Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Effect of the fatty acid composition of acclimated oenological *Lactobacillus plantarum* on the resistance to ethanol

B.M. Bravo-Ferrada[1], A. Gómez-Zavaglia[2], L. Semorile[1] and E.E. Tymczyszyn[1]

1 Laboratorio de Microbiología Molecular, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Bernal, Argentina
2 Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) (CONICET La Plata, UNLP), La Plata, Argentina

Significance and Impact of the Study: Understanding the adaptation of oenological strains to ethanol has strong impact on winemaking as it allows defining the most appropriate conditions to maintain bacteria viability and improve the efficiency of malolactic fermentation. The acclimation of oenological *Lact. plantarum* in media containing ethanol produces a drastic decrease of C18:1 with an increase in the content of saturated short-chain-length membrane fatty acids that are correlated with a higher resistance to the adverse environmental conditions of wine.

Keywords
acclimation, ethanol stress, fatty acid, *Lactobacillus plantarum*, wine.

Correspondence
Emma Elizabeth Tymczyszyn, Roque Saenz Peña 352, Buenos Aires B1876BXD, Argentina.
E-mail: elitym@yahoo.com.ar

2014/1615: received 4 August 2014, revised 22 October 2014 and accepted 27 October 2014

doi:10.1111/lam.12350

Abstract

The aim of this work was to evaluate the changes due to acclimation to ethanol on the fatty acid composition of three oenological *Lactobacillus plantarum* strains and their effect on the resistance to ethanol and malic acid consumption (MAC). *Lactobacillus plantarum* UNQLp 133, UNQLp 65.3 and UNQLp 155 were acclimated in the presence of 6 or 10% v/v ethanol, for 48 h at 28°C. Lipids were extracted to obtain fatty acid methyl esters and analysed by gas chromatography interfaced with mass spectroscopy. The influence of change in fatty acid composition on the viability and MAC in synthetic wine was analysed by determining the Pearson correlation coefficient. Acclimated strains showed a significant change in the fatty composition with regard to the nonacclimated strains. Adaptation to ethanol led to a decrease in the unsaturated/saturated ratio, mainly resulting from an increase in the contribution of short-length fatty acid C12:0 and a decrease of C18:1. The content of C12:0 was related to a higher viability after inoculation of synthetic wine. The MAC increased at higher contents in saturated fatty acid, but its efficiency was strain dependent.

Introduction

Malolactic bacteria are lactic acid bacteria that are able to carry out malolactic fermentation (MLF). The control of their activity is an important technological aspect in the commercial production of wine (Bastianini *et al.* 2000). The viability of malolactic starters must be high enough to ensure that the harsh wine conditions are not detrimental for MLF (Henick-Kling 1995).

The acclimation of starter cultures with sublethal concentrations of ethanol and low pH (from 3·5 to 4·6) is necessary to increase their ethanol resistance during winemaking (Cecconi *et al.* 2009; Solieri *et al.* 2009; Lerm *et al.* 2010).

Even when *Oenococcus oeni* is the main species used as commercial starter for MLF, some *Lactobacillus plantarum* strains can also survive in the harsh conditions of wine (Lerm *et al.* 2011; Bravo-Ferrada *et al.* 2013). In addition, different studies have demonstrated that *Lact. plantarum* strains are able to grow in wine conditions (Du Plessis *et al.* 2004; G-Alegría *et al.* 2004; López *et al.* 2008; Miller *et al.* 2011) and have resistance mechanisms to tolerate high concentrations of ethanol and low pHs (G-Alegría *et al.* 2004; van Bokhorst-van de Veen *et al.* 2011; Lee

et al. 2012). The viability of bacterial cells depends on their adaptation capacity to the environment, which is mainly regulated by the membrane fluidity (da Silveira *et al.* 2003; Chu-Ky *et al.* 2005). Several events are related with the ability of cells to survive in wine conditions and metabolize malic acid, one of them being the synthesis of small heat-shock proteins and membrane proteins (Guzzo *et al.* 1997; Silveira *et al.* 2004). Another strategy is the adjustment of the membrane lipid composition. Different authors investigated the effect of stress factors on the lipid composition of *O. oeni.* In this regard, it has been reported that the increase in the unsaturated/saturated (US/S) fatty acids ratio counteract the toxic effect of ethanol (Garbay *et al.* 1995; Bastianini *et al.* 2000; da Silveira *et al.* 2003). Nevertheless, Grandvalet *et al.* 2008 found a decrease in the US/S fatty acids ratio when *O. oeni* was grown in the presence of ethanol.

The increase in the resistance to ethanol has been associated with high concentrations of cycC19:0 fatty acid (Garbay *et al.* 1995; Bastianini *et al.* 2000; Teixeira *et al.* 2002; Grandvalet *et al.* 2008). CycC19:0 is produced by enzymatic conversion of C18:1. However, the role of cycC19:0 is controversial. Some authors consider this fatty acid as unsaturated and responsible for the increase of membrane fluidity (Garbay *et al.* 1995; Bastianini *et al.* 2000). On the contrary, other authors ascribe its protective effect against ethanol to its capacity to rigidize lipid membranes, thus reducing bacterial membrane permeability (Grandvalet *et al.* 2008).

In our previous study, we reported that acclimation of oenological strains of *Lact. plantarum* with 6 and 10% v/v ethanol prevents membrane damage after inoculation in wine conditions (Bravo-Ferrada *et al.* 2014). However, to our knowledge, the effect of acclimation on the fatty acid composition of *Lact. plantarum* has not been studied. For this reason, the aim of this work was to evaluate the changes due to acclimation to ethanol on the membrane fatty acid composition of three oenological strains of *Lact. plantarum* and correlate these changes with the malic acid consumption (MAC) and the bacterial resistance to harsh wine environment.

Results and discussion

Different authors reported alterations in membrane properties of *O. oeni* grown in the presence of ethanol (da Silveira *et al.* 2002, 2003; Chu-Ky *et al.* 2005; da Silveira and Abee 2009). It was also reported that acclimation of *Lact. plantarum* leads to decrease in membrane damage when grown in the presence of ethanol (Bravo-Ferrada *et al.* 2014). Lipid membranes are the main target of damage by ethanol and micro-organisms have different strategies to prevent this damage (da Silveira *et al.* 2003, Silveira *et al.* 2004). One of them is the modification of the fatty acid composition to regulate the membrane fluidity (Garbay *et al.* 1995; Teixeira *et al.* 2002; da Silveira *et al.* 2003; Grandvalet *et al.* 2008). However, to our knowledge, a correlation between the fatty acid composition of *Lact. plantarum* strains and acclimation has not been reported hereto.

The fatty acid composition of *Lact. plantarum* UNQLp 133, 65.3 and 155 nonacclimated and acclimated in the presence of 6 and 10% v/v ethanol is depicted in Fig. 1a–c (controls: nonacclimated strains). The fatty acid composition of the nonacclimated strains was similar, 16:0 and 18:1 being the most abundant fatty acids. CycC19:0, a typical lactobacilli fatty acid, was ~10–20%.

Upon acclimation, important changes were observed in the fatty acid composition. Acclimation of *Lact. plantarum* UNQLp 133 with 6 and 10% v/v ethanol led to an increase of 12:0 and a decrease of 16:0, 18:1 and cycC19:0 (Fig. 1a). A significant increase of C14:0 was observed

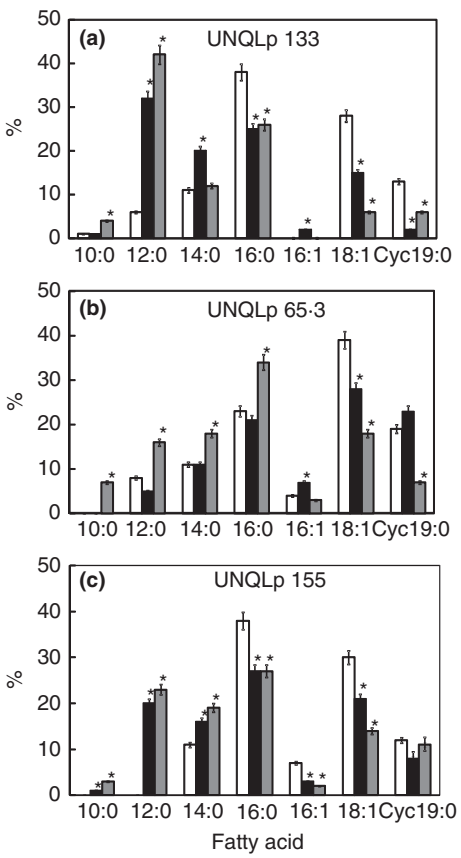

Figure 1 Fatty acid composition of *Lactobacillus plantarum* strains nonacclimated (white bars) or acclimated at 28°C for 48 h in the presence of 6% (black bars) and 10% (grey bars) v/v ethanol. (a) *Lact. plantarum* UNQLp 133, (b) UNQLp 65.3 and (c) UNQLp 155. *Significant different from nonacclimated cells ($P < 0{\cdot}05$).

after acclimation with ethanol 6%, and C10:0 significantly increased when UNQLp 133 was acclimated with ethanol 10% ($P < 0{\cdot}05$). *Lactobacillus plantarum* 65.3 acclimated with 6% v/v ethanol showed a decrease in the content of 18:1 and an increase in the content of C16:1 ($P < 0{\cdot}05$) (Fig. 1b). When acclimated with 10% v/v ethanol, an increase of 10:0, 12:0, 14:0 and 16:0 and decrease of 18:1 and cycC19:0 was observed ($P < 0{\cdot}05$). Strain UNQLp 155 showed an increase of C10:0, C14:0 and C12:0 fatty acids ($P < 0{\cdot}05$). However, the contribution of cycC19:0 in strain 155 did not change upon acclimation ($P > 0{\cdot}05$) (Fig. 1c).

Determining the contribution of saturated and unsaturated fatty acids allowed a generalization on the consequences of acclimation on the fatty acid composition of the three strains (Table 1). The decrease of 18:1 fatty acid in the three acclimated strains led to a decrease of the unsaturation degree (Table 1). This effect was also reflected in the US/S ratio, which was more drastic when strains were acclimated with 10% v/v ethanol (Table 1). This decrease of the US/S ratio was concomitant with a decrease in the hydrocarbon chain lengths (CL), also in the three strains (Table 1).

To investigate the influence of fatty acid composition on the technological properties of strains, the alterations occurred upon acclimation were correlated with the viability and MAC previously reported (Bravo-Ferrada *et al.* 2014). The log CFU ml^{-1} reported in Table 1 corresponds to the viability of strains after 15 days of incubation at 28°C (initial inoculum: ~10^7 CFU ml^{-1}).

Pearson correlation coefficient was performed to establish a relationship between viability and MAC, with the fatty acid composition of the three strains analysed. A significant negative correlation between C12:0 and C18:1, and between C12:0 and cyc C19:0 was observed (Table 2). In contrast, the content of C12:0 had a positive correlation with viability. In addition, the content of cycC19:0 showed a negative relation with C14:0 and a positive one with the content of C18:1 (Table 2). No correlation was found between MAC and fatty acid composition (Table 2). However, when the MAC was analysed for each strain, a negative correlation was found between MAC and the content of C18:1 (Fig. 2a), the US/S ratio (Fig. 2b) and the chain length of fatty acids (Fig. 2c). These results indicate that the consumption of malic acid is strain specific, and this metabolic activity is favoured in the presence of high contents of saturated short fatty acids and low percentage of C18:1.

In *O. oeni*, acclimation leads to an increase of Cyc C19:0 (Bastianini *et al.* 2000; Teixeira *et al.* 2002). However, the mechanisms of adaptation in *Lact. plantarum* seem to be different. The alterations observed in the fatty acid composition of *Lact. plantarum* upon acclimation demonstrated that the US/S ratio plays a fundamental role on the adaptation to ethanol. Different authors reported the increase of the US/S after growing *Escherichia coli* and *O. oeni* in the presence of ethanol (Garbay *et al.* 1995; da Silveira *et al.* 2002; Teixeira *et al.* 2002). In spite of that, we observed a great decrease of the US/S in the three *Lact. plantarum* strains assayed. The higher ethanol concentration, the lower the US/S ratio (Table 1). These results are in agreement with data reported by van Bokhorst-van de Veen *et al.* (2011) for a strain of *Lact. plantarum* grown in MRS containing 8% v/v ethanol. This resistance mechanism leads to the decrease of membrane fluidity, making cells less permeable to ethanol and providing them the capacity to growth in wine and increase the consumption of L-malic acid.

Table 1 Change in the fatty acids parameters of *Lactobacillus plantarum* strains acclimated in presence of ethanol or nonacclimated. Consequences of acclimation on the viability and MAC after 15 days of incubation in synthetic wine at 28°C

Strain	Accl Conditions	USD*	US/S†	Chain length‡	Log CFU ml^{-1}§¶	MAC (%)¶
Lactobacillus plantarum UNQLp133	Non-Accl	0·28 ± 0·01	0·41 ± 0·05	15·95 ± 0·80	6·8 ± 0·15	37·8 ± 2·9
	Accl Eth 6%	0·17 ± 0·01	0·21 ± 0·05	14·14 ± 0·71	8·0 ± 0·17	48·9 ± 3·5
	Accl Eth 10%	0·06 ± 0·00	0·07 ± 0·05	13·50 ± 0·68	8·0 ± 0·17	54·0 ± 3·7
Lactobacillus plantarum UNQLp65.3	Non-Accl	0·43 ± 0·02	0·70 ± 0·05	17·45 ± 0·88	6·3 ± 0·30	44·4 ± 3·8
	Accl Eth 6%	0·35 ± 0·02	0·58 ± 0·05	16·03 ± 0·80	7·3 ± 0·18	71·1 ± 3·2
	Accl Eth 10%	0·21 ± 0·01	0·26 ± 0·05	15·63 ± 0·78	7·4 ± 0·22	90·0 ± 2·9
Lactobacillus plantarum UNQLp155	Non-Accl	0·37 ± 0·02	0·61 ± 0·05	16·42 ± 0·82	7·3 ± 0·21	55·6 ± 4·0
	Accl Eth 6%	0·24 ± 0·01	0·33 ± 0·05	14·84 ± 0·74	8·3 ± 0·15	67·3 ± 3·5
	Accl Eth 10%	0·16 ± 0·01	0·19 ± 0·05	14·97 ± 0·75	8·4 ± 0·14	71·8 ± 3·2

MAC, malic acid consumption; US/S, unsaturated/saturated; USD, unsaturation degree.

*Unsaturation degree = Σ(C18:1 + C16:1)/100.

†Unsaturated to saturated fatty acid ratio = Σ(C18:1 + C16:1)/(10:0 + 12:0 + 14:0 + 16:0 + 19:0cyc).

‡Mean fatty acid chain length = Σ(P × *C*)/100 (P = percentage contribution of each fatty acid and C = corresponding number of carbon atoms).

§Initial inoculum 1 × 10^7 CFU ml^{-1}.

¶Results previously reported in Bravo-Ferrada *et al.* 2014.

Table 2 Correlation values between fatty acids composition, viability and MAC of acclimated and nonacclimated *Lactobacillus plantarum* UNQLp 133, 65·3 and 155 grown in the presence of 6 and 10% v/v ethanol

	C10:0	C12:0	C14:0	C16:0	C16:1	C18:1	Cyc19:0	Viability	MAC†
10:0	1·00								
12:0	0·46	1·00							
14:0	0·47	0·51	1·00						
16:0	0·20	−0·37	−0·12	1·00					
16:1	−0·43	−0·66	−0·29	−0·11	1·00				
18:1	−0·63	**−0·88****	−0·57	0·10	0·57	1·00			
cycC19:0	−0·50	**−0·72***	**−0·67***	−0·24	0·58	**0·74***	1·00		
Viability	0·28	**0·67***	0·64	−0·20	−0·24	**−0·82****	−0·61	1·00	
MAC†	0·63	0·04	0·46	−0·07	0·28	−0·32	−0·06	0·40	1·00

MAC, malic acid consumption. Correlation values above 0·65 were indicated in bold.
*Correlation is significant at the 0·05 level.
**Correlation is significant at the 0·01 level.
†MAC (%) after 15 days in synthetic wine.

The relationship between resistance to ethanol and increase of the short-length fatty acid reflects the complexity of the mechanisms involved. van Bokhorst-van de Veen *et al.* (2011) reported that ethanol stress affects the expression of *Lact. plantarum* genes associated with fatty acids biosynthesis. They found that in the presence of ethanol, genes coding for fatty acid elongation and acyl carrier protein down-regulate, with a concomitant induction of the operons involved in the initiation phase of fatty acid biosynthesis. These results may explain the decrease of C18:1 and increase of short fatty acid content observed in the present work for *Lact. plantarum* strains acclimated in the presence of different concentrations of ethanol.

The regulation of the chain length of fatty acids, also related with membrane fluidity, could be other metabolic strategy leading to maintain bacteria homoeostasis. In this regard, it was reported that short-chain-length fatty acids fluidize membranes when certain micro-organisms are exposed to ethanol (Weber and de Bont 1996). In addition, short-chain-length fatty acids have been described to counteract the inter-digitations produced by ethanol on lipid bilayers, thereby maintaining the membrane permeability (Weber and de Bont 1996). This could be the mechanism of adaptation of *Lact. plantarum* in the acclimation conditions reported in this work (especially the high correlation between the drastic decrease of C18:1 and the increase of cell viability after ethanol stress).

The selection of efficient malolactic starters for winemaking requires a deep knowledge on the acclimatization conditions. *Lactobacillus plantarum* is widely used in food biotechnology of fermented products and has been recently proposed as a functional strain to improve winemaking conditions (Du Plessis *et al.* 2004; López *et al.* 2008; Lerm *et al.* 2011; Miller *et al.* 2011; Bravo-Ferrada *et al.* 2013). The results presented in this work showed that *Lact. plantarum* strains isolated from red wines have different strategies to regulate the membrane lipid composition, and thus overcome the harsh wine conditions.

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) (GenBank 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. Cells 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.

Acclimation conditions of LAB strains

Cells in the stationary phase (approximately 10^9 CFU ml^{-1}) were harvested by centrifugation at 4000 ***g*** for 10 min, suspended in the same volume (10 ml) of an acclimation medium containing 6 and 10% v/v ethanol and incubated at 28°C for 48 h. The composition of the acclimation medium was: 50 g l^{-1} MRS, 40 g l^{-1} D(-) fructose, 20 g l^{-1} D(-) glucose, 4 g l^{-1} L-malate, 1 g l^{-1} Tween 80, 0·1 mg l^{-1} piridoxina, and 6% v/v or 10% v/v ethanol (pH: 4·6) (Bravo-Ferrada *et al.* 2014).

Lipid extraction

Lipids were extracted according to the modified Bligh and Dyer method (Marinetti 1993). Briefly, cell pellets were suspended in chloroform–methanol–water (1 : 2 : 0·8 v/v/v) (4·75 ml per g of cells) for 12 h at 4°C, and then

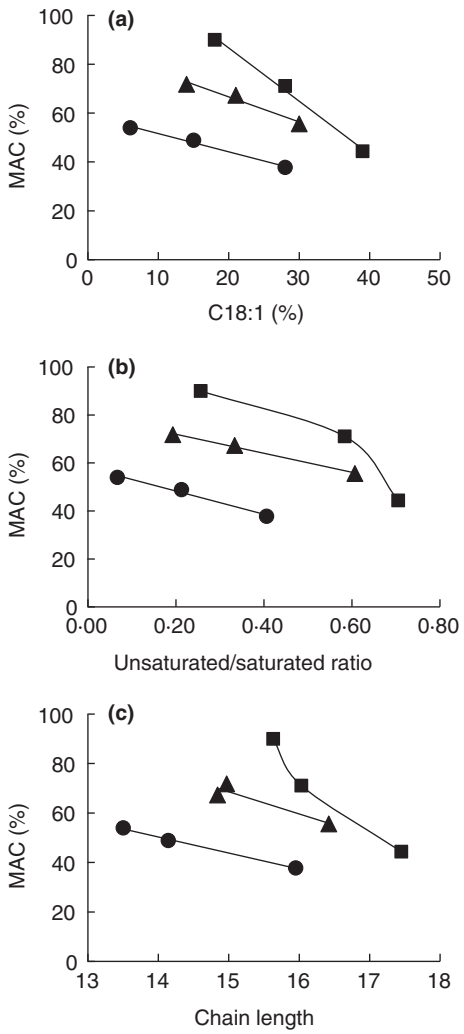

Figure 2 Percentage of malic acid consumption (MAC) as function of the content of: (a) C18:1, (b) Unsaturated/saturated ratio and (c) Chain length of fatty acids for *Lactobacillus plantarum* UNQLp133 (●), UNQLp 65.3 (■) and UNQLp 155 (▲).

centrifugated 10 min at 8000 ***g*** at 10°C. The supernatant was collected, and a second extraction on the bacterial pellet was performed. Both supernatants were mixed, and chloroform–water (1 : 1) was added (12·5 ml per g of cell culture). The final mixture was centrifuged at 8000 ***g*** for 20 min. The chloroform phase was collected and dried under vacuum (Rotavapor® RE 120—Büchi, Flawil, Switzerland). Lipids were dissolved in chloroform (final concentration 3 mg ml^{-1}) and stored at −20°C for up to 2 weeks.

Characterization of fatty acids

The fatty acids methyl esters (FAME) were prepared by adding 2 ml sulphuric acid (20 g l^{-1} in methanol) to 3 mg bacterial lipids, heating at 60°C for 2 h, extracting the esters with 1 ml chloroform–water (2 : 0·7 v/v) and washing twice with 0·7 ml water. The obtained FAME were analysed on a gas chromatograph interfaced with a mass spectrometer detector (Thermo Scientific EM/DSQ II and Trace GC Ultra AI3000, Austin, TX) using capillary column Rxi-5 ms (30 m × 0·25 mm). The analysis conditions were: injection temperature 250°C, detector temperature 280°C and column temperature initially 60°C increased to 290°C at 10°C min^{-1}. The FAME were identified by mass spectrometry (GC-MS). The fatty acid composition was determined by considering the relative contribution of each peak area.

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 (5 g l^{-1} tartaric acid, 4·5 g l^{-1} malic acid, 0·6 g l^{-1} acetic acid, 2 g l^{-1} glucose, 2 g l^{-1} fructose and 14·0% v/v ethanol, pH 3·5.) and incubated at 28°C without shaking. Bacterial growth was determined by plating on MRS agar after 15 days. The MAC was determined using a malic acid enzymatic assay (L-Malic Acid MegaQuant™ Format enzymatic kit, Megazyme International, Wicklow, Ireland). Nonacclimated cells were inoculated in synthetic wine as a control.

Statistical analysis

Determinations were performed in duplicate from three independent cultures of each bacterial strain studied. The relative differences were reproducible, independently of the culture used. The effect of fatty acid composition on the viability and MAC was analysed on the three strains, by determining the Pearson correlation coefficient. Correlation was significant for $P < 0{\cdot}05$. Analysis of variance (ANOVA) was carried out using Tukey's HDS test and if $P < 0{\cdot}05$ the differences were considered statistically significant. ANOVA and Pearson correlation were carried out using the statistical program Statistix 8 (Analitical Software, Tallahassee, FL).

Acknowledgements

This work has been funded by grants from the National University of Quilmes (Program for Fundamental Molecular Microbiology and Biotechnological Applications) and National Agency for Scientific and Technological Promotion (ANPCyT, Argentina, PICT SU 2012 No 2804, PICTO-UNQ 2006 No 36474). BMBF is recipient of a fellowship from the National Research Council (CONICET, Argentina). EET and AGZ are members of the Research

Career from CONICET. LS is member of the Research Career from the Commission for Scientific Research of the Buenos Aires Province (CIC-PBA).

Conflict of Interest

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

References

Bastianini, A., Granchi, L., Guerrini, S. and Vincenzini, M. (2000) Fatty acid composition of malolactic *Oenococcus oeni* strains exposed to pH and ethanol stress. *Ital J Food Sci* **12**, 333–342.

van Bokhorst-van de Veen, H., Abee, T., Tempelaars, M., Bron, P.A., Kleerebezem, M. and Marco, M.L. (2011) Short- and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*. *Appl Environ Microbiol* **77**, 5247–5256.

Bravo-Ferrada, B.M., Hollmann, A., Delfederico, L., Valdés La Hens, D., Caballero, A. and Semorile, L. (2013) Patagonian red wines: selection of *Lactobacillus plantarum* isolates as potential starter cultures for malolactic fermentation. *World J Microbiol Biotechnol* **29**, 1537–1549.

Bravo-Ferrada, B.M., Tymczyszyn, E.E., Gómez-Zavaglia, A. and Semorile, L. (2014) Effect of acclimation medium on cell viability, membrane integrity and ability to consume malic acid in synthetic wine by oenological *Lactobacillus plantarum* strains. *J Appl Microbiol* **116**, 360–367.

Cecconi, D., Milli, A., Rinalducci, S., Zolla, L. and Zapparoli, G. (2009) Proteomic analysis of *Oenococcus oeni* freeze-dried culture to assess the importance of cell acclimation to conduct malolactic fermentation in wine. *Electrophoresis* **30**, 2988–2995.

Chu-Ky, S., Tourdot-Maréchal, R., Maréchal, P.A. and Guzzo, J. (2005) Combined cold, acid, ethanol shocks in *Oenococcus oeni*: effects on membrane fluidity and cell viability. *Biochim Biophys Acta* **1717**, 118–124.

De Man, J.O., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Du Plessis, H.W., Dicks, L.M.T., Pretorius, I.S., Lambrechts, M.G. and du Toit, M. (2004) Identification of lactic acid bacteria isolated from South African brandy base wines. *J Food Microbiol* **91**, 19–29.

G-Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C. *et al.* (2004) High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiol Lett* **230**, 53–61.

Garbay, S., Rozes, N. and Lonvaud-Funel, A. (1995) Fatty acid compositions of *Leuconostoc oenos*, incidence of growth conditions and relationship with malolactic efficiency. *Food Microbiol* **12**, 387–395.

Grandvalet, C., Assad-García, J.S., Chu-Ky, S., Tollot, M., Guzzo, J., Gresti, J. and Tourdot-Maréchal, R. (2008) Changes in membrane lipid composition in ethanol- and acid-adapted *Oenococcus oeni* cells: characterization of the cfa gene by heterologous complementation. *Microbiology* **154**, 2611–2619.

Guzzo, J., Delmas, F., Pierre, F., Jobin, M.P., Samyn, B., Van Beeumen, J., Cavin, J.F. and Divies, C. (1997) A small heat shock protein from *Leuconostoc oenos* induced by multiple stresses and during stationary growth phase. *Lett Appl Microbiol* **24**, 393–396.

Henick-Kling, T. (1995) Control of malolactic fermentation in wine: energetic, flavour modification and methods of starter culture preparation. *J Appl Bacteriol Suppl* **79**, 29S–37S.

Lee, S.G., Lee, K.W., Park, T.H., Park, J.Y., Han, N.S. and Kim, J.H. (2012) Proteomic analysis of proteins increased or reduced by ethanol of *Lactobacillus plantarum* ST4 isolated from *Makgeoli*, traditional Korean rice wine. *J Microbiol Biotechnol* **22**, 516–525.

Lerm, E., Engelbrecht, L. and du Toit, M. (2010) Malolactic fermentation: the ABC′s of MLF. *S Afr J Enol Vitic* **31**, 186–192.

Lerm, E., Engelbrecht, L. and du Toit, M. (2011) Selection and characterization of *Oenococcus oeni* and *Lactobacillus plantarum* South African wine isolates for use as malolactic starter cultures. *S Afr J Enol Vitic* **32**, 280–295.

López, I., López, R., Santamaría, P., Torres, C. and Ruiz-Larrea, F. (2008) Performance of malolactic fermentation by inoculation of selected *Lactobacillus plantarum* and *Oenococcus oeni* strains isolated from Rioja red wines. *Vitis* **47**, 123–129.

Marinetti, G.V. (1993) Isolation, analysis and identification of lipids. In *Techniques of Lipidology*, ed. Kates, M., Burdon, H.R. and Knippemberg, P.H. pp. 220–227. Amsterdam: Elsevier.

Miller, B.J., Franz, C.M., Cho, G.S. and du Toit, M. (2011) Expression of the malolactic enzyme gene (*mle*) from *Lactobacillus plantarum* under winemaking conditions. *Curr Microbiol* **62**, 1682–1688.

da Silveira, M.G. and Abee, T. (2009) Activity of ethanol-stressed *Oenococcus oeni* cells: a flow cytometric approach. *J Appl Microbiol* **106**, 1690–1696.

Silveira, M.G., Baumgartner, M., Rombouts, F.M. and Abee, T. (2004) Effect of adaptation to ethanol on cytoplasmic and membrane protein profiles of *Oenococcus oeni*. *Appl Environ Microbiol* **70**, 2748–2755.

da Silveira, M.G., San Romão, V., Loureiro-Dias, M.C., Rombouts, F.M. and Abee, T. (2002) Flow cytometry assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. *Appl Environ Microbiol* **68**, 6087–6093.

da Silveira, M.G., Golovina, E.A., Hoekstra, F.A., Rombouts, F.M. and Abee, T. (2003) Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells. *Appl Environ Microbiol* **69**, 5826–5832.

Solieri, L., Genova, F., De Paola, M. and Giudici, P. (2009) Characterization and technological properties of *Oenococcus oeni* strains from wine spontaneous malolactic fermentations: a framework for selection of new starter cultures. *J Appl Microbiol* **108**, 285–298.

Teixeira, H., Gonçalves, M.G., Rozés, N., Ramos, A. and San Romão, M.V. (2002) Lactobacillic acid accumulation in the plasma membrane of *Oenococcus oeni*: a response to ethanol stress? *Microb Ecol* **43**, 146–153.

Weber, F.J. and de Bont, J.A.M. (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim Biophys Acta* **1286**, 225–245.