 2014; Garofalo et al. 2015).
229 With the aim to enrich our collection of Patagonian oenological LAB strains, *L.*
230 *plantarum* and *O. oeni* were surveyed from a Pinot noir wine, 2012 vintage, suffering
231 spontaneous MLF, and a genetic screening of aroma-related enzymes was performed
232 with the aim to select those with possible effect on sensorial quality of wine.
233 A total of sixty isolates were identified as LAB from MRS culture, and thirty from
234 MLO culture, by morphology, Gram positive staining and catalase negative reaction.
235 Twenty-seven isolates were presumptively identified as belonging to *L. plantarum*
236 species and thirty isolates as *O. oeni* by 16S-ARDRA (Rodas et al. 2003). The other
237 LAB species identified were *Lactobacillus brevis* and *Pediococcus acidilactici* (data not
238 shown). After this presumptive identification, a clustering analysis was performed
239 because a pool of different genotypes reduces the number of isolates to be studied and
240 simplifies the evaluation of the implantation capacity and malic acid consumption. For
241 this purpose, RAPD-PCR profiles with Coc primer were obtained from 30 *O. oeni* and

242 27 *L. plantarum* isolates. The clonal relationship between each single genomic
243 fingerprinting of the *L. plantarum* and *O. oeni* isolates is shown in the UPGMA
244 dendogram of **Fig 1**. Considering an arbitrary percentage similarity of 80.5% for *L.*
245 *plantarum* isolates, they were grouped into 4 clusters (**Fig 1a**). Clusters 2 and 4 contain
246 the largest number of members (6 and 8 strains, respectively), while clusters 1 and 3
247 contain 2 and 3 members respectively. All the clusters included members which showed
248 a 100% of similarity among them.
249 For *O. oeni* isolates, and considering an arbitrary percentage similarity of 86.5%, they
250 were grouped into 4 clusters **(Fig 1b)**. Cluster 2 has the largest number of members (7)
251 and three pairs of them have a 100% of similarity. Clusters 1 and 3 have 4 members,
252 while cluster 4 contains 3 members.
253 The 19 biotypes discriminated from *L. plantarum* isolates and the 18 from *O. oeni*
254 isolates, contained in a single wine, suggest a notable intraspecific diversity for both
255 LAB species. These results are in agreement with other reports which indicated a rich
256 biodiversity of *L. plantarum* strains of oenological origin, even higher than *O. oeni*
257 (López et al. 2008, Testa et al. 2014; Berbegal et al. 2016). On the other hand, different
258 RAPD patterns of *O. oeni* isolates were often recovered in most of wine samples
259 analyzed by Solieri et al. (2010). Similar results were reported using Pulsed-Field Gel
260 Electrophoresis (PFGE), confirming that several strains can occur in a single
261 spontaneous MLF (Ruiz et al. 2008, González-Arenzana et al. 2012, Solieri et al. 2010).
262

263 ***3.1. PCR detection of genes encoding enzymes of oenological interest***

264 One strain of each cluster of *O. oeni* (UNQOe 6, UNQOe 17, UNQOe 31b, UNQOe
265 24b) and *L. plantarum* (UNQLp 11, UNQLp 12a, UNQLp 22, UNQLp 27) were chosen
266 to investigate the presence of genes coding aroma-related enzymes and their ability to

ACCEPTED MANUSCRIPT

267 conduct MLF in laboratory conditions. This selection was made according to the
268 isolates ability to grow in MLO or MRS broth supplemented with 10% (v/v) ethanol
269 (Bravo-Ferrada et al., 2013, 2016) in order to reduce the number of isolates used for
270 vinification assays, considering that a best adaptation to ethanol is an important factor to
271 survive in the wine harsh conditions. In order to confirm the presumptive identification
272 of these strains, the *16S rRNA* gene was sequenced (GenBank Accession Numbers
273 KU693340, KU693341, KU985242, KU985241, KU693338, KU693339, KU985239
274 and KU985240 for strains UNQLp 11, UNQLp 22, UNQLp 27, UNQLp 12a, UNQOe
275 6, UNQOe 31b, UNQOe 17 and UNQOe 24, respectively) confirming the previous
276 identification. The absence of genes involved in biogenic amines synthesis was tested,
277 and none of the eight strains showed the presence of these genes (data not shown). The
278 inability to produce biogenic amines is an important characteristic for any strain to be
279 used as starter culture, since these compounds have a negative impact on wine
280 wholesomeness (Lerm et al. 2011, Mtshali et al. 2010).
281 The presence of genes coding β-glucosidase, phenolic acid decarboxylase (PAD), citrate
282 lyase and proline aminopeptidase (PAP) enzymes were also screened. Although the
283 presence of these genes does not guarantee its expression during a vinification process,
284 the PCR screening allows knowing which strains have the potential to synthesizing
285 enzymes related to aroma production in wine. The screening of aroma-related genes is
286 showed in **Table 1**. *L. plantarum* strains showed the higher presence of these genes,
287 being UNQLp 11 and UNQLp 22 positive for the four genes studied. In contrast,
288 UNQOe 31b and UNQOe 6 were positive for two of them, citrate lyase and beta-
289 glucosidase genes. These results are in agreement with the genetic screening of aroma-
290 related enzymes accomplished from South African *O. oeni* and *L. plantarum* strains
291 (Lerm et al. 2011), and with the enzyme activities detected in other Patagonian *O. oeni*

ACCEPTED MANUSCRIPT

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

ACCEPTED MANUSCRIPT

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-

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

372

373 ## *4*. Conclusion

374 In this study, *L. plantarum* and *O. oeni* strains obtained from Patagonian Pinot noir wine
375 were selected, based on the presence of aroma-related genes, and successfully adapted
376 to survive and conduct MLF in a sterile Pinot noir wine with high ethanol content (>
377 14% v/v). Although *O. oeni* has been reported as the main LAB starter culture for MLF,
378 the results obtained in this work show that some *L. plantarum* strains have some
379 potential advantages, such as the presence of more flavor-relative genes, a higher ability
380 to consume malic acid with a smaller inoculum size and without pre-acclimation
381 treatment, with the consequent economic advantages on the production of the
382 indigenous starter cultures. More studies at medium and high scales and the effect of
383 storage conditions are necessary to determine the effectiveness of these strains.

384

385 ## 5. Acknowledgments

386 This work was funded by grants from Universidad Nacional de Quilmes (Programa
387 Microbiología Molecular Básica y Aplicaciones Biotecnológicas), Comisión de
388 Investigaciones Científicas de la Provincia de Buenos Aires (CIC-PBA), and ANPCyT
389 (PICTO UNQ 2006 Nº 36474, PICT SU 2012 Nº 2804, PICT 2013 Nº 1481, PICT 2014
390 N° 1395). NB is a fellow of Consejo Nacional de Investigaciones Científicas y Técnicas
391 (CONICET). LS is a member of the Research Career of CIC-BA; BMBF, AH and ET

ACCEPTED MANUSCRIPT

392 are members of the Research Career of CONICET. All authors have agreed to submit
393 this manuscript to the "LWT – Food Science and Technology”.

394

395 **6. References**

396 Apostol, B.L., Black, W.C.I.V., Miller, B.R., Reiter, P. & Beaty, B.J. (1993). Estimation of the number of
397 full sibling families at an oviposition site using RAPD – PCR markers: applications to the
398 mosquito *Aedes aegypti*. *Theoretical Applied Genetics,* 86, 991-1000.

399 Arena, M.E., de Nadra, M.C.M., & Muñoz, R. (2002). The arginine deiminase pathway in the wine lactic
400 acid bacterium Lactobacillus hilgardii X 1 B: structural and functional study of the arcABC genes.
401 *Gene,* 301(1), 61-66.

402 Bartowsky, E. J., & Henschke, P. A. (1999).Use of a polymerase chain reaction for specific detection of
403 the malolactic fermentation bacterium Oenococcus oeni (formerly Leuconostoc oenos) in grape
404 juice and wine samples. *Australian Journal of Grape and Wine Research*, 5(2), 39-44.

405 Beneduce, L., Romano, A., Capozzi, A., Lucas, P., Barnavon, L., Bach, B., Vuchot, P., Grieco, F. &
406 Spano, G. (2010). Biogenic amines in regional wines. *Annals of Microbiology,* 60, 573-578.

407 Berbegal, C., Peña, N., Russo, P., Grieco, F., Pardo,I.,Ferrer,S.,Spano, G., & Capozzi, V. (2016)
408 Technological properties of Lactobacillus plantarum strains isolated from Apulia wines. *Food*
409 *Microbiology*, 57, 187-194.

410 Bekal, S., Van Beeumen, J., Samyn, B., Garmyn, D., Henini, S., Diviès, C., & Prévost, H. (1998).
411 Purification of Leuconostoc mesenteroides Citrate Lyase and Cloning and Characterization of the
412 citCDEFG Gene Cluster. *Journal of Bacteriology,* 180, 647-654.

413 Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A. (2014). Microbial biogeography of wine
414 grapes is conditioned by cultivar, vintage, and climate. *Proceedings of the National Academy of*
415 *Sciences,* 111, 139-148.

416 Bordas, M., Araque, I., Bordons, A., & Reguant, C. (2015). Differential expression of selected
417 *Oenococcus oeni* genes for adaptation in wine-like media and red wine. *Annals of Microbiology,*
418 65, 2277-2285.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

419 Bravo-Ferrada, B.M., Delfederico. L., Hollmann, A., Valdés La Hens, D., Curilén, Y., Caballero, A. &
420 Semorile, L. (2011). *Oenococcus oeni* from Patagonian red wines: isolation, characterization and
421 technological properties. *International Journal of Microbiology Research,* 3, 48-55.

422 Bravo-Ferrada, B.M., Hollmann, A., Delfederico, L., Valdés La Hens, D., Caballero, A. & Semorile, L.
423 (2013). Patagonian red wines: selection of *Lactobacillus plantarum* starter cultures for malolactic
424 fermentation. *World Journal of Microbiology and Biotechnology,* 29, 1537-1549.

425 Bravo-Ferrada, B.M., Tymczyszyn, E.E., Gómez-Zavaglia, A. & Semorile, L. (2014).Effect of
426 acclimation medium on cell viability, membrane integrity and ability to consume malic acid in
427 synthetic wine by oenological *Lactobacillus plantarum* strains. *Journal of Applied Microbiology,*
428 116, 360-367.

429 Bravo-Ferrada, B.M., Gómez-Zavaglia, A., Semorile, L. & Tymczyszyn, E. (2015a). Effect of the fatty
430 acid composition of acclimated oenological *Lactobacillus plantarum* on the resistance to ethanol.
431 *Letter in Applied Microbiology*, 60, 155-161.

432 Bravo-Ferrada, B.M., Gonçalves, S., Semorile, L., Santos, N.C., Tymczyszyn, E.E. & Hollmann, A.
433 (2015b). Study of surface damage on cell envelope assessed by AFM and flow cytometry of
434 *Lactobacillus plantarum* exposed to ethanol and dehydration. *Journal of Applied Microbiology*,
435 118, 1409–1417.

436 Bravo-Ferrada, B.M., Brizuela, N., Gerbino, E., Gómez-Zavaglia, A., Semorile, L., Tymczyszyn, E.E.
437 (2015c). Effect of protective agents and previous acclimation on ethanol resistance of frozen and
438 freeze-dried *Lactobacillus plantarum* strains. *Cryobiology*, 71, 522-528.

439 Bravo-Ferrada, B.M., Hollmann, A., Brizuela, N., Valdés La Hens, D., Tymczyszyn, E.E. & Semorile, L.
440 (2016). Growth and consumption of L-malic acid in wine-like medium by acclimated and non-
441 acclimated cultures of Patagonian *Oenococcus oeni* strains. *Folia Microbiologica,* 61, 365–373.

442 Capozzi, V., Russo, P., Beneduce, L., Weidmann, S., Grieco, F., Guzzo, J. & Spano, G., (2010).
443 Technological properties of *Oenococcus oeni* strains isolated from typical southern Italian wines.
444 *Letter in Applied Microbiology*, 50, 327-334.

445 Carreté, R., Reguant, C., Rozés, N., Constantí, M. & Bordons, A. (2006). Analysis of *Oenococcus oeni*
446 strains in simulated microvinifications with some stress compounds. *American Journal of Enology*
447 *and Viticulture,* 57, 356-362.

ACCEPTED MANUSCRIPT

448 Caspritz, G., & Radler, F. (1983). Malolactic enzyme of *Lactobacillus plantarum*. Purification, properties,
449 and distribution among bacteria. *Journal of Biological Chemistry*, 258(8), 4907-4910.
450 Cavin, J.F., Andioc, V., Etievant, P.X., & Divies, C. (1993). Ability of wine lactic acid bacteria to
451 metabolize phenol carboxylic acids. *American Journal of Enology and Viticulture,* 44(1), 76-80.
452 Cecconi, D., Milli, A., Rinalducci, S., Zolla, L. & Zapparoli, G. (2009).Proteomic analysis of *Oenococcus*
453 *oeni* freeze-dried culture to assess the importance of cell acclimation to conduct malolactic
454 fermentation in wine. *Electrophoresis,* 30, 2988‑2995.
455 Chu-Ky, S., Tourdot-Marechal, R., Marechal, P.A., Guzzo, J. (2005). Combined cold, acid, ethanol
456 shocks in *Oenococcus oeni*: effects on membrane fluidity and cell viability. *Biochimica et*
457 *Biophysica Acta*, 1717, 118–124.
458 Cocconcelli, P.S., Porro. D., Galandini, S. & Senini, L. (1995). Development of RAPD protocol for
459 typing of strains of lactic acid bacteria and enterococci. *Applied Microbiology,* 21, 376-379.
460 Delfederico, L., Hollmann, A., Martínez, M., Iglesias, N.G., De Antoni, G. & Semorile, L. (2006).
461 Molecular identification and typing of lactobacilli isolated from kefir grains. *Journal of Dairy*
462 *Research*, 73, 20–27.
463 De Man, J. C., Rogosa, D., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal*
464 *of Applied Bacteriology*, 23(1), 130-135.
465 Du Toit, M., Engelbrecht, L., Lerm, E., & Krieger-Weber, S. (2011). Lactobacillus: the next generation of
466 malolactic fermentation starter cultures—an overview. *Food and Bioprocess Technology,* 4(6),
467 876-906.
468 Garofalo, C., El Khoury, M., Lucas, P., Bely, M., Russo, P., Spano, G. & Capozzi, V. (2015).
469 Autochthonous starter cultures and indigenous grape variety for regional wine production. *Journal*
470 *of Applied lMicrobiology*, 118, 1395-1408.
471 G-Alegría, E., López, I., Ignacio Ruiz, J., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C. &
472 Ruiz-Larrea, F. (2004). High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni*
473 strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS*
474 *Microbiology Letters*, 230, 53-61.
475 Grimaldi, A., McLean, H., & Jiranek, V. (2000). Identification and partial characterization of glycosidic
476 activities of commercial strains of the lactic acid bacteria *Oenococcus oeni*. *American Journal of*
477 *Enology and Viticulture*, 51, 362-369.

478 González-Arenzana, L., López, R., Santamaría, P., Tenorio, C., López-Alfaro, I. (2012). Dynamics of
479 indigenous lactic acid bacteria populations in wine fermentations from la Rioja (Spain) during
480 three vintages. *Microbial Ecology,* 63, 12-19.
481 Joosten, H.M.L.J., & Northolt, M.D. (1989). Detection, growth, and amine-producing capacity of
482 lactobacilli in cheese. *Applied and Environmental Microbiology*, 55(9), 2356-2359.
483 Landete, J.M., Arena, M.E., Pardo, I., Manca de Nadra, M.C. & Ferrer, S. (2010). The role of two
484 families of bacterial enzymes in putrescine synthesis from agmatine via agmatine deiminase.
485 *International Microbiology*, 13, 169-177.
486 Le Jeune, C., Lonvaud-Funel, A., Ten Brink, B., Hofstra, H., van der Vossen, J.M.B.M. (1995).
487 Development of a detection system for histidine decarboxilating lactic acid bacteria based on DNA
488 probes, PCR and activity test. *Applied and Environmental Microbiology*, 78, 316-326.
489 Lerm, E., Engelbrecht, L., du Toit, M. (2010). Malolactic fermentation: the ABC´s of MLF. *South African*
490 *Journal for Enology and Viticulture,* 31, 186-212.
491 Lerm, E., Engelbrecht, L., du Toit, M. (2011). Selection and characterization of *Oenococcus oeni* and
492 *Lactobacillus plantarum* South African wine isolates for use as malolactic fermentation starter
493 cultures. *South African Journal for Enology and Viticulture,* 32, 280-295.
494 Liu, S.Q. (2002). Malolactic fermentation in wine: beyond deacidification. *Journal of Applied*
495 *Microbiology*, 92, 598-601.
496 Lonvaud-Funel, A. (1999). Lactic acid bacteria in the quality improvement and depreciation of wine.
497 *Antonie van Leeuwenhoek*. 76,317-331.
498 López, I., López, R., Santamaría, P., Torres, C. & Ruiz-Larrea, F. (2008). Performance of malolactic
499 fermentation by inoculation of selected *Lactobacillus plantarum* and *Oenococcus oeni* strains
500 isolated from Rioja red wine. *VitiS,* 47, 123-129.
501 Lucas, P., Landete, J., Coton, M., Coton, E. & Lonvaud-Funel, A. (2003). The tyrosine decarboxylase
502 operon of *Lactobacillus brevis* IOEB 9809: characterization and conservation in tyramine-
503 producing bacteria. *FEMS Microbiololy Letters*, 229, 65-71.
504 Maicas, S., Pardo, I. & Ferrer, S. (2000). The effects of freezing and freeze-drying of *Oenococcus oeni*
505 upon induction of malolactic fermentation in red wine. *International Journal of Food Science and*
506 *Technology*, 35, 75-79.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

507 Matos, J., Nardi, M., Kumura, H., & Monnet, V. (1998). Genetic Characterization of pepP, Which
508 Encodes an Aminopeptidase P Whose Deficiency Does Not Affect *Lactococcus lactis* Growth in
509 Milk, Unlike Deficiency of the X-Prolyl Dipeptidyl Aminopeptidase. *Applied and Environmental*
510 *Microbiology*, 64(11), 4591-4595.
511 Mtshali, P. S., Divol, B., Van Rensburg, P., & Du Toit, M. (2010). Genetic screening of wine ‐ related
512 enzymes in Lactobacillus species isolated from South African wines. *Journal of Applied*
513 *Microbiology*, 108(4), 1389-1397.
514 Olguín, N., Bordons, A., & Reguant, C. (2010). Multigenic expression analysis as an approach to
515 understanding the behaviour of *Oenococcus oeni* in wine-like conditions. *International Journal of*
516 *Food Microbiology*, 144(1), 88-95.
517 Pozo-Bayón, M.A., G-Alegría, E., Polo, M.C., Tenorio, C., Martín-Álvarez, P.J., Calvo De La Banda,
518 M.T., & Moreno-Arribas, M.V. (2005). Wine volatile and amino acid composition after malolactic
519 fermentation: effect of Oenococcus oeni and Lactobacillus plantarum starter cultures. *Journal of*
520 *Agricultural and Food Chemistry,* 53(22), 8729-8735.
521 Reguant, C., Bordons, A., Arola, L., & Rozes, N. (2000). Influence of phenolic compounds on the
522 physiology of *Oenococcus oeni* from wine. *Journal of Applied Microbiology*, 88(6), 1065-1071.
523 Ribereau-Gayon, P., Glories, Y., Maujean, A. & Dubourdieu, D. (2006). Handbook of Enology, Volume
524 2: The chemistry of wine stabilization and treatments. 2nd Ed. John Wiley & Sons, New York
525 Rodas, A.M., Ferrer, S., Pardo, I. (2003). 16S-ARDRA, a tool for identification of lactic acid bacteria
526 isolated from grape must and wine. *Systematic and Applied Microbiology*, 26, 412-422.
527 Ruiz, P., Izquierdo, P.M., Seseña, S., & Palop, M.L. (2008). Intraspecific genetic diversity ofl actic acid
528 bacteria from malolactic fermentation of Cencibel wines as derived from combined analysis of
529 RAPD-PCR and PFGE pattern. *Food Microbiology*, 25, 942–948.
530 Ruiz, P., Seseña, S., Izquierdo, P.M., Llanos Palop, M. (2010). Bacterial biodiversity and dynamics
531 during malolactic fermentation of Tempranillo wines as determined by a culture-independent
532 method (PCR-DGGE). *Applied Microbiology and Biotechnology*, 86, 1555–1562.
533 Silveira, M.G., Baumgartner, M., Rombouts, F.M. & Abee, T. (2004). Effect of adaptation to ethanol on
534 cytoplasmic and membrane protein profiles of *Oenococcus oeni*. *Applied and Environmental*
535 *Microbiology*, 70, 2748–2755.

536 Solieri, L., Genova, F., De Paola, M. & Giudici, P. (2010). Characterization and technological properties
537 of *Oenococcus oeni* strains from wine spontaneous malolactic fermentations: a framework for
538 selection of new starter cultures. *Journal of Applied Microbiology*, 108, 285-298.
539 Spano, G., Rinaldi, A., Ugliano, M., Moio, L., Beneduce, L., & Massa, S. (2005). A β-glucosidase gene
540 isolated from wine *Lactobacillus plantarum* is regulated by abiotic stresses. *Journal of Applied*
541 *Microbiology*, 98(4), 855-861.
542 Spano, G., & Massa, S. (2006) Environmental stress response in wine Lactic Acid Bacteria: beyond
543 Bacillus subtilis. *Critical Reviews in Microbiology*, 32, 77-86.
544 Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., & Rattray, F. (2010)
545 Biogenic amines in fermented food. *European Journal of Clinical Nutrition*, 64, 95-100.
546 Swiegers, J.H., Bartowsky, E.J., Henschke, P.A. & Pretorius, I.S. (2005). Yeast and bacteria modulation
547 of wine aroma and flavor. *Australian Journal of Grape and Wine Research*, 11, 139-173.
548 Ulrike, E., Rogall, T., Blocker, H., Emde, M., Bottger, E.C. (1989). Isolation and direct complete
549 nucleotide determination of entire genes. Characterization of a gene encoding for 16S ribosomal
550 RNA. *Nucleic Acids Research,* 17, 7843-7853.
551 Valdés La Hens, D., Bravo-Ferrada, B., Delfederico, L., Caballero, A., Semorile, L. (2015). Prevalence of
552 *Lactobacillus plantarum* and *Oenococcus oeni* during spontaneous malolactic fermentation in
553 Patagonian red wines revealed by polymerase chain reaction-denaturing gradient gel
554 electrophoresis with two targeted genes. *Australian Journal of Grape and Wine Research,* 21, 49-
555 56.
556 Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. & Lee, T.H. (1985). Occurrence and growth of
557 lactic acid bacteria in wine: A review. *American Journal of Enology and Viticulture,* 36, 302-313.
558
559

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

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-

ACCEPTED MANUSCRIPT

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

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

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

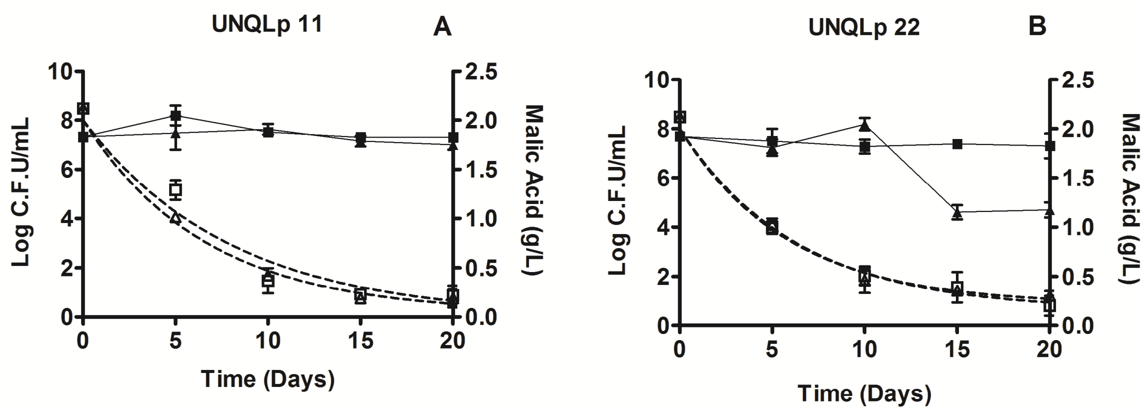
ACCEPTED MANUSCRIPT

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.**

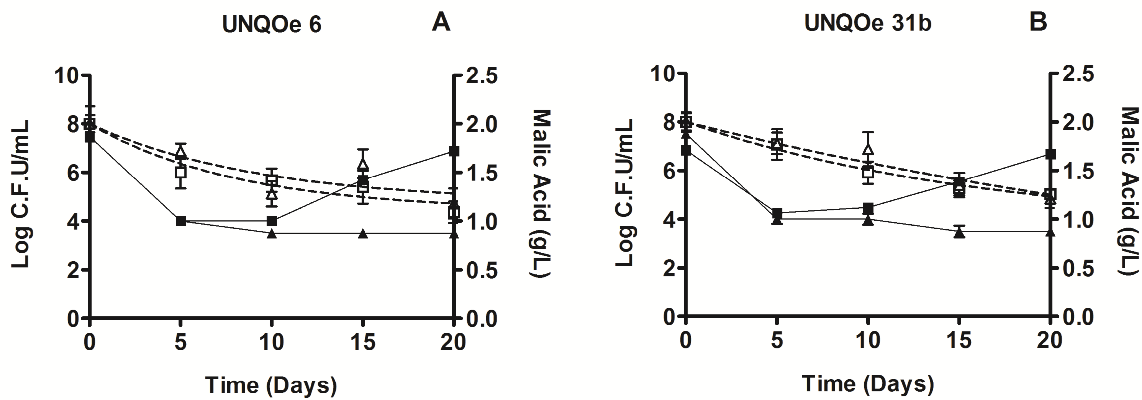
	UNQLp 11		**UNQLp 22**		**UNQOe 6**		**UNQOe 31b**	
	Non Accl	Accl	Non Accl	Accl	Non Accl	Accl	Non Accl	Accl
N_0 (CFU/mL)	2.00E+07	2.00E+07	5.01E+07	5.01E+07	3.16E+08	3.16E+08	3.16E+08	5.01E+08
Log N/N_0	-0.30	-0.02	-3.00	-0.30	-1.59	-0.24	-0.57	0.16
MAC (%)	91.04 ± 3.12^{ab}	89.62 ± 2.69^{ab}	85.84 ± 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 ($P < 0.05$).

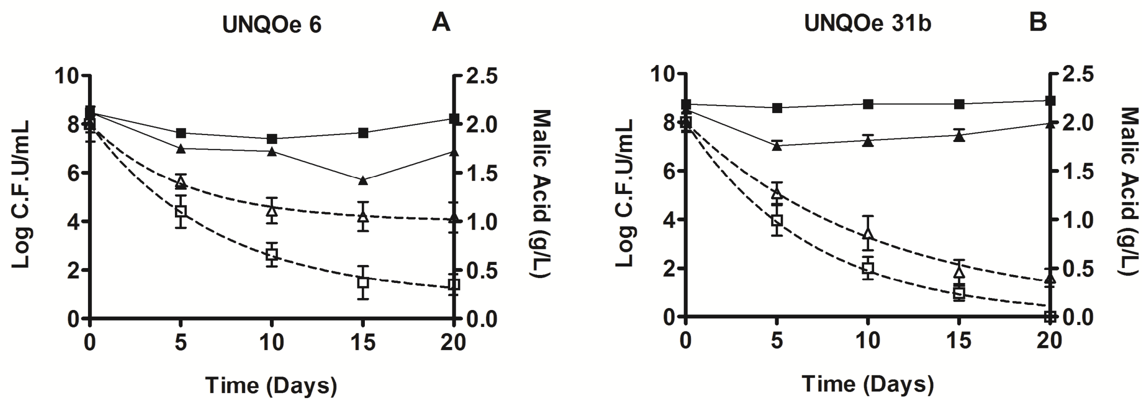
ACCEPTED MANUSCRIPT



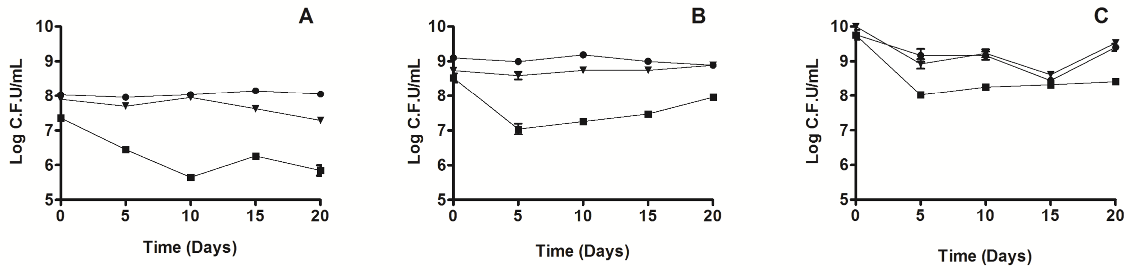
ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT

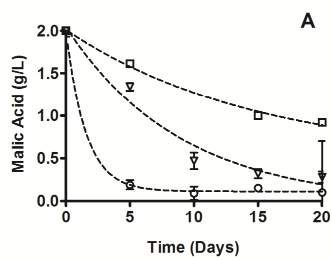


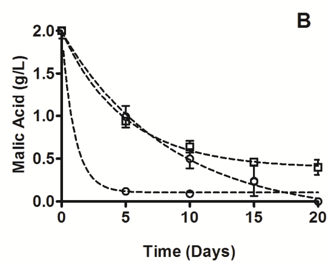
ACCEPTED MANUSCRIPT

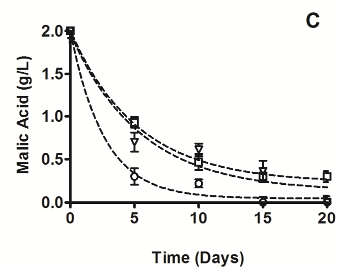


ACCEPTED MANUSCRIPT

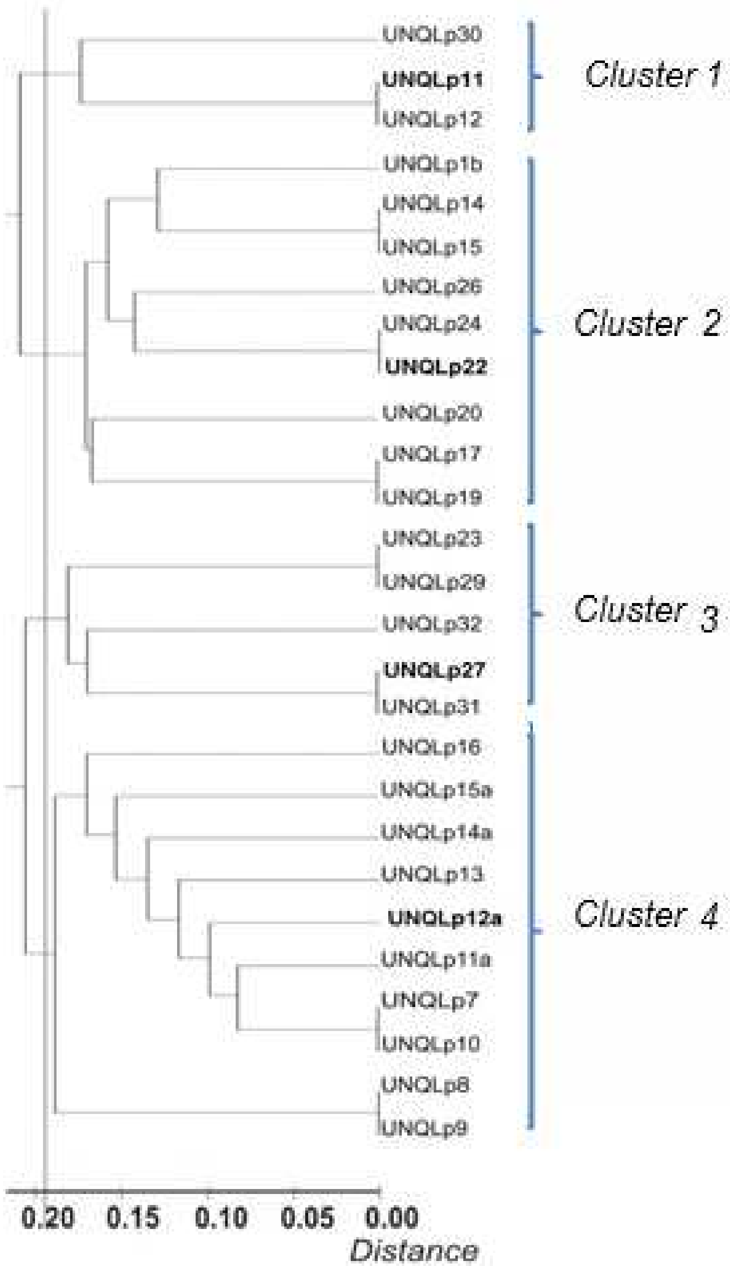
ACCEPTED MANUSCRIPT

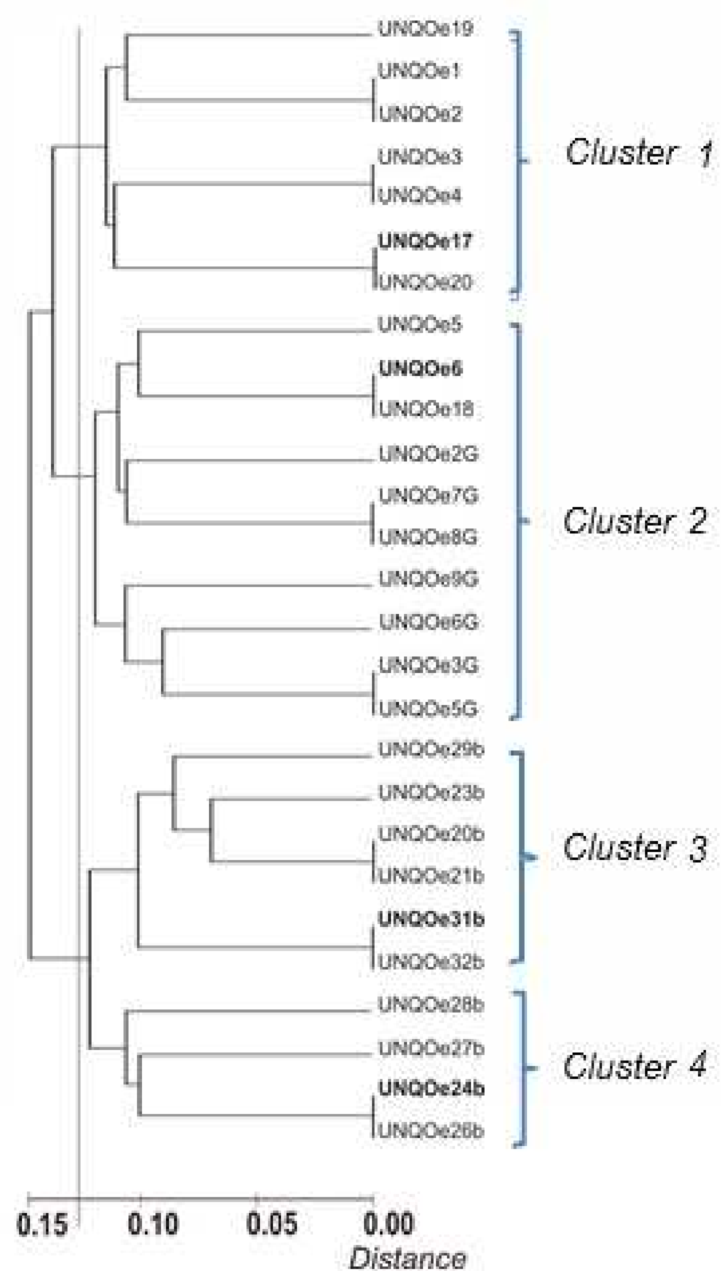






ACCEPTED MANUSCRIPT


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

ACCEPTED MANUSCRIPT

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

ACCEPTED MANUSCRIPT