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ORIGINAL ARTICLE

Effect of acclimation medium on cell viability, membrane integrity and ability to consume malic acid in synthetic wine by oenological *Lactobacillus plantarum* strains

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Keywords
acclimation medium, ethanol stress, *Lactobacillus plantarum*, malic acid consumption, membrane damage, wine.

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Abstract

Aims: The aim of this work was to evaluate the effect of acclimation on the viability, membrane integrity and the ability to consume malic acid of three oenological strains of *Lactobacillus plantarum*.

Methods and Results: Cultures in the stationary phase were inoculated in an acclimation medium (Accl.) containing 0, 6 or 10% v/v ethanol and incubated 48 h at 28°C. After incubation, cells were harvested by centrifugation and inoculated in a synthetic wine, containing 14% v/v ethanol and pH 3·5 at 28°C. Viability and membrane integrity were determined by flow cytometry (FC) using carboxyfluorescein diacetate (cFDA) and propidium iodide. Bacterial growth and malic acid consumption were monitored in a synthetic wine during 15 days. In nonacclimated strains, the damage of bacterial membranes produced a dramatic decrease in microbial viability in synthetic wine. In contrast, survival of strains previously acclimated in Accl. with 6 and 10% v/v ethanol was noticeable higher. Therefore, acclimation with ethanol increased the cultivability in synthetic wine and consequently, the consumption of L-malic acid after 15 days of growth.

Conclusion: Acclimation of oenological strains in media containing ethanol prior to wine inoculation significantly decreases the membrane damage and improves viability in the harsh wine conditions. The role of membrane integrity is crucial to warrant the degradation of L-malic acid.

Significance and Impact of the Study: The efficiency of multiparametric FC in monitoring viability and membrane damage along with the malic acid consumption has a strong impact on winemaking because it represents a useful tool for a quick and highly reliable evaluation of oenological parameters.

Introduction

The efficiency of starter cultures in wine industry is largely based on the microbiological control during winemaking. The activity of lactic acid bacteria (LAB) in malolactic fermentation (MLF) is nowadays essential for the production of commercial wines (da Silveira and Abee 2009). Therefore, inoculating starters of LAB to the grape must or wine ensures the success of fermentation (Davis *et al.* 1985; Lerm *et al.* 2010) reducing the risks associated with spontaneous MLF (i.e. potential spoilage bacteria or bacteriophages (Bauer and Dicks 2004)) and encouraging the flavour (Lerm *et al.* 2010).

However, the harsh wine environment is not appropriate for LAB growth. Different physicochemical and biological parameters (i.e. low pH, high ethanol concentration, SO_2 tolerance, yeasts-bacteria interactions) can inhibit bacterial growth, undergoing a high rate of mortality, and consequently, the failure of MLF (Cecconi *et al.* 2009).

For this reason, the viability of malolactic starters must be high enough to ensure that the harsh wine conditions will not be detrimental for MLF (Henick-Kling 1995).

The main target for ethanol injury is the cytoplasmic membrane. Ethanol is a noncharged molecule of low molecular weight that can disrupt the membrane integrity, leading to dissipation of the membrane electrochemical gradient (Leão and van Uden 1984; da Silveira *et al.* 2002) and loss of intracellular compounds (i.e. enzymatic cofactors and ions essential for cell growth and fermentation) (Osman and Ingram 1985). Bacterial exposure to stress factors such as low pH, ethanol or low temperatures, induces membrane changes that are well documented in the literature. These membrane changes include the decrease in the membrane fluidity as a result of alterations in the fatty acid composition (Bastianini *et al.* 2000; Chu-Ky *et al.* 2005), or the induction of small heat shock proteins LO18 (Guzzo *et al.* 1997), and accordingly modification of both cytoplasmic and membrane protein profiles (Silveira *et al.* 2004).

Multiparametric flow cytometry (FC) is a powerful technique that allows the discrimination of LAB in different physiological states. Two fluorescent probes have been used for this purpose: carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) (Rault *et al.* 2007). cFDA passively diffuses into cells, and its acetate groups are cleaved by intracellular esterases to yield highly green fluorescent carboxyfluorescein succinimidyl ester. The charged red fluorescent dye PI labels DNA, but only penetrates bacterial cells when membranes are damaged. Therefore, cells with intact membranes are permeable to cFDA but impermeable to PI and become green. On this basis, the combined use of cFDA and PI allows the discrimination of viable, dead and permeabilized (damaged) cells. Multiparameter FC analysis represents an important tool in winemaking because it allows monitoring industrial fermentation in real time. According to our knowledge, it has been already used to monitor alcoholic fermentation by *Saccharomyces cerevisiae* in winemaking (Farthing *et al.* 2007), ethanol stresses in *Oenococcus oeni* (da Silveira *et al.* 2002) and MLF in cider carried out by *Lactobacillus hilgardii* (Quirós *et al.* 2009).

Oenococcus oeni is probably the species of LAB best adapted to overcome the harsh environmental wine conditions and therefore is present in most of the commercial MLF starters. *Lactobacillus plantarum* is widely used in food biotechnology of fermented products. In the last years, it has been reported that some strains of *Lact. plantarum* can survive (Lerm *et al.* 2011) and grow in the harsh wine conditions (Du Plessis *et al.* 2004; López *et al.* 2008; Miller *et al.* 2011) and possess resistance mechanisms to high ethanol concentrations and low pHs (G-Alegría *et al.* 2004; Lee *et al.* 2012). This LAB species also shows a more diverse enzymatic profile than *O. oeni* (Mathews *et al.* 2004; Spano *et al.* 2005), which could play an important role in the modification of the wine aroma profile (Swiegers *et al.* 2005). This information supports their potential use as starter cultures.

The aim of this work was to evaluate the effect of an acclimation medium containing different concentrations of ethanol on the viability and membrane integrity of three oenological strains of *Lact. plantarum* when grown in a synthetic wine medium containing 14% v/v ethanol. These strains were obtained from Patagonian red wines and selected for possible use as starter cultures for MLF due to their oenological and technological properties (Bravo-Ferrada *et al.* 2013). The viability and membrane integrity of *Lact. plantarum* strains were measured by multiparametric FC. Acclimated and nonacclimated *Lact. plantarum* strains were inoculated in synthetic wine, and their FC parameters were correlated with cultivability and ability to consume malic acid.

Materials and methods

Strains, medium and growth condition

Lactobacillus plantarum UNQLp 133, UNQLp 65·3 and UNQLp 155 were isolated from Patagonian Pinot noir red wine (Bravo-Ferrada *et al.* 2013) (GeneBank Accession Numbers *rpoB* gene KC679065, KC679060 and KC679067, respectively; *16S rRNA* gene KC562905 for UNQLp 133, KC679066 for UNQLp 65·3 and KC652904 for UNQLp 155). Cultures were grown in 10 ml of MRS broth (Biokar Diagnostics, Beauvais, France) (De Man *et al.* 1960), at 28°C and pH 6·5 for 48 h in anaerobic conditions (AnaeroPack - Mitsubishi Gas Chemical America, Inc., New York, NY).

Culture conditions of LAB strains prior to inoculation in synthetic wine

Cells in the stationary phase (approximately 10^9 CFU ml^{-1}) were harvested by centrifugation at 4000 ***g*** for 10 min and suspended in the same volume (10 ml) of a modified acclimation medium (Accl.), containing 0, 6 and 10% v/v of ethanol concentration. The composition of Accl. was defined according to Lerm *et al.* (2011) and is shown in Table 1. After incubation at 28°C for 48 h, the acclimated cells were harvested by centrifugation and inoculated in a synthetically prepared wine. The composition of the synthetic wine was similar to that described by Ugliano *et al.* (2003) (adapted to the composition of the Patagonian wines) and is indicated in Table 2.

Staining procedures

Cultures before and after acclimation treatment and also after 24 h incubation in a synthetic wine were harvested

Table 1 Composition of the acclimation culture medium (Accl.)

Components	Concentration
MRS	50 g l^{-1}
D(-) fructose	40 g l^{-1}
D(-) glucose	20 g l^{-1}
L-malate	1 g l^{-1}
Tween 80	1 ml
Pyridoxine	0·1 g l^{-1}
Ethanol	0, 6 or 10% v/v
pH	4·6

MRS: de Man, Rogosa, Sharpe broth (Biokar Diagnostics, Beauvais, France).

Table 2 Composition of the synthetic wine

Components	Concentration
Tartaric acid	5 g l^{-1}
Malic acid	4·5 g l^{-1}
Acetic acid	0·6 g l^{-1}
Glucose	2 g l^{-1}
Fructose	2 g l^{-1}
Yeast extract	2 g l^{-1}
NaCl	0·2 g l^{-1}
$(NH_4)_2SO_4$	1 g l^{-1}
K_2HPO_4	2 g l^{-1}
$MgSO_4{\cdot}7H_2O$	0·2 g l^{-1}
$MgSO_4$	0·05 g l^{-1}
Ethanol	14% v/v
pH	3·5

by centrifugation at 4000 ***g*** for 10 min, and washed twice with sterile PBS. The concentration of cell suspensions was adjusted to approximately 10^6 CFU ml^{-1}. One millilitre of cell suspensions was incubated with 2 μl cFDA (Molecular Probes, Leiden, the Netherlands) (50 μg μl^{-1}) 30 min at 28°C in the dark. Then, PI (Molecular Probes) was added to a final concentration of 0·5 mg ml^{-1} and incubated 5 min at room temperature.

Flow cytometry analyses

FC analysis was performed with a FACSCalibur instrument using the CellQuest software (Becton Dickinson, Mountain View, CA, USA) according to Rault *et al.* (2007). Samples were processed so that 10 000 events were collected for each sample, and the event rate was less than 300 events s^{-1}. All parameters were collected as logarithmic signals. FL1 channel (530 nm) was used to set the green fluorescence of cFDA and FL3 channel (670), to set the red fluorescence of PI dye. Mixtures of heat killed (80°C for 30 min) and freshly harvested cells were stained with cFDA and PI both in double-staining assays. These mixtures served as controls, to set the flow cytometer detectors and compensation to distinguish the four regions: Q1 (dead cells): PI+ and cFDA−; Q2 (membrane damages cells): PI+ and cFDA+; Q3 (debris): PI− and cFDA− and Q4 (viable cells): PI− and cFDA+. The percentage of each population was determined as (i/(Q1 + Q2 + Q4))/100, where i is Q1, Q2 or Q4. Nonfluorescent debris in Q3 was excluded (Hiraoka and Kimbara 2002).

Bacterial plate counts

Cultivable cells were determined by plating onto MRS agar (Biokar Diagnostics) before and after acclimation treatment, and also after 1-, 3-, 6- and 24-h incubation in a synthetic wine. Bacteria were suspended in 1 ml 0·7% NaCl w/v, serially diluted and plated on MRS agar plates. Cell counts were determined after 48-h incubation at 28°C.

Ability of acclimated bacterial cells to grow and consume malic acid in a synthetic wine

Cells previously acclimated were inoculated (1×10^7 CFU ml^{-1}) in 50 ml of synthetic wine and incubated at 28°C without shaking during 15 days. Remaining malic acid concentration was determined with a malic acid enzymatic assay (L-malic Acid MegaQuant™ Format enzymatic kit, Megazyme International, Wicklow, Ireland). Bacterial growth was monitored by plating on MRS agar, sampling at days 0, 1, 3, 5, 10 and 15.

Reproducibility of results

Determinations were carried out in duplicate from three independent cultures of each bacterial strain studied. The relative differences were reproducible, independently of the culture used. Analysis of variance (ANOVA) was carried out using the statistical program STATISTIX 8 Software (Analytical Software, Tallahassee, FL, USA). All pairwise comparisons were performed using Tukey's HDS test, and if $P < 0{\cdot}05$, the difference was considered statistically significant.

Results

Membrane damage and cell viability

In preliminary experiments, *Lact. plantarum* UNQLp 133, UNQLp 65·3 and UNQLp 155 were incubated in a synthetic wine. After 3-h incubation, the PI uptake was higher than 80% for all the three strains. At the same time, a noticeable decrease in microbial cultivability was observed (decrease of *ca.* 6 log CFU ml^{-1} after 3-h incubation).

To improve the bacterial resistance in such a harsh environment, strains were acclimated in a medium containing 0, 6 and 10% v/v ethanol (Accl. Eth. 0, 6 and 10%).

Cultivability before and after incubation in a synthetic wine (24 h at 28°C) is presented in Table 3. Cultivability in Accl. and in MRS showed no significant differences ($P > 0{\cdot}05$). After inoculation in a synthetic wine, no significant differences were observed in the CFU ml^{-1} of strains acclimated with 6 and 10% v/v ethanol before and after synthetic wine inoculation ($P > 0{\cdot}05$). In contrast, nonacclimated strains and strains acclimated without ethanol (Accl. Eth 0%) showed a dramatic reduction in cultivability in all cases (Table 3).

Multiparametric FC using cFDA and PI was assayed to evaluate the effect of Accl. on viability, membrane damage and dead cells. Immediately after acclimation and before inoculation in synthetic wine, acclimated and nonacclimated cells showed no significant differences ($P > 0{\cdot}05$). In this situation, the percentage of viable cells (cFDA+ and PI−) was higher than 85% for the three strains (data not shown). In contrast, after 24 h incubation at 28°C in synthetic wine, broad differences were observed between acclimated and nonacclimated bacteria (Table 4). Acclimation with 6 or 10% v/v ethanol produced a noticeable increase in viability in all the strains (higher % viable cells and lower % of permeabilized cells (PI− and cFDA+)). On the contrary, Accl. Eth 0% led to a huge increase in PI+ and cFDA+ cells, indicating membrane damage. For nonacclimated cells grown in MRS, the decrease in viability was concomitant with a tremendous increase in cells PI+ cFDA− (dead), which is in agreement with results of cultivability (Table 3). The relationship between viability by FC and cultivability by plate count was analysed by linear regression of % of viable cells (Table 4) as function of log CFU ml^{-1} (Table 3). For the three strains, the coefficient of determination (R^2) was higher than 0·93.

Table 3 Cultivability of *Lactobacillus plantarum* strains before and after incubation in a synthetic wine 24 h at 28°C. Strains were previously acclimated in ethanol, 6% v/v (Eth 6%), 10% v/v (Eth 10%), without ethanol (Eth 0%) or nonacclimated (MRS broth)

Strain	Condition	Before wine inoculation* (Log CFU ml^{-1})	After wine inoculation* (Log CFU ml^{-1})
UNQLp 133	MRS	$10{\cdot}9 \pm 0{\cdot}5^{(a)}$	$5{\cdot}0 \pm 0{\cdot}2^{(b)}$
	Accl. Eth 0%	$9{\cdot}7 \pm 0{\cdot}2^{(a)}$	$6{\cdot}0 \pm 0{\cdot}4^{(b)}$
	Accl. Eth 6%	$9{\cdot}4 \pm 0{\cdot}7^{(a)}$	$8{\cdot}2 \pm 0{\cdot}2^{(a)}$
	Accl. Eth 10%	$8{\cdot}0 \pm 0{\cdot}7^{(a)}$	$8{\cdot}6 \pm 0{\cdot}2^{(a)}$
UNQLp 65·3	MRS	$11{\cdot}3 \pm 0{\cdot}2^{(a)}$	$3{\cdot}78 \pm 0{\cdot}5^{(b)}$
	Accl. Eth 0%	$9{\cdot}6 \pm 0{\cdot}4^{(a)}$	$5{\cdot}00 \pm 0{\cdot}4^{(b)}$
	Accl. Eth 6%	$9{\cdot}0 \pm 0{\cdot}8^{(a)}$	$7{\cdot}70 \pm 0{\cdot}4^{(a)}$
	Accl. Eth 10%	$9{\cdot}3 \pm 0{\cdot}8^{(a)}$	$8{\cdot}53 \pm 0{\cdot}4^{(a)}$
UNQLp 155	MRS	$10{\cdot}9 \pm 0{\cdot}3^{(a)}$	$3{\cdot}4 \pm 0{\cdot}4^{(b)}$
	Accl. Eth 0%	$10{\cdot}2 \pm 0{\cdot}4^{(a)}$	$5{\cdot}2 \pm 0{\cdot}3^{(b)}$
	Accl. Eth 6%	$10{\cdot}0 \pm 1{\cdot}3^{(a)}$	$8{\cdot}9 \pm 0{\cdot}4^{(a)}$
	Accl. Eth 10%	$9{\cdot}8 \pm 1{\cdot}3^{(a)}$	$8{\cdot}8 \pm 0{\cdot}4^{(a)}$

*Different letters (a, b, c and d) denote statistically significant differences ($P < 0{\cdot}05$).

Ability of acclimated cells to grow in a synthetic wine

The ability of *Lact. plantarum* strains to grow in a synthetic wine was then monitored during 15 days. At day 1, the viability of acclimated cells remained constant or showed a slight decrease, whereas that of the nonacclimated strains decreased 0·5–1 log CFU ml^{-1} (Fig. 1). After day 1, the strains acclimated in Accl. Eth. 6% and Accl. Eth. 10% were able to grow in synthetic wine but with a different cultivability. After 15 days of growth in synthetic wine, the cultivability of *Lact. plantarum* UNQLp 133, UNQLp 65·3 and UNQLp 155 increased 1, 0·5 and 1·5 log CFU ml^{-1}, respectively. No significant differences were observed for strains previously acclimated in Accl. Eth 6% and Accl. Eth 10% (Fig. 1).

A completely different behaviour was observed for the nonacclimated strains (grown in MRS) and the acclimated strains without ethanol (Accl. Eth 0%). The cultivability of *Lact. plantarum* UNQLp 133 showed a slight decrease up to fifth day of incubation. After that, it increased up to $\approx 10^6$ CFU ml^{-1} (Fig. 1a). A similar behaviour was observed when acclimated in Accl. Eth 0%. Nonacclimated *Lact. plantarum* UNQLp 65·3 (grown in MRS) showed a continuous decrease in viability (3×10^5 CFU ml^{-1} at day 15) (Fig. 1b). When acclimated in Accl. Eth. 0%, *Lact. plantarum* UNQLp 65·3 showed higher level of cultivability. After a 0·7 log decrease during the first 5 days, it remained constant up to day 15. Finally, not acclimated strain *Lact. plantarum* UNQLp 155 (grown in MRS) was unable to grow in synthetic wine (Fig. 1c), whereas cells acclimated in Accl. Eth. 0% showed a moderate increase in cultivability (Fig. 1c).

Ability of acclimated cells to consume malic acid in synthetic wine

The evolution of L-malic acid consumption in a synthetic wine is shown in Fig. 2. Both nonacclimated and acclimated strains were able to consume L-malic acid, but in a different extent (Fig. 2).

After acclimation in Accl. Eth. 6%, *Lact. plantarum* UNQLp 133, UNQLp 65·3 and UNQLp 155 were able to reduce the L-malic acid concentration of synthetic wine to values slightly below 2·5, 1·3 and 2 g l^{-1}, respectively at day 15. The nonacclimated strains (grown in MRS)

Table 4 Percentages of viable (cFDA+), permeabilized (cFDA+ and PI+) and dead (PI+) of different *Lactobacillus plantarum* strains grown in MRS and pre-acclimated in different concentrations of ethanol (0, 6 and 10%) after incubation in synthetic wine for 24 h at 28°C

Strain	Condition	Viable* (%)	Permeabilized* (%)	Dead* (%)
UNQLp 133	MRS	20·6 ± 1·5$^{(b)}$	20·4 ± 1·8$^{(b)}$	59·0 ± 2·8$^{(a)}$
	Accl. Eth 0%	21·4 ± 2·1$^{(b)}$	57·5 ± 3·2$^{(a)}$	21·2 ± 1·3$^{(b)}$
	Accl. Eth 6%	68·6 ± 3·4$^{(a)}$	19·5 ± 1·8$^{(b)}$	11·9 ± 2·0$^{(b,c)}$
	Accl. Eth 10%	78·4 ± 2·8$^{(a)}$	16·1 ± 1·4$^{(b)}$	5·5 ± 1·4$^{(c)}$
UNQLp 65·3	MRS	17·8 ± 1·9$^{(b)}$	12·1 ± 0·9$^{(b)}$	70·2 ± 3·5$^{(a)}$
	Accl. Eth 0%	16·1 ± 1·0$^{(b)}$	53·5 ± 3·0$^{(a)}$	31·4 ± 2·4$^{(b)}$
	Accl. Eth 6%	63·8 ± 2·3$^{(a)}$	21·4 ± 2·5$^{(b)}$	14·8 ± 1·9$^{(c)}$
	Accl. Eth 10%	68·6 ± 1·5$^{(a)}$	19·1 ± 2·1$^{(b)}$	12·3 ± 1·6$^{(c)}$
UNQLp 155	MRS	9·1 ± 0·9$^{(c)}$	68·2 ± 2·4$^{(a)}$	22·7 ± 2·2$^{(a)}$
	Accl. Eth 0%	9·1 ± 1·4$^{(c)}$	78·8 ± 5·5$^{(a)}$	12·1 ± 1·4$^{(b)}$
	Accl. Eth 6%	67·7 ± 2·4$^{(b)}$	29·9 ± 2·1$^{(b)}$	2·5 ± 1·6$^{(c)}$
	Accl. Eth 10%	83·4 ± 3·0$^{(a)}$	15·1 ± 1·5$^{(b)}$	1·5 ± 0·6$^{(c)}$

*Different letters (a, b, c and d) denote statistically significant differences ($P < 0·05$).

were much less efficient to consume L-malic acid since its concentration remained slightly below 3·2 g l^{-1} at day 15. For strains acclimated in Accl. Eth. 0%, only *Lact. plantarum* UNQLp155 increased the L-malic acid consumption, but in a lesser extent than the acclimated in Accl. Eth. 6 and 10%. It is important to point out that only *Lact. plantarum* UNQLp 65·3 previously acclimated in Accl. Eth. 10% (Fig. 2) was able to consume almost all the L-malic acid present in the synthetic wine (4·5 g l^{-1}). Under the same conditions, the other two strains only consumed half of L-malic acid content (Fig. 2).

Discussion

Three strains of *Lact. plantarum* isolated from red Patagonian wines were selected as potential starter cultures for MLF because of their oenological and technological properties (Bravo-Ferrada *et al.* 2013). However, their incubation in synthetic wine led to a rapid decrease in cultivability concomitant to a drastic disruption of membrane integrity.

As the resistance of malolactic starters to wine conditions is crucial for a successful of MLF, acclimation of starter cultures with sublethal concentrations of ethanol becomes necessary. Previous papers reported that acclimation in media containing ethanol within 4 and 10% v/v (Cecconi *et al.* 2009; Solieri *et al.* 2009; Lerm *et al.* 2010) and low pH (from 3·5 to 4·6) can increase the ethanol resistance during winemaking. The results obtained in this work showed that acclimation in the presence of 6 and 10% v/v ethanol improves the cultivability and L-malic acid consumption in synthetic wine. It is recommended that besides ethanol and acids, the acclimation medium must be rich in nutrients and growth factors promoting the expression of resistance mechanisms (Lerm *et al.* 2011). Moreover, high concentrations of glucose and fructose favour the development of biomass in adverse conditions (Maicas *et al.* 2000).

Ethanol interacts with the lipid bilayer and induces an increase in membrane fluidity and proton permeability (Leão and van Uden 1984) and leading to the loss of intracellular compounds (Osman and Ingram 1985; da Silveira *et al.* 2002). The proton efflux affects pH-dependent physiological processes, such as ATP synthesis and the L-malate uptake. Deleterious effect in the MLF by *O. oeni* inoculated in wine is due the combined effect of ethanol and low pH (da Silveira *et al.* 2002). Capucho and San Romao (1994) reported that MLF at pH 5 was not affected at ethanol concentrations lower than 20%. However, a successful MLF at pH 3 requires concentrations of ethanol lower than 12%. Moreover, da Silveira *et al.* (2002) reported that ethanol concentrations higher than 8% induce an extensive membrane disorganization. This leads to the efflux of compounds with high molecular weight (i.e. NAD^+, NADH and AMP) that are cofactors of malolactic activity.

In addition, it has been reported that acclimation with ethanol at low pH, induces a decrease in membrane fluidity as a result of the changes in fatty acid composition (Chu-Ky *et al.* 2005; Grandvalet *et al.* 2008). This indicates that during acclimation the expression of several cytoplasmic and membrane proteins is affected (Teixeira *et al.* 2002). In this work, the percentage of dead cells after acclimation without ethanol (Accl. Eth. 0%) was lower than that of nonacclimated cells (grown in MRS) (Table 3). However, the acclimation in this condition was not sufficient to protect membrane integrity, as observed by the high percentage of PI+ cFDA+ cells (damaged) (Table 4). This membrane damage affected both cultivability (Table 3 and Fig. 1) and ability to consume malic acid (Fig. 2). On the contrary, acclimation in Accl. Eth. 6 and 10% contributed for the integrity of membranes, as

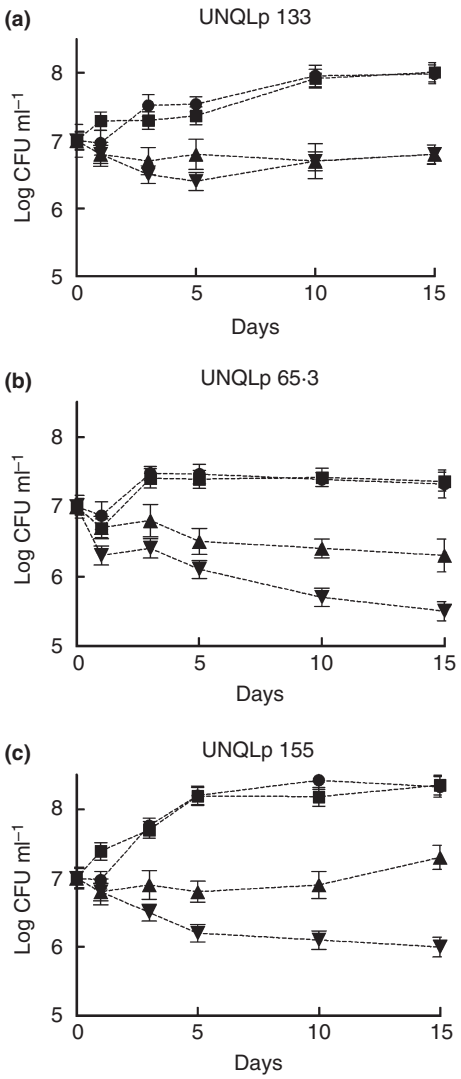

Figure 1 Evolution of cultivability of *Lactobacillus plantarum* strains in a synthetic wine at 28°C during 15 days. (●) cells previously acclimated in Accl. Eth. 6%, (■) Accl. Eth. 10%, (▲) Accl. Eth. 0% and (▼) nonacclimated (grown in MRS). (a) *Lact. plantarum* UNQLp 133, (b) UNQLp 65·3 and (c) UNQLp 155.

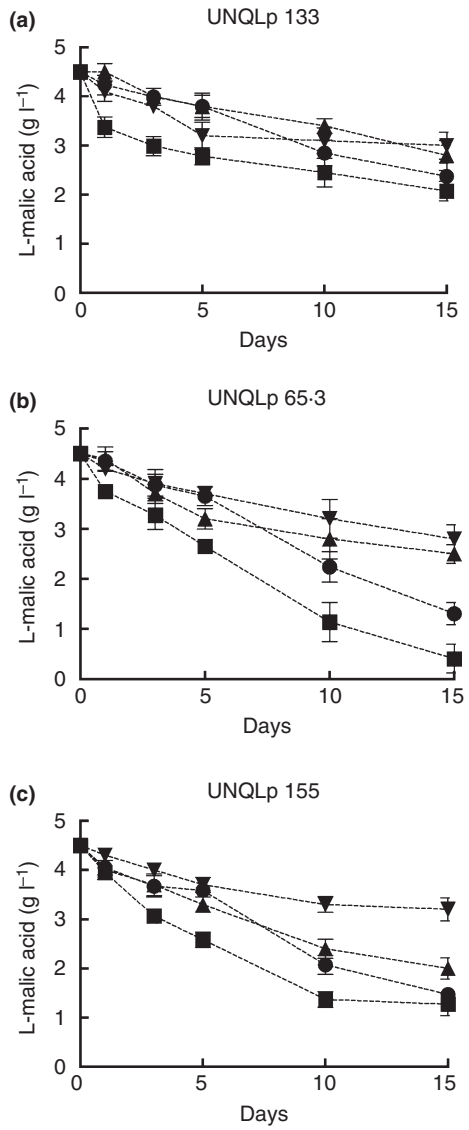

Figure 2 Evolution of L-malic acid concentration in a synthetic wine at 28°C during 15 days. (●) cells previously acclimated in Accl. Eth. 6%, (■) Accl. Eth. 10%, (▲) Accl. Eth. 0% and (▼) nonacclimated (grown in MRS). (a) *Lactobacillus plantarum* UNQLp 133, (b) UNQLp 65·3 and (c) UNQLp 155.

observed by the high % of PI cells (viable) (Table 4), which was consistent with their higher cultivability (Table 3 and Fig. 1). These results are in agreement with those obtained by da Silveira *et al.* (2002) for *O. oeni*. They observed that *O. oeni*. grown in the presence of 8% v/v ethanol are more resistant to a 25 min 16% v/v ethanol exposure than nonacclimated strains. The critical importance of acclimation on both cell viability and membrane integrity was reflected in Tables 3 and 4 of this work. In this regard, the high correlation between cultivability measured by plate count and % of viable cells by FC is in agreement with results reported by Rault *et al.* (2007).

Different authors have studied the effect of ethanol on membrane fluidity and lipid composition of *O. oeni*. However, this effect has been scarcely addressed on *Lact. plantarum*. The effect of ethanol *on O. oeni* is somewhat controversial. da Silveira *et al.* (2002) reported an increase in the bilayer rigidity together with an increase in unsaturated lipid ratio concomitant with an increase in membrane-associated proteins. Grandvalet *et al.* (2008) found an increase in the saturated/unsaturated fatty acids ratio, ascribing the increased ethanol resistance to the increase in lactobacillic acid levels (Teixeira *et al.* 2002). An increase

in the saturated/unsaturated fatty acids ratio has also been reported for *Lact. plantarum* grown in MRS supplemented with 8% v/v of ethanol (van Bokhorst-van de Veen *et al.* 2011). These changes are ascribed to *de novo* fatty acids biosynthesis. In this work, we observed that acclimation increases the resistance of cytoplasmic membrane. More studies should be conducted to know the mechanism involved in such this adaptation.

Considering that malate decarboxylation by LAB requires an intact membrane for the transport of protons inside the cell (da Silveira *et al.* 2002), the integrity of the membrane before winemaking becomes critical. The results found in the present work support this hypothesis. In this regard, *O. oeni* grown in the presence of ethanol increases the order at the membrane lipid–water interface and decreases the permeability as a response to the fluidizing effect of ethanol (da Silveira *et al.* 2003). These ethanol-adapted cells are able to retain more efficiently intracellular compounds, which justify the efficient MLF in ethanol-adapted cells (da Silveira and Abee 2009).

Conclusion

The results reported in this work showed the high correlation between multiparametric FC assay (with cFDA and PI) and the cultivability after wine inoculation, supporting its usefulness as a quick analytical screening method of strains destined to winemaking.

In addition, the oenological properties of *Lact. plantarum* strains (Bravo-Ferrada *et al.* 2013) together with the acclimation conditions that improve the bacterial resistance to high ethanol concentrations present in wine are promising for their successful implementation as malolactic starters in the wine industry.

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Conflict of interest

Authors state that there are no conflict of interest that might bias this work.

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