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Effect of protective agents and previous acclimation on ethanol resistance of frozen and freeze-dried *Lactobacillus plantarum* strains

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ABSTRACT

The aim of this work was to study the protective effect of sucrose, trehalose and glutamate during freezing and freeze-drying of three oenological *Lactobacillus plantarum* strains previously acclimated in the presence of ethanol. The efficiency of protective agents was assessed by analyses of membrane integrity and bacterial cultivability in a synthetic wine after the preservation processes. No significant differences in the cultivability, with respect to the controls cells, were observed after freezing at −80 °C and −20 °C, and pre-acclimated cells were more resistant to freeze-drying than non-acclimated ones. The results of multiparametric flow cytometry showed a significant level of membrane damage after freeze-drying in two of the three strains. The cultivability was determined after incubation in wine-like medium containing 13 or 14% v/v ethanol at 21 °C for 24 h and the results were interpreted using principal component analysis (PCA). Acclimation was the most important factor for preservation, increasing the bacterial resistance to ethanol after freezing and freeze-drying. Freeze-drying was the most drastic method of preservation, followed by freezing at −20 °C. The increase of ethanol concentration from 6 to 10% v/v in the acclimation medium improved the recovery of two of the three strains. In turn, the increase of ethanol content in the synthetic wine led to a dramatic decrease of viable cells in the three strains investigated. The results of this study indicate that a successful inoculation of dehydrated *L. plantarum* in wine depends not only on the use of protective agents, but also on the cell acclimation process prior to preservation, and on the ethanol content of wine.

1. Introduction

Malolactic fermentation (MLF) usually occurs in the winemaking process after alcoholic fermentation (AF), and is carried out by indigenous lactic acid bacteria (LAB). MLF consists of the decarboxylation of L-malic acid into L-lactic acid reducing the acidity of wine. MLF also improves microbiological stability and organoleptic characteristics [14]. To control this fermentation process the use of starter cultures is a common practice. *Oenococcus oeni* is the major LAB used to stimulate MLF in commercial starter cultures. However, some *Lactobacillus plantarum* strains have also displayed the ability to survive the harsh wine conditions and commercial cultures of this LAB species have recently been used [13].

In red wines produced in North Patagonia – Argentina, MLF usually occurs spontaneously and randomly; any delay in the starting of this process can lead to an alterations of wine quality. To overcome this problem, the use of starter cultures is widely recommended. However, the harsh environment of wine can inhibit the bacterial growth. The success of starter cultures depends on the bacterial strain and it is conditioned by different factors, including the adaptation to the winemaking practices of each wine. Thus, the use of indigenous bacterial cultures, able to adapt to conditions of a specific wine-producing area, has been recommended by various authors [7,19].

The high ethanol concentration in wines (from 12 to 15% v/v) is the main cause for the decrease of LAB viability, being cell membranes the first target of damage. To overcome this harmful condition, LAB can be pre-adapted to grow in these environments. This adaptation consists in a previous incubation of bacteria in acclimation media containing low ethanol concentrations. In a previous

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paper, we reported that Patagonian *L. plantarum* strains, acclimated in presence of ethanol 6 or 10% v/v, were able to grow in a synthetic wine and metabolize malic acid in a quicker way than non-acclimated ones. Moreover, multiparametric flow cytometry analysis indicated that membranes of acclimated cells were more resistant to ethanol than non-acclimated ones [2]. This higher resistance was correlated with alterations in the composition of lipid membranes, namely an increase of the membrane saturated fatty acids and a decrease of their chain length [3].

It should further be noted that MLF starter cultures must withstand preservation processes, such as freezing and freeze-drying. Frozen and freeze-dried cultures should ideally have the same technological properties than before being preserved. However, during these processes, the reduction of water activity produces damages on the cell structures and thus, microorganisms may die [20,21]. To avoid these damages, sugars or amino acids are usually added to the preservation medium as cryo or lyoprotectants, trehalose, sucrose and glutamate being the most frequently used [5,23–25]. From a chemical point of view, protective agents generally have amino or hydroxyl groups that can replace water on the macromolecules. This replace of water molecules protects proteins and lipid membranes during preservation and thus, improves cell recovery [22].

The recovery of membrane properties after freeze-thawing or dehydration–rehydration processes is essential to successfully inoculate MLF starter cultures in wines with high ethanol concentrations. With this background, the aim of the present work was to study the efficiency of trehalose, sucrose and glutamate as protective compounds of three *L. plantarum* strains, as well as the effect of acclimation on their recovery after freezing (at −20 and −80 °C) and freeze-drying. Cultivability and membrane damage were assessed after these preservation processes. The presence of sub-lethal damage was evaluated by inoculating MLF starter cultures in a synthetic wine containing 13 or 14% v/v ethanol. Principal component analysis (PCA) enabled the correlation between the effect of the different treatments (acclimation, preservation methods, protective agents and ethanol concentration) and bacterial cultivability under wine-like conditions. The results obtained represent a strong background supporting the adequate preservation and recovery of MLF starter cultures.

2. Materials and methods

2.1. Strains, medium and growth conditions

L. plantarum UNQLp 133, UNQLp 65.3, and UNQLp 155 were isolated from Patagonian Pinot noir red wine [1] (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). Bacterial cells were grown in 10 mL of MRS broth (Biokar Diagnostics, Beauvais, France) [8], at 28 °C and pH 6.5 for 48 h.

2.2. Acclimation conditions of LAB strains

Cells in the stationary phase (approximately 10^{10} 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 or 10% v/v ethanol and incubated at 21 °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, g L^{-1} Tween 80, 0.1 mg L^{-1} pyridoxine, and 6% v/v or 10% v/v ethanol (pH: 4.6) [2].

2.3. Freeze-drying and freezing conditions

Bacterial cells acclimated with 6% or 10% v/v ethanol, and non-acclimated were harvested by centrifugation (13,000 × g for 6 min) at room temperature under aseptic conditions. Then, pellets were washed once with 50 mM potassium phosphate buffer solution (pH 6.5), centrifuged again, and resuspended in 1 mL of 2.5% w/v sodium glutamate (pH 6.0) [5], 20% w/v sucrose or 20% w/v trehalose [6,25] to attain ~1 × 10^{10} CFU mL^{-1}.

After that, bacterial suspensions were frozen overnight in glass vials in a static state at −80 °C. Samples were then desiccated in a freeze-dryer (Labconco freeze dryer system/Freezone 4.4, Kansas City, MO, USA) for 24 h (condenser temperature: −50 °C; chamber pressure: 0.06 mbar). Then, vials were sealed and samples were stored at 4 °C for 48 h.

For freezing preservation, the bacterial pellets were washed once with 50 mM potassium phosphate buffer solution (pH 6.5), centrifuged again, concentrated to ~ 1 × 10^{10} CFU mL^{-1} and then frozen at −20 or −80 °C for 30 days, using glutamate, sucrose and trehalose in the same conditions as those used for freeze-drying. Before the corresponding assays, samples were thawed at 28 °C for 15 min.

2.4. Ethanol tolerance

Freeze-dried and freeze-thawed bacterial cells, previously acclimated or not, were inoculated in a synthetic wine containing 13 or 14% v/v ethanol and incubated at 21 °C for 24 h. The composition of the synthetic wine was: 5 g L^{-1} tartaric acid, 4.5 g L^{-1} L-malic acid, 0.6 g L^{-1} acetic acid, 2 g L^{-1} glucose, 2 g L^{-1} fructose, 2 mg L^{-1} yeast extract, 0.2 mg L^{-1} sodium chloride, 1 mg L^{-1} ammonium sulphate, 2 mg L^{-1} potassium phosphate, 0.2 mg L^{-1} magnesium sulphate heptahydrate, 0.05 mg L^{-1} manganese sulphate and 13 or 14% v/v ethanol (pH: 3.5) [2].

2.5. Staining procedures

After freeze-thawing and freeze-drying, bacterial cells were washed twice with sterile phosphate buffered saline (PBS). One milliliter of cell suspensions containing approximately 10^6 CFU mL^{-1}, were incubated with 2 μL 5-carboxyfluorescein diacetate (cFDA) (Molecular Probes, Leiden, Netherlands) (50 μg μL^{-1}) for 10 min at 28 °C in the dark. Then, propidium iodide (PI) (Molecular Probes, Leiden, Netherlands) was added to a final concentration of 0.5 mg mL^{-1} and incubated 5 min at room temperature.

2.6. Flow cytometry analyses (FC)

FC analyses were performed with a FACS Calibur instrument using the CellQuest software (Becton Dickinson, Mountain View, CA, USA) according to Rault et al. [18]. Samples were processed so that 10000 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 dead (thermally treated at 80 °C for 30 min) and freshly harvested cells were stained with cFDA and PI both in double-staining assays. These mixtures were used as controls, to set the flow cytometer detectors and compensation to distinguish the four regions: Q1 (dead cells): PI+ and cFDA-; Q2 (membrane damaged 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. Non-fluorescent debris in Q3 was excluded [11].

2.7. Bacterial plate counts

Cultivable cells were determined by plating onto MRS agar (Biokar Diagnostics) before and after acclimation treatments, before and after preservation treatments and after incubation in a synthetic wine containing 13 or 14% v/v ethanol. Cell counts were determined after 48 h incubation at 28 °C. Cells survival was expressed as Log N/N_0, where N is the CFU mL^{-1} after a given treatment and N_0 is the CFU mL^{-1} of culture before the treatment.

2.8. Principal component analysis (PCA)

In order to compare the differences among treatments, principal component analysis (PCA) was performed taking into account the information provided by both flow cytometry and plate counting for all the preservation conditions and treatments (freezing at −20 and −80 °C, freeze-drying, non-acclimation or acclimation with 6 or 10% v/v ethanol, use of trehalose, sucrose or glutamate as protective compounds) [9,16]. PCA was carried out using The Unscrambler® software (version 9.8, CAMO, Norway).

2.9. Statistical analyses

Determinations were carried out in duplicate from three independent cultures of each bacterial strain studied. Analysis of variance (ANOVA) was carried out using the statistical program STATISTIX 8 Software (Analytical Software, Tallahassee, FL, USA). Two-sided Dunnett's Multiple Comparisons with a Control was performed and if $P < 0.05$ the difference was considered statistically significant.

3. Results

The effect of sucrose, trehalose and glutamate on freeze-thawed and freeze-dried *L. plantarum* strains is shown in Fig. 1. In general, the three strains were highly resistant to freezing at −80 and −20 °C in the conditions assayed (acclimated or not, and using trehalose, sucrose and glutamate as cryoprotectants). In terms of bacterial cultivability, freezing at −20 or −80 °C were the worst conditions for non-acclimated UNQLp 155 strain.

As expected, freeze-drying had a more detrimental effect on cells. Bacterial treatments carried out before this preservation process demonstrated to have an important role on the subsequent cell recovery. In the presence of protective agents cultivability of non-acclimated freeze-dried cells significantly decreased with regard to non-freeze-dried cells (Fig. 1). Prior bacterial acclimation with low ethanol concentrations led to a better cell recovery after freeze-drying in the presence of protective compounds. It must be pointed out that the efficiency of protective agents during freeze-drying was strain dependent (Fig. 1). After acclimation in 10% v/v ethanol, the three protective agents were equally efficient for strain UNQLp 133 ($P > 0.05$). In turn, for strain UNQLp 65.3, trehalose and glutamate were more efficient than sucrose ($P < 0.05$) and for strain UNQLp 155, sucrose and glutamate were more efficient than trehalose ($P < 0.05$) (Fig. 1).

Considering that membrane integrity is an important issue in bacterial preservation, the efficiency of sucrose, trehalose and glutamate to prevent sub-lethal membrane damages was assessed using multiparametric flow cytometry. Fig. 2 displays the percentage of viable, damaged and dead cells after freezing and freeze-

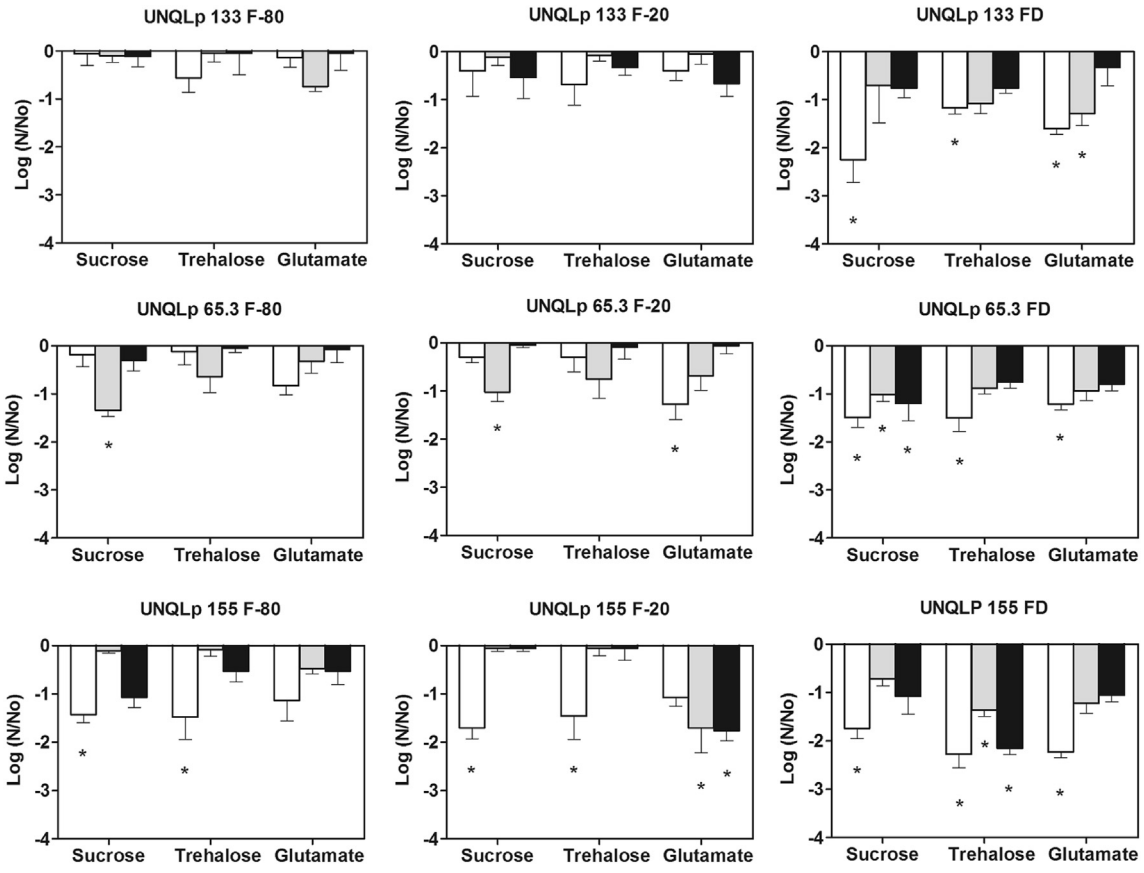

Fig. 1. Log N/N_0 of UNQLp 133, UNQLp 65.3 and UNQLp 155 *L. plantarum* strains, after freezing at −80 °C (F-80, column on the left), freezing at −20 °C (F-20, column in the middle) and freeze-drying (FD, column on the right) in presence of 20% w/v sucrose, 20% w/v trehalose or 2.5% w/v glutamate. For each protective agent, bars indicate the bacterial treatments before preservation as follows: white bars denote non-acclimated strains, grey bars, strains acclimated in 6% v/v ethanol and black bars, strains acclimated in 10% v/v ethanol. (*) indicate significant differences with regard to fresh cultures ($P < 0.05$).

drying the three strains. More than 60% viable cells were obtained after freezing at −80 °C and −20 °C in all conditions, regardless the strain investigated (Fig. 2 A and B). No significant differences were observed with regard to the controls (non-frozen cells) ($P > 0.05$). For frozen cultures in the absence of protective agents, the percentage of viable cells was lower than 50% (data not shown).

With regard to freeze-drying, strain UNQLp 65.3 was more resistant than the other two strains. Indeed, also in this strain, more than 60% of cells had their membranes undamaged after freeze-drying in all conditions (Fig. 2 CII). The situation in strains UNQLp 133 and 155 was different. In strain UNQLp 133, acclimated in 6% v/v ethanol and further freeze-dried in presence of trehalose or sucrose, ≈ 70% cells were viable. When this strain was acclimated in 10% v/v ethanol, sucrose and glutamate were the best lyoprotectants as the percentage of viable cells was 62 and 75%, respectively (Fig. 2 CI). Sucrose, trehalose and glutamate were not efficient agents to prevent membrane damage in freeze-dried non-acclimated cells. In fact, the percentage of viable cells dropped to 40, 14 and 25% when these protective agents were used, with a consequent increase of damaged cells.

In strain UNQLp 155, the worst scenario was observed when trehalose or glutamate were used as lyoprotectants during freeze-drying. In this situation, no matter acclimation was carried out or not, less than 40% of cells were viable after freeze-drying. In addition, it is worth to mention than in cells acclimated with 10% v/v ethanol, an increase of damaged and dead cells after freeze-drying with trehalose or glutamate was observed.

In order to know the effect of acclimation and preservation processes on the technological properties, frozen and freeze-dried cells were inoculated in a synthetic wine containing 13 or 14% v/v ethanol, incubated at 21 °C, and the cultivability was evaluated after 24 h. Fig. 3 displays the loss of viability of bacterial cells grown in a wine-like medium after the different treatments, with respect to fresh cultures.

The highest survival was observed for bacteria frozen at −80 °C and the lowest one, for the freeze-dried ones. In strains UNQLp 133 and 65.3, acclimation in 6% or 10% v/v ethanol prior preservation led to an increase of the bacterial survival after wine-like incubation, acclimation in 10% v/v ethanol being better than acclimation in 6% v/v. In contrast, for strain UNQLp 155, acclimation in 10% v/v ethanol was worse than in 6% v/v for some of the conditions assayed (freezing at −80 °C or at −20 °C in presence of sucrose). Strain UNQLp 155 was the most sensitive one to freezing and freeze-drying processes. On the other hand, the increase in the ethanol content from 13 to 14% v/v led to a drastic decrease of cultivability for all the preservation processes and for all the strains analyzed.

Finally, a PCA was carried out to rationalize the effect of each

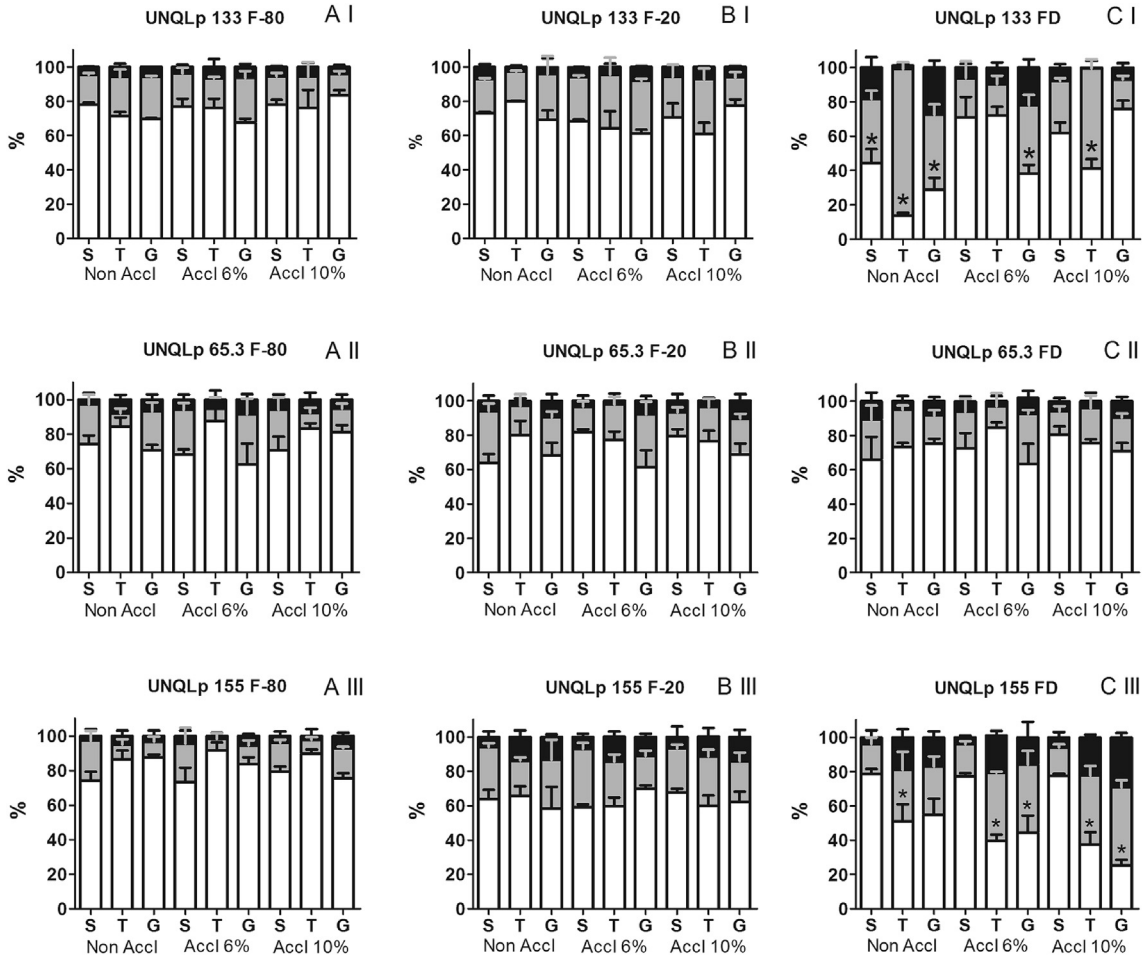

Fig. 2. Percentage of viable, damaged and dead cells of strains UNQLp 133, UNQLp 65.3 and UNQLp 155 *L. plantarum*, after different preservation treatments as determined by multiparametric flow cytometry. Graphs on the left column correspond to cells frozen at −80 °C (F-80), graphs in the middle column, to cells frozen at −20 °C (F-20), and graphs on the right column, to freeze-dried cells (FD). The preservation treatments were carried out in presence of 20% w/v sucrose (**S**), 20% w/v trehalose (**T**) or 2.5% w/v glutamate (**G**). Bacterial cells were acclimated in 6% (Accl 6%) or 10% (Accl 10%) v/v ethanol or non-acclimated (Non Accl). Viable, damaged and dead cells were plotted in white, grey and black bars, respectively.

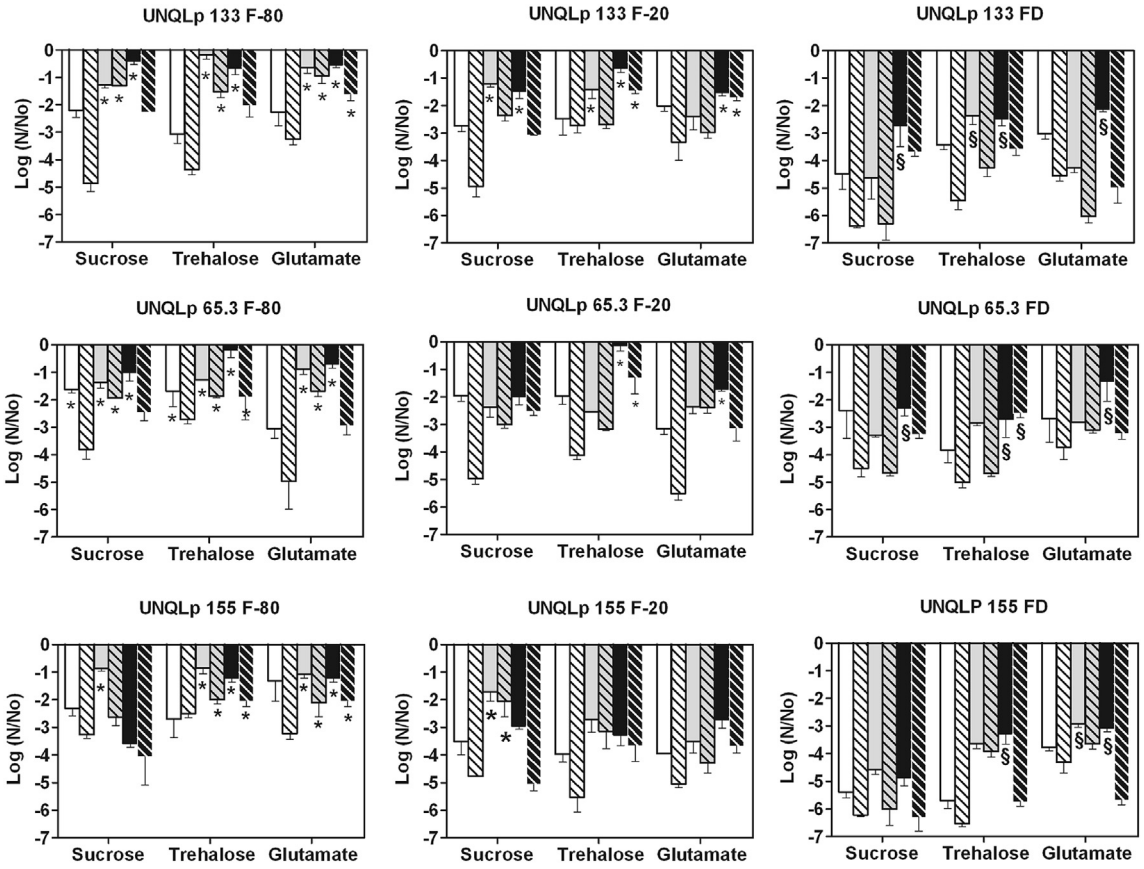

Fig. 3. Log N/N_0 of UNQLp 133, UNQLp 65.3 and UNQLp 155 *L. plantarum* strains after preservation processes and incubation in wine-like medium. Freezing at −80 °C (F-80, column on the left), at −20 °C (F-20, column in the middle) or freeze-drying (FD, column on the right) in presence of 20% w/v sucrose, 20% w/v trehalose or 2.5% w/v glutamate. For each protective agent, bars indicate the bacterial treatments before preservation as follows: white bars denote non-acclimated strains incubated in wine-like medium containing 13% (solid bars) or 14% v/v ethanol (crossed bars), grey bars denote strains acclimated in 6% v/v ethanol incubated in wine-like medium containing 13% (solid bars) or 14% v/v ethanol (crossed bars), black bars denote strains acclimated in 10% v/v ethanol incubated in wine-like medium containing 13% (solid bars) or 14% v/v ethanol (crossed bars). (*) indicate no significant differences with respect to fresh cultures ($P < 0.05$), (§) Indicate the best condition for freeze-dried microorganism.

variable on the resistance to wine-like conditions. Fig. 4 shows a bidimensional scores' map corresponding to two principal components (PCs) of PCA: PC2 *vs* PC1. This scores plot is especially useful since these two components explain a higher percentage of variance than any other pair of components (62–63% of the total variance for all the strains investigated). Three groups explaining 35%–36% of the variance were observed along PC1. Each of these groups corresponds to different acclimation conditions (acclimation with 10%, 6% v/v ethanol and not acclimated strains). Along PC2, other three groups, explaining 26–28% of the variance were observed. In this case, the observed groups could be ascribed to different preservation conditions: full black circles correspond to strains frozen at −80 °C, full grey circles to strains frozen at −20 °C, and opened circles, to freeze-dried strains. Note, that along PC1 bacterial resistance increased from right to left, and along PC2, the harmfulness of preservation processes increased from down to up. This indicates that PCA analysis enables an accurate correlation between bacterial physiological states and harmful effects of preservation processes at a first glance.

4. Discussion

In order to assess the best conditions for MLF starter cultures preservation, the effect of trehalose, sucrose and glutamate as protective agents, and the effect of acclimation treatments on freezing and freeze-drying of three *L. plantarum* strains were studied.

The effectiveness of sugars and amino acids to protect LAB applied to dairy products has been extensively reported [5,6,23,24]. However, little information is available about preservation processes in oenological strains. Trehalose, sucrose and glutamate have been described as effective lyoprotectant agents during freeze-drying of *Oenococcus oeni* and *Lactobacillus brevis* strains [27], glutamate being the most efficient agent to protect *O. oeni* strains [10,15,28]. The results obtained in this work show that the three compounds investigated were efficient protectants during freezing and freeze-drying of oenological strains, although their efficiency is strain and process dependent.

During freezing and freeze-drying, cells are exposed to low water activity that can lead to damage cell structures [4,25,26]. According to the results presented in Fig. 1, freeze-drying was the most drastic process, leading to a lower recovery than freezing. This can be explained considering that during freeze-drying, cells are exposed to vacuum dehydration, and cell envelopes are exposed to a hydrophobic environment which may alter membrane permeability in a more drastic way than freeze-thawing [17,26]. The higher percentage of damaged membranes corresponding to freeze-dried cells (Fig. 2) confirmed this detrimental effect.

The effect of pH, pre-incubation at different temperatures and freezing rates on bacterial recovery after freeze-drying was reported by Zhao and Zhang (2009) [28]. The results depicted in Fig. 1 indicate that pre-acclimation in the presence of low ethanol

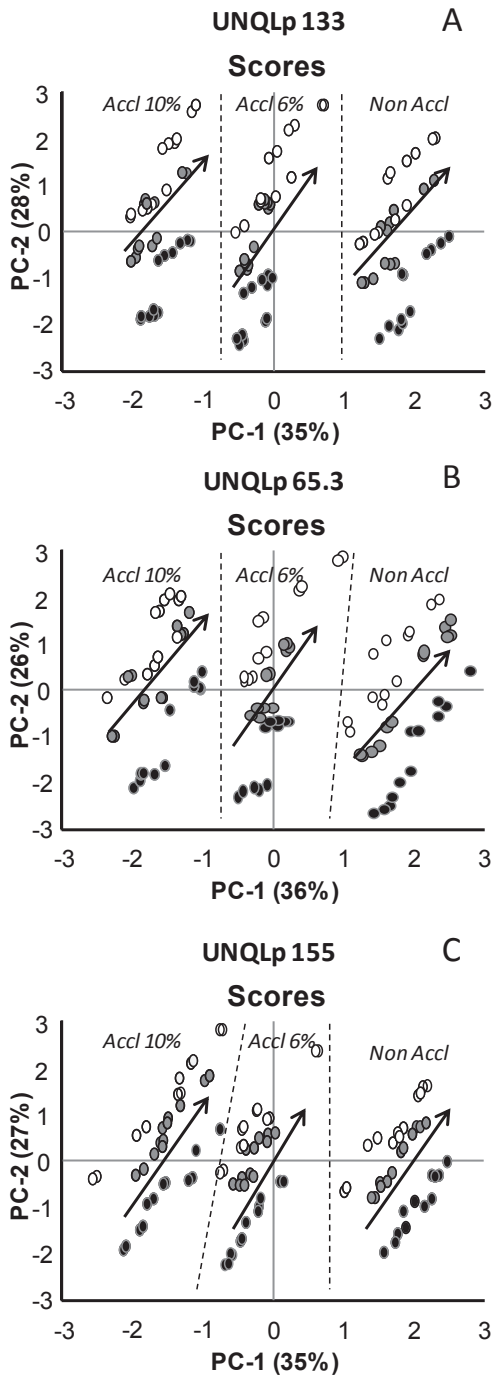

Fig. 4. Score plots from the PCA performed on strains UNQLp 133 (A), UNQLp 65.3 (B) and UNQLp 155 (C) frozen at −80 °C (black circles), frozen at −20 °C (grey circles) or freeze-dried (white circles) in presence of protective agents. Before preservation, bacterial cells were acclimated in 6% v/v or 10% v/v ethanol, or non-acclimated. After preservation, cultures were incubated in wine-like medium containing 13% v/v or 14% v/v ethanol for 24 h at 21 °C. The arrows indicate the increase of ethanol concentration.

concentrations increased the resistance to freeze-drying. We have previously reported that acclimation of the studied *L. plantarum* strains with 6 or 10% v/v ethanol leads to an increase of saturated fatty acids and a decrease of their chain length. Taking into account that membranes are the first target of damage during preservation processes, this change in the membrane composition could be responsible for the higher recovery after preservation of acclimated strains. Similar results were found by Hau et al. (2009) [12], who ascribed a higher recovery of *O. oeni* after freeze-drying to an increased content of Cyc 19:0 fatty acid among microorganisms grown at low pH.

Freezing rate is another factor related with cell damage, high drying rates (freezing at −80 °C) being less harmful than low ones (−20 °C) [28]. This explains the relation between bacterial cultivability after wine-like inoculation and the previous preservation process. Even when the number of viable cells did not significantly decrease after freezing (both at −20° and −80 °C) and freeze-drying, after wine-like inoculation the loss of viability was higher for cells frozen at −20 °C and for freeze-dried cells, than for cells frozen at −80 °C (Fig. 3).

Resistance to ethanol after acclimation [2] remained unaltered after preservation processes (Fig. 3). In particular, acclimated cells frozen at −80 °C did not show significant differences in cultivability after wine-like inoculation with regard to the controls. In contrast, the ethanol sensibility of non-acclimated cells frozen at −80 °C was similar than that observed before freezing [2]. The beneficial effect of acclimation was also observed for cells frozen at −20 °C and for freeze-dried cells. Acclimation had a particularly beneficial effect to increase the cultivability after inoculation of wine-like medium containing 14% v/v ethanol (Fig. 3). In this regard, Maicas et al. (2000) [15] reported that the efficiency of a strain of *O. oeni* to conduct malolactic fermentation after freezing and freeze-drying is increased if the strain is previously grown in an acclimation medium or in wine.

In general, cell cultivability in wine containing 13% v/v ethanol was higher for all conditions assayed. Acclimation with 10% v/v ethanol was more efficient than with 6% v/v ethanol for strains UNQLp 133 and 65.3. In contrast, strain UNQLp 155 acclimated with 10% v/v ethanol presented a lower survival when it was inoculated in synthetic wine containing 14% v/v ethanol. Previous AFM assays carried out by our group indicate that acclimation with high ethanol concentrations leads to changes on the bacterial surface of strain UNQLp 155 (increase of zeta-potential), making cells more susceptible to surface damage after freeze-drying [15].

PCA carried out in this work clearly summarized the information obtained in Figs. 1–3, explaining within 62 and 63% of the total variance. The effect of both acclimation and harmfulness of preservation processes could be easily noticed from the PCA plot. As previously mentioned, the protective effect of acclimation on the recovery of *L. plantarum* strains is the result of the adaptation of the fatty acid composition to harsh environments. Therefore, the groups observed on PC1 also provide an indirect information about physiological changes taking place on bacterial cells upon exposure to ethanol. On the other hand, the groups observed along PC2 provided information related with the harsh effects of preservation processes on bacterial cultivability. Taking all this information as a whole, the importance of PCA is that it enables the obtaining of complete information about both bacterial preservation and physiological states at a first glance. This is particularly important when large amounts of samples and preservation conditions are to be analyzed. As a consequence, this approach provides support to define in the future, supervised multivariate methods enabling the prediction bacterial behavior after acclimation and/or preservation methods for unknown samples.

According to the results obtained in the present work, the best condition for malolactic starter cultures preservation was freezing at −80 °C, although freezing at −20 °C and freeze-drying were also adequate methods. All the protective agents assayed were able to protect cell structures and increase cell survival after wine inoculation. On the other hand, acclimation treatment is essential to increase the resistance of bacterial cells before inoculation in wine. Finally, a successful inoculation of starter cultures in wine depends on a large extent to the ethanol content of wine, as it was

demonstrated by the great differences observed between synthetic wines containing 13 or 14% v/v ethanol. This underlines the critical importance of a prior knowledge of physicochemical parameters of wine to decide the right moment to add malolactic starter cultures during winemaking.

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Conflict of interest

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

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