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ORIGINAL ARTICLE

Study of surface damage on cell envelope assessed by AFM and flow cytometry of *Lactobacillus plantarum* exposed to ethanol and dehydration

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Abstract

Aims: In this work, we evaluated freeze-drying damage at the surface level of oenological strain *Lactobacillus plantarum* UNQLp155, as well as its ability to grow in a synthetic wine with and without pre-acclimation.
Methods and Results: Damage on cell surface was studied by flow cytometry, zeta potential and atomic force microscopy, and cell survival was analysed by plate count. Results showed that beside cells acclimated at lower ethanol concentration (6% v/v) became more susceptible to drying than nonacclimated ones, after rehydration they maintain their increased ability to grow in a synthetic wine. Acclimation at a higher ethanol concentration (10% v/v) produces several damages on the cell surface losing its ability to grow in a synthetic wine.
Conclusions: In this work, we showed for the first time that sublethal alterations on bacterial surface induced by a pre-acclimation with a low ethanol concentration (6%), upon a freeze-drying process, result in a better bacterial adaptation to the stress conditions of wine-like medium, as well as to the preservation process.
Significance and Impact of the Study: Understanding the adaptation to ethanol of oenological strains and their effects on the preservation process has a strong impact on winemaking process and allows to define the most appropriate conditions to obtain malolactic starters cultures.

Introduction

Freeze-drying is usually used in the preservation of lactic acid bacteria (LAB) commercial starters. However, this technique brings about side effects, such as changes in the physical state of membrane lipids and structure of sensitive proteins, decreasing cell viability (Leslie *et al.* 1995).

Many factors are related with microbial cell survival throughout freeze-drying and storage, growth factors being a critical parameter which determines the physiological state of bacteria (Carvalho *et al.* 2004). Cytoplasmic membrane integrity and damage on the cell surface have been related to cell viability after drying (Broadbent and Lin 1999; Santivarangkna *et al.* 2006; Tymczyszyn *et al.* 2008). The cell surface is formed by different components, including lipoteichoic acids, polysaccharides, S-layer proteins and glycoproteins (Schar-Zammaretti and Ubbink 2003). These components have important physiological roles, for instance in cell adhesion, and on the retention of heavy metals and toxins. Therefore, drying conditions, as well as preparation of

cell suspensions and powder formulations, have to be carefully evaluated to obtain high survival rates of viable bacteria. Sugars are added in freeze-drying media to protect membrane and improve cell recovery, being trehalose one of the best cryoprotectants reported (Gomez Zavaglia *et al.* 2003; Tymczyszyn *et al.* 2007b).

Malolactic fermentation (MLF) is conducted by lactic acid bacteria (LAB) and often follows the alcoholic fermentation during winemaking. MLF is essential to decrease acidity resulting from the decarboxylation of L-malic acid to L-lactic acid and CO_2. For this reason, it has placed a great emphasis on the development of malolactic starter cultures and processes of preserving them (Maicas *et al.* 2000).

However, wine is not an appropriate environment for LAB due to their physicochemical and biological parameters (*e.g.* low pH, ethanol concentration, SO_2 tolerance and yeast-bacteria interactions) that are able to inhibit bacterial growth and, consequently malolactic activity. When these conditions occur, the starter cultures inoculated into wine undergo a high rate of mortality, thus leading to the failure of MLF (Cecconi *et al.* 2009). In a previous work, we reported that acclimation of *Lactobacillus plantarum* cells in a media containing 6 or 10% v/v ethanol and low pH (3·5) was able to increase the ethanol resistance of the Patagonian oenological strain UNQLp 155 in wine-like medium. By multiparametric flow cytometry, we demonstrated that the acclimated strains showed lower damage after wine inoculation than nonacclimated strains (Bravo-Ferrada *et al.* 2014). In addition, the increase in resistance to ethanol was correlated with change in fatty acid composition of lipid membrane, with an increase in saturation degree and decrease in the chain length of lipids (Bravo-Ferrada *et al.* 2015).

Expression of heat shock proteins and changes in membrane protein profile have also been reported as adaptation mechanism to ethanol (Da Silveira *et al.* 2003; Silveira *et al.* 2004; Da Silveira and Abee 2009). Additionally, cell growth of *Lact. plantarum* in the presence of ethanol leads to changes in the cell wall, as observed by transmission electronic microscopy (TEM) (van Bokhorst-van de Veen *et al.* 2011). However, the effect of acclimation treatment on the dehydration and its effects on the cell surface have not been studied yet.

With this background, the aim of this work was to evaluate the effects that different acclimation treatments and subsequent freeze-drying, in the presence of trehalose as protectant, on cell viability, resistance to ethanol, damage at the cell surface and membrane integrity. For this purpose, flow cytometry, atomic force microscopy (AFM) and zeta potential techniques were applied. *Lactobacillus plantarum* UNQLp 155 strain isolated from a red Patagonian wine was selected as potential starter culture for MLF due to their previously described oenological and technological properties (Bravo-Ferrada *et al.* 2013).

Material and methods

Strain, medium and growth conditions

Lactobacillus plantarum UNQLp 155 was isolated from Patagonian Pinot noir red wine (Bravo-Ferrada *et al.* 2013). The identification of *Lact. plantarum* was verified by amplifying and sequencing the beta subunit of the rpoB gene (GenBank Accession Numbers KC679067), and sequence of 16S rRNA gene (GenBank Accession Numbers KC562904). 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 (AnaeroPack - Mitsubishi Gas Chemical America, Inc., New York, NY).

Acclimation conditions

Cells in the early stationary phase (approx. 10^9 CFU ml^{-1}) were harvested by centrifugation at 5000 ***g*** for 10 min and suspended in the same volume (10 ml) of a modified acclimation medium (Accl.) containing 0%, 6% or 10% v/v ethanol. The Accl. composition was defined according to Lerm *et al.* (2011) and Bravo-Ferrada *et al.* (2014). After incubation at 28°C for 48 h, the acclimated cells were harvested by centrifugation and prepared for freeze-drying.

Sample preparation for freeze-drying

The cells in 1 ml of a culture, previously acclimated or nonacclimated, were harvested by centrifugation at 5000 ***g*** for 10 min. Pellets were washed twice with 0·85% NaCl w/v, and resuspended in 1 ml of 20% (w/w) aqueous solution of trehalose, previously sterilized using 0·2 μm pore sterile filters (Tymczyszyn *et al.* 2007a).

Freeze-drying procedure

Aliquots of 1 ml containing cell suspension in trehalose were transferred into 5 ml glass vials under aseptic conditions and frozen for 24 h at −80°C. A freeze-dryer Labconco Freeze Dry System/Freezone 4·4 (Labconco Corporation, Kansas, MO) operated with condenser at −50°C at a chamber pressure of 0·05 mbar was used. The freeze-drying process lasted for 24 h. The residual water content of the dried powders was 5·8 ± 0·1 % (dry

basis) and it was determined in a vacuum oven at 70°C until a constant weight was attained.

Determination of cell viability

The number of viable cells before and after the freeze-drying process was determined as colony forming units (CFU). Decimal dilutions were prepared from the suspension before freezing and plated on MRS agar. Freeze-dried samples were rehydrated to their original volume with 0·85% NaCl w/v, incubated at room temperature for 15 min, and subsequently diluted and plated on MRS agar. Plates were incubated at 28°C for 48 h and the colonies counted. Viability was expressed as CFU ml^{-1}.

Tolerance to synthetic wine

Bacterial tolerance to environment conditions was studied at laboratory scale using 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). The composition of the wine-like medium was similar to that described by Ugliano *et al.* (2003) but adapted to the composition of the Patagonian red wines (low pH and high ethanol concentration) (Bravo-Ferrada *et al.* 2014). Ten millilitre of synthetic wine was inoculated with the rehydrated cells (previously acclimated in the presence of 0, 6 or 10% v/v ethanol), at 21°C without shaking, during 24 h. After that, bacterial viability was monitored by plating on MRS agar at 28°C for 48 h.

Flow cytometry analysis

Cells were incubated with the DNA-binding probe propidium iodide (PI), which penetrates cells only when membranes are damaged. Stock solutions of PI (Molecular Probes, Leiden, Netherlands) were prepared in distilled water to a final concentration of 10 g l^{-1} and stored in the dark at 4°C. PI was added to a final concentration of 0·5 g l^{-1} and incubated for 5 min at room temperature. For the flow cytometry analysis, the cell concentration in the samples was adjusted to approx. 10^6 CFU ml^{-1}. PI uptake was followed by flow cytometry (FACSCALIBUR, CELLQUEST software; Becton Dickinson, Mountain View, CA), according to a previously reported procedure (Hugo *et al.* 2012). Samples were analysed before and after the freeze-drying process, as well as after incubation in synthetic wine at 21°C for 24 h. For each sample, 10 000 events were collected, keeping the event rate below 300 events s^{-1}. Untreated stained cells were used as negative control and cells heated for 3 min at 80°C as positive control.

Atomic force microscopy imaging

Direct observation of ethanol-induced effects on the morphology of bacterial cells was conducted by AFM. UNQLp 155 cells were acclimated at 0, 6 or 10% v/v ethanol and then freeze-dried. Control samples were grown in MRS broth for 48 h at 28°C. Cell suspensions with 1×10^9 cells ml^{-1} were spun down at 3000 ***g*** for 10 min and washed twice with 0·85% NaCl to remove the media. A 100 μl droplet of each sample was applied onto a glass slide and allowed to rest at 25°C for 1 h. After deposition, the sample was rinsed 10 times with Milli-Q water and air-dried at 25°C. On average, five individual bacterial cells were imaged at high resolution for each sample. AFM images were acquired using a JPK NanoWizard II (Berlin, Germany) mounted on a Zeiss Axiovert 200 inverted microscope (Göttingen, Germany). Measurements were carried out in intermittent contact mode using ACT silicon cantilevers from AppNano (Huntingdon, UK), with a nominal tip radius of 6 nm. ACT cantilevers displayed typical frequencies of 300 kHz and a spring constant of 40 N/m. All images were obtained with similar AFM parameter (setpoint, scan rate and gain) values. Height and error signals were collected and images were analysed with the JPK Instruments image processing software ver. 4.2.53.

Surface roughness analysis

The data generated from the AFM height images were used to calculate the surface quadratic roughness of the bacterial cell surface. Using the software GWYDDION ver. 2.19 (Czech Metrology Institute, Brno, Czech Republic), the bacterial cell shape was estimated through the application of a mean filter to the raw data. Subtraction of the treated image from the original height image generated a flattened representation of the bacterial cell surface; the surface roughness of a selected area of this flattened image was then calculated from the height standard deviation, i.e. the root-mean-square value (rms) of the height distribution in Equation 1,

$$\mathrm{rms} = \sqrt{\sum_{i=1}^{n} \frac{(z_i - z_m)^2}{N-1}} \qquad (1)$$

where, N is the total number of data points, z_i is the height of the i-th point and z_m is the mean height (Girasole *et al.* 2007; Alves *et al.* 2010). Roughness values were measured over the entire bacterial cell surface on areas with a fixed size of 75×75 nm^2.

Zeta potential

Zeta potential studies were performed at 25°C on a Zetasizer Nano ZS (Malvern Instruments, Worcestershire,

UK), equipped with a 633-nm He–Ne laser. Cell dilutions were prepared using 10 mmoles l^{-1} HEPES buffer, pH 7·4, containing 150 mmoles l^{-1} NaCl, and then filtered using a 0·22 μm pore-size filter. The bacterial suspensions were dispensed into disposable zeta potential cells with gold electrodes and allowed to equilibrate for 15 min at 25°C. The zeta potential for each sample was calculated from the measured value of electrophoretic mobility using the Smoluchowski equation (Hunter 1981). The complete experiment was carried out twice for each sample, using independently grown cultures.

Reproducibility of results and statistical analysis

Measurements were done in duplicate from three independent cultures of each group studied. Analysis of variance (ANOVA) was carried out using the statistical software PRISMA GRAPH. Data are showed as mean value.

Results

Membrane damage and cell viability

Cell viability of UNQLp 155 strain, grown in MRS broth or previously acclimated in different conditions, was determined before and after the freeze-drying process (Fig. 1). No significant differences were observed before and after preservation when *Lact. plantarum* cells were grown in MRS, or acclimated in 0 or 6% ethanol. However, a significant decrease in cell viability was observed when the cells acclimated in 10% ethanol were freeze-dried.

To evaluate the effect of acclimation and freeze-drying on the technological properties, acclimated and nonacclimated *Lact. plantarum* cells were incubated in a synthetic wine (14% v/v ethanol, pH 3·5), before and after freeze-drying process (Fig. 2). Before preservation, cells acclimated in 6 or 10% ethanol showed a higher resistance to wine-like conditions than cells that were nonacclimated or acclimated in 0% ethanol. After the preservation process, cultures nonacclimated or acclimated in 0% ethanol showed the same number of viable cells as the cultures previous to freeze-drying. On the other hand, freeze-dried cells acclimated in 6 or 10% ethanol exhibited a decrease in viability after wine-like inoculation. However, cells acclimated in 6% ethanol showed a higher resistance to wine-like conditions than cells acclimated in 10% ethanol.

PI uptake (%) in UNQLp 155 strain cells, after different treatments, is showed in Fig. 3. Acclimated cells (in 0, 6 or 10% v/v ethanol) showed an increase in PI uptake, indicating the induction of membrane damage during the acclimation treatments. After freeze-drying, a higher increase in PI uptake was observed for all treatments, and no differences were observed when rehydrated cells were incubated in synthetic wine. These results suggest that the freeze-drying process may impair membrane integrity.

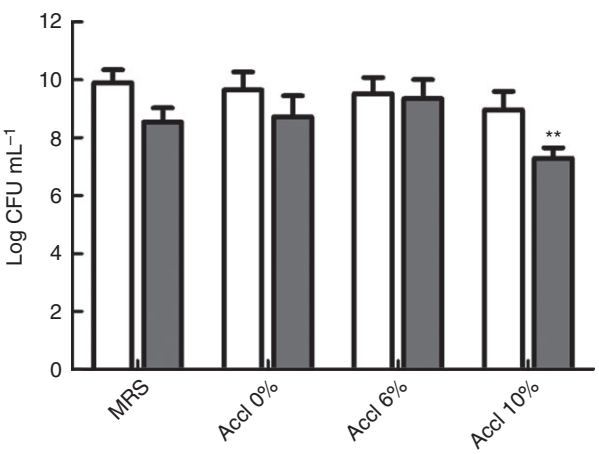

Figure 1 Cultivability of *Lactobacillus plantarum*UNQLp 155 cells nonacclimated (MRS) and acclimated in the presence of ethanol 0% (Accl 0%), 6% (Accl 6%) or 10% v/v (Accl 10%), before (white bars) and after (grey bars) freeze-drying. Each point is the mean ± SD. $^{**}P < 0{\cdot}01$, one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs* the control column (MRS before freeze-drying).

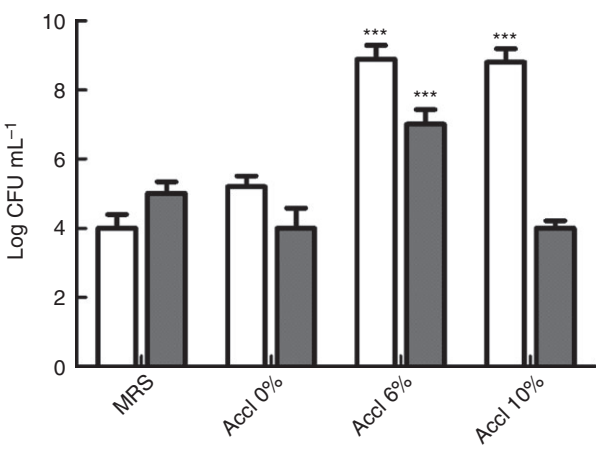

Figure 2 Cultivability of *Lactobacillus plantarum*UNQLp 155 cells nonacclimated (MRS) and acclimated in the presence of ethanol 0% (Accl 0%), 6% (Accl 6%) or 10% v/v (Accl 10%), before (white bars) and after (grey bars) freeze-drying and incubation for 24 h in a synthetic wine with 14% ethanol, at 21°C. Each point is the mean ± SD. $^{***}P < 0{\cdot}001$, one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs* the control column (MRS wine 14%).

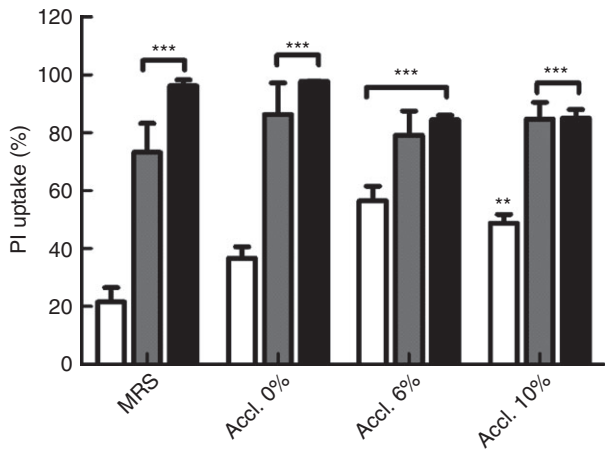

Figure 3 Percentages of PI uptake in *Lactobacillus plantarum* UNQLp 155 cells grown in MRS and acclimated in different ethanol concentrations (0, 6 and 10%), before freeze-drying (white bars), after freeze-drying (grey bars) and after freeze-drying, followed by wine-like incubation for 24 h with 14% ethanol, at 21°C (solid black bars). Each point is the mean ± SD. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs* the control column (MRS Control).

Atomic force microscopy

Cell envelope damage of *Lact. plantarum* strain was imaged by AFM. Figures 4a,b show, as expected, smooth cell surfaces before freeze-drying (fresh culture control). The surface remained relatively smooth after drying without previous acclimation treatment (Fig. 4c,d) and when cells were acclimated in a medium with 0% ethanol, indicating no obvious damage on the cell envelope (Fig. 4e, f). When the cells were acclimated in 6% ethanol, some disruptions in the bacterial surface were observed (Fig. 4g,h). Finally, clear damages on the cell surface appeared in cells acclimated in 10% ethanol, together with an increase on the overall surface roughness and, in some cases, membrane disruption and extensive leakage of intracellular content (Fig. 4i,j). Cracks on the cell surface or lysis were found in other cells (Fig. 4e,f).

To quantify the damage exerted by the freeze-drying process, the roughness of the *Lact. plantarum* cell surface was measured and treated to correlate the functional behaviour of this bacterial strain with topographic properties by using roughness parameters. Surface roughness measurements of untreated cells (1·37 ± 0·45 nm) are in good agreement with previous publication (Andre *et al.* 2011), serving as control. Figure 5 shows the average surface roughness calculated for the bacterial cells in the different study conditions. Values of surface roughness were not significantly affected by the freeze-drying of nonacclimated cells (1·05 ± 0·74 nm) and acclimated in 0% ethanol (1·23 ± 0·43 nm) in relation to the control. In contrast, the values of surface roughness for freeze-dried cells acclimated in 6 or 10% ethanol were significantly affected (2·64 ± 0·96 nm and 6·65 ± 1·87 nm respectively) (Fig. 5).

Zeta potential

The zeta potential is a measure of the charge that develops at the interface between a surface and its liquid environment (Hunter 1981). It is determined by the surface macromolecules and their structure, and it has been employed to determine the physiological cell state after drying and rehydration (Fernandez-Murga *et al.* 2000; Gómez Zavag lia *et al.* 2003; Tymczyszyn *et al.* 2007a). The variation in zeta potential of cells has been measured before and after the freeze-drying process, and has been shown to be related with the preservation process (Tymczyszyn *et al.* 2008). In this work, the Zeta potential of control cells, grown in MRS broth, was close to −14 mV (Fig. 6).

Zeta potential values were not significantly affected in freeze-dried cells nonacclimated (−13·9 ± 1·04 mV) or acclimated in 0 or 6% ethanol (−14·1 ± 1·40 mV and −13·8 ± 0·46 mV, respectively) relative to the control (−14·0 ± 0·30). In contrast, the values of zeta potential for freeze-dried cells acclimated in 10% ethanol were significantly different, reaching more negative values, close to −21 mV (Fig. 6).

Discussion

The obtention of malolactic starter cultures requires the previous oenological and technological characterization of LAB strains, and depends strongly on the preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in term of viability and activity (Carvalho *et al.* 2004). Also, the knowledge of acclimation conditions is needed, in order to improve cell growth in wine and the performance of MLF after the preservation process (Solieri *et al.* 2010; Lerm *et al.* 2011).

In a previous work, we showed that acclimation with sub-lethal ethanol concentrations improve the cell viability of three Patagonian *Lact. plantarum* strains in wine-like medium (Bravo-Ferrada *et al.* 2014). In this work, we studied the effect of preservation on the behaviour and ability to grow in a wine-like media of one of those strains.

Our results showed that, whereas in the absence of cryoprotectant agent the cell recovery was lower than 10^4 CFU ml^{-1}, the freeze-drying process in the presence of trehalose as cryoprotector, results in a higher cell recovery ($\approx 10^9$ CFU ml^{-1}); only the acclimation in 10% ethanol produced a significant decrease in cultivability (Fig. 1). Also, the acclimation in 10% ethanol lead to a decrease in cell viability in the order of 5 log units after

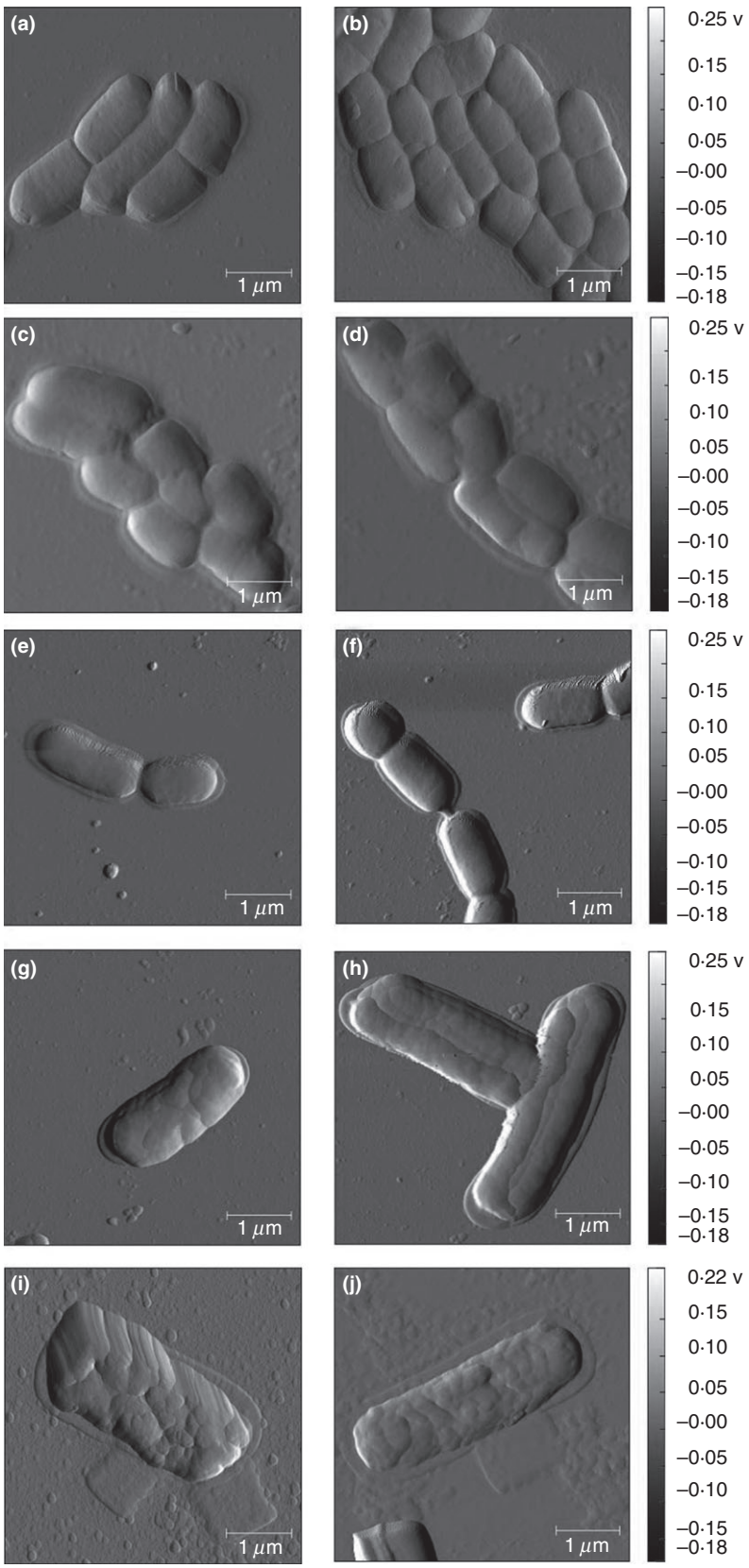

Figure 4 Atomic force microscopy error images of *Lactobacillus plantarum*UNQLp 155 cells. (a) and (b) Control (cells grown in MRS without freeze-drying). (c) and (d) Cells grown in MRS and freeze-dried. (e) and (f) Cells acclimated in 0% ethanol and freeze-dried. (g) and (h) Cells acclimated in 6% ethanol and freeze-dried. (i) and (j) Cells acclimated in 10% ethanol and freeze-dried.

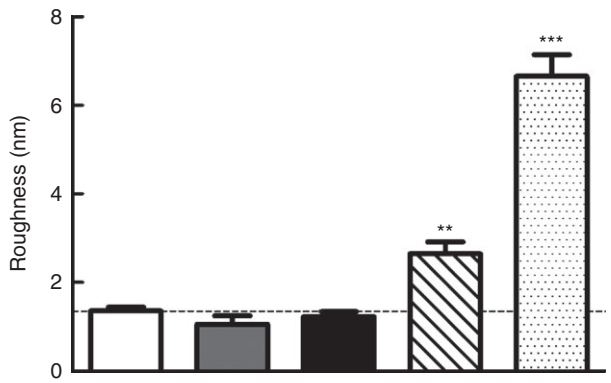

Figure 5 Surface roughness (calculated based on the atomic force microscopy images) of *Lactobacillus plantarum* UNQLp 155 cells submitted to different conditions. Fresh culture (white bars), cells grown in MRS and freeze-dried (grey bars), cells acclimated in 0% ethanol and freeze-dried (solid black bars), cells acclimated in 6% ethanol and freeze-dried (bars with diagonal lines), cells acclimated in 10% ethanol and freeze-dried (white bars with black dots). Each point is the mean ± SD. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs* the control column (MRS Control).

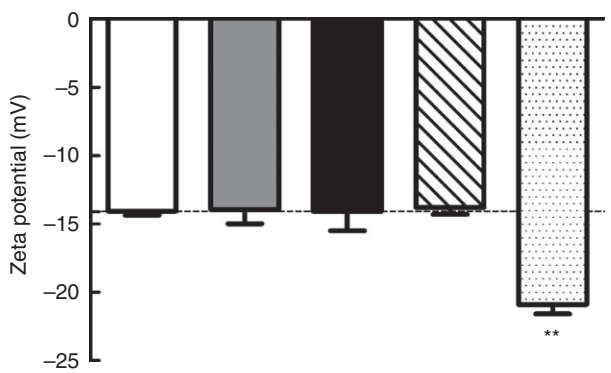

Figure 6 Zeta potential measurements of *Lactobacillus plantarum* UNQLp 155 cells submitted to different conditions. Fresh culture (white bars), cells grown in MRS and freeze-dried (grey bars), cells acclimated in 0% ethanol and freeze-dried (solid black bars), cells acclimated in 6% ethanol and freeze-dried (bars with diagonal lines), cells acclimated in 10% ethanol and freeze-dried (bars with black dots). Each point is the mean ± SD. $^{**}P < 0{\cdot}01$, one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs* the control column (MRS Control).

freeze-drying and inoculation into a wine-like medium with 14% ethanol for 24 h. Nonacclimated *Lact. plantarum* cells showed a similar viability reduction in a wine-like medium (14% ethanol). However, the acclimation treatment in the presence of lower ethanol concentration (6% v/v) proved to be the best condition for a subsequent growth of bacterial cells in a wine-like medium similar to Patagonian Pinot noir wines (pH 3·5, 14% ethanol), showing an increase in cultivability relative to nonacclimated bacterial cells (2 log units).

To correlate the viability results with membrane damage, PI uptake assays were performed. As it can be seen on Fig. 3, all the conditions tested showed higher membrane disruption after the dehydration process, before and after wine-like incubation.

Acclimation treatments induce changes in the cell membrane, including changes in the fatty acids and proteins composition, making the membrane less permeable to ethanol (Guzzo *et al.* 1997; Da Silveira *et al.* 2003; Chu-Ky *et al.* 2005). However, these changes are not sufficient to preserve membrane permeability after the drying and rehydration processes.

According to results showed in Figs 1 and 2, the membrane damage induced by ethanol is not lethal, and the cells can repair it and be able to grow, in good agreement with previous reports (Santivarangkna *et al.* 2006; Tymczyszyn *et al.* 2007a).

In a previous work, we reported that changes in the zeta potential were correlated with a decrease in the cell viability of members of the *Lactobacillus* genera (Tymczyszyn *et al.* 2008). Other authors have pointed that AFM could also be used to evaluate cell integrity (Santivarangkna *et al.* 2007). Thus, in this work, AFM imaging combined with zeta potential measurements were employed to complement the PI uptake data, in order to dissect the effects that different acclimation processes and freeze-drying itself induce on the cell envelope.

The AFM data obtained clearly demonstrate the details of the damage suffered by the *Lact. plantarum* cells following the acclimation treatments under different conditions, and freeze-drying process (Fig. 4). AFM images obtained immediately after rehydration of freeze-dried cells reveal that acclimation in 10% ethanol seem to be more aggressive, leading to a massive damage in the cell envelope, with loss of cellular content, in good agreement with the reduction in cell viability observed by plate count (Figs 1 and 2). Besides, the use of 6% ethanol in the acclimation media also induces some stress on the cell envelopes, but from the viability data (Fig. 2), we may conclude that this damage is not lethal. This sublethal stress on the cells could be related with the better adaptation to growth in the wine-like medium.

For a better evaluation of the effects on the membrane, other quantitative parameters were obtained. Surface roughness measurements, derived from AFM images, confirmed that acclimation in 10% ethanol increases cell roughness. Acclimation in 6% ethanol also induces an increase in surface roughness, but much less noticeable than the observed for 10% ethanol. Finally, in cells nonacclimated or acclimated in a medium without ethanol, no significant differences were found when compared

with control cell, confirming that damages on the cell envelope and its concomitant increase in the surface roughness should be ascribed to changes induced by ethanol during the acclimation process.

Zeta potential data reveal alterations on the cell surface only in bacteria acclimated in 10% ethanol, without significant changes in the remaining groups. These results indicate that cell growth at high ethanol concentrations produce changes at the surface level as an adaptation mechanism, but these changes are negative determinants for the drying process. To our best knowledge, this is the first time that changes on cell surface after acclimation treatments with ethanol are analysed.

From this study, we may report that nonacclimated cells and cells acclimated without ethanol present a higher resistance to the drying process but, without ethanol pretreatment, cells showed a poor ability to grow in wine-like conditions. On the other hand, acclimation treatments in the presence of 10% ethanol produce a physiological adaptation that improves cell growth in wine-like conditions, but also induce some changes on the bacterial surface that reduce its ability to support the freeze-drying process. The present work support the idea that a pre-acclimation with a lower ethanol concentration (6%) is able to induce sublethal alterations on bacteria that, upon a subsequent freeze-drying process, result in a better bacterial adaptation to the stress conditions of wine-like medium, as well as to the preservation process.

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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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