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Effect of Lipid Composition on the Stability of Cellular Membranes during Freeze–Thawing of *Lactobacillus acidophilus* Grown at Different Temperatures

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Lactobacillus acidophilus **CRL 640 grown at the optimal temperature of 37°C (M37) appeared more sensitive to freeze-thawing than when it was grown at 25°C (M25). In the first case, 87% of the cells died, in contrast to 33% for cells grown at 25°C. All the surviving M37 cells showed sensitivity to NaCl. However, among the surviving M25 cells, only 85% were sensitive to NaCl. The rest of the cells were considered uninjured. Freeze-thawing in cells grown at 25°C showed a liberation of nucleic acids and proteins. However, the leakage was higher in M37 cells after freeze–thawing. The greater fraction of damaged cells were observed in M25 culture after freeze–thawing. A relative increase of 81% in cardiolipid (CL), with respect to total phospholipids and 72% triglycosyldiglyceride (TGDG) with respect to the total glycolipids was observed in M37. In addition, a decrease of palmitoyl (C16:0), oleoyl (C18:0) fatty acids at CL, phosphatidylglycerol (PG), and diglicosyldiglyceride (DGDG) fractions and the increase of C19 cyc and C18:0, 10-OH fatty acids in neutral lipid, and CL fractions was also apparent. In M25 cells, the concentration of DGDG and PG was higher than in M37 cells. The difference in cryotolerance between the frozen cultures emphasizes the importance of selecting appropriate conditions of growth of microorganisms for use as dietary adjuncts.**

Key Words: Lactobacillus acidophilus; **cryotolerance; cellular membrane; lipid composition.**

When an aqueous solution is frozen, only a proportion of the water undergoes transition to ice. Thus, gases and solutes in the residual aqueous solution become more concentrated. Under these conditions, cells suspended in the solution are exposed to a hypertonic stress, as a result of the gradient in the chemical potential of water between the cytosol and the extracellular solution. In consequence, the cell volume decreases due to an outflux of water. This sequence of events occurs due to the semipermeable characteristics of the plasma membrane—that is, its ability to constitute a selective permeability barrier to solutes in the outer and the inner cellular solutions. Hence, the integrity of the membrane structure with those semipermeable properties is a prerequisite for survival (1). The membrane structure has semipermeable properties when lipids arrange in a bilayer conformation. Lipids organize in a bilayer when the areas of the head group region and the hydrocarbon chains are comparable. In other cases, lipids stabilize in nonbilayer arrangements with a loss of the barrier properties (2).

It is known that, although freezing is a widely used procedure to store bacterial cultures, freezing and subsequent thawing is lethal to a large fraction of a given population (3), being the cytoplasmic membrane the principal site of lethal damage (4). This lethal damage could be due to changes in the bilayer conformation and in the permeability properties to ions induced by hydration or curvature stress. Thus, an understanding of the stability properties of the membrane in order to enhance cell cryopreservation can be reached in connection to the lipid components achieved during the growth conditions.

The response of cells to freeze-thawing can be modified by the growth conditions before freezing such as temperature (1, 5). Cryotolerance appears to be enhanced when bacteria are adapted to suboptimal tem-

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peratures. This phenomenon has been related to changes in the lipid composition of the cellular membrane (6–8). Although cryotolerance conditions have been investigated in psycrophylic and mesophylic bacteria (6), no studies have been performed in thermophilic lactic acid bacteria in correlation to cell survival, membrane damage and lipid composition.

Within the lactic group, *Lactobacillus acidophilus*, a Gram-positive microorganism, has been proposed as a dietary adjunct because of its claimed beneficial properties on health (9). Usually, starter cultures are grown at the optimal temperature for using in freeze–thawing studies. However, the high sensitivity of *L. acidophilus* to cryogenic treatments results in structural and physiological injury that makes difficult its preservation.

The aim of this paper is to investigate the membrane alterations occurring during freeze–thaw of *L. acidophilus* CRL 640 grown at minimal and optimal temperatures and its correlation with the fatty acid composition and the polar lipid species appearing in each condition of growth.

MATERIALS AND METHODS

Microorganism, culture conditions, and harvesting. *L. acidophilus* CRL 640 was obtained from the CERELA stock culture collection and was previously isolated from fermented milks. The cultures were grown in MRS broth from Merck (Darmstadt, Germany), (22) at 25 and 37°C for 72 and 16 h, respectively, in a fermentor (Bioflo C32, New Brunswick) with rotatory shaking at 20 rpm. The pH was automatically controlled with NH_4OH to pH 6.0. The specific growth rate (μ) was determined by the slope of a semilogarithmic plot of A_{560} vs time. The cells were harvested by centrifugation at 5000*g* during 20 min at 4°C.

Freezing and thawing. Cells in the stationary phase were harvested by centrifugation at 5000*g* during 20 min and suspended in 0.85% NaCl to reach a concentration of 2×10^9 CFU ml^{-1}. Aliquots of 5 ml of the cell suspension were placed in glass tubes of boro silicate (15 × 1.5 cm). Two of them were taken as control in which lipids were analyzed. The rest was frozen at −20°C, and stored at the same temperature during 24 h. The samples were thawed by immersion during 5 min in a water bath at 37°C. Unfrozen samples were used as control.

Viability determinations. Viability of the control and freeze–thawed samples were determined by the plate dilution method. Cell suspensions were serially diluted and aliquots were pour-plated in mass simultaneously in MRS[2] agar and MRS supplemented with 2% NaCl (MRSNa). The plates were incubated at 37°C for 72 h and the resulting colonies were counted.

Determinations of dead and injured cells. The differences in colony counts in MRS agar (rich medium) between those obtained before and those after freeze–thawing were used to calculate the number of dead cells (10). The differences between the number of colony forming units per ml (CFU.ml^{-1}) in MRS, and in MRSNa (selective media) after freeze–thawing represent the amount of injured cells among the survivors (11). The results were expressed as percentage (%) of dead and injured cells.

Lipid extraction. Lipids were extracted from pellets of bacteria harvested by centrifugation in the stationary state before freezing following the Bligh and Dyer method (13) modified as described by Fernández Murga *et al.* (14).

One milliliter with 40–50 mg cells was mixed with 3.75 ml of methanol:chloroform (2:1) v:v. The lipid residue was approximately 17 mg per gram of cell.

The lipid residue was dissolved in 1 ml of chloroform:methanol 1:1. From this total lipid extraction the percentage of each phospholipid specie was calculated. Similarly, the percentage of the glycolipid species was calculated with respect to the total of glycolipids per gram of cells.

Thin-layer chromatography. The polar lipids were characterized by their migrations on aluminium-backed Silica Gel 60 thin-layer-chromatographic (TLC) plates (Merck, Darmstadt, Germany). Chloroform/methanol/acetic acid/water (65:25:4:2, v/v/v/v) was employed as mobile phase. Lipids were identified with specific reagents following the techniques described previously (14).

Fatty acid methyl esters (FAMEs). Methylesters of the fatty acids were obtained by acid methanolysis. After being transferred into a screw cap Pyrex test tube, the dried sample was mixed with 1 ml of 2% HCl in methanol (v/v) and kept at 60°C for 2 h. After cooling, 1 ml of water was added. The fatty acid methyl esters (FAMEs) were extracted with dichloromethane (3 × 0.5 ml). The organic layer was washed with water and evaporated under nitrogen. Details of the determination of the unsaturated fatty acids are given in references (14, 20).

Gas-liquid chromatography (GLC). Analytical GLC was carried out on a Hewlett-Packard 5890 gas chromatograph interfaced with a mass spectrometer (Trio-2 VG Masslab, Manchester, UK). The following operating conditions were used: FAMEs: capillary column HP-5 (25 m × 0.31 mm × 0.17 μm); carrier gas nitrogen; oven temperature 100 to 280°C at 10°C/min. Electron impact mass spectra were obtained at 70 eV and individual FAME were identified by their fragmentation patterns and ion masses.

RESULTS

Effect of Freeze–Thawing on Cellular Membranes

An overnight culture of *L. acidophilus* CRL 640 grown at 37°C was shifted to 25 and to 37°C. Under these growth conditions, the generation time at 37°C was 1.4 h, whereas it increased to 24 h at 25°C. Cells in the stationary state of both cultures were freeze–thawed following the protocol described under Materials and Methods.

L. acidophilus CRL 640 grown at 37°C (M37) appeared very sensitive to freeze–thawing since an 87% of the cells died (Fig. 1). In contrast, the reduction after freeze–thawing, in the total viable counts in MRS, was only 33% for cells grown at 25°C (M25). The percentage of surviving cells grown at 25°C was 67%. Within this percentage, 85% was sensitive to NaCl. The rest of the cells was considered uninjured. In contrast, all the surviving cells of M37 cultures showed sensitivity to NaCl (Fig. 1). The extent of death and injury in *L. acidophilus* CRL 640 during freeze–thawing were studied by plating the samples in a rich medium (MRS) to obtain total viable counts, and selective medium (MRSNa) to determine the number of damaged cells (11). No differences in colony counts between the plat-

[2] Abbreviations used: CL, cardiolipin; PG, phosphatidyglycerol; DGDG, diglycosyldiacylglycerol; TGDG, triglicosyldiacylglycerol; C18-10OH, 10 hidroxi-oleic acid; MRS, broth according to Ref. 22.

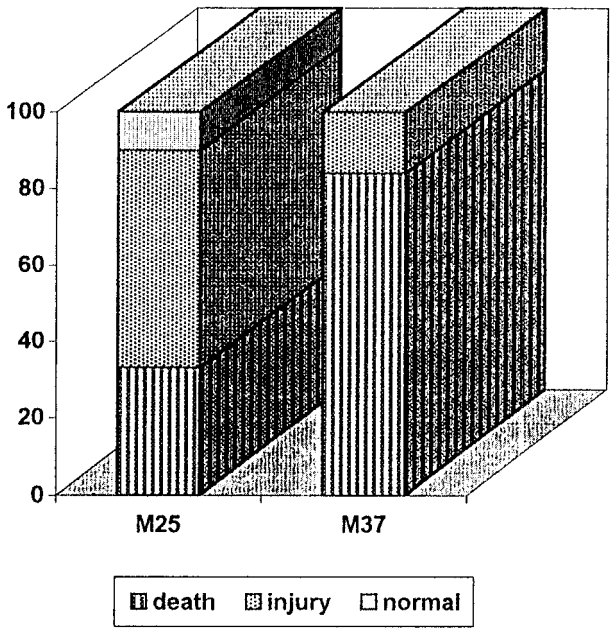

FIG. 1. Death and cellular damage of *Lact. acidophilus* CRL 640 grown at 25 and 37°C after freezing/thawing. Note: The maximum variations from the mean values were less than 5%.

ing media were detected before freezing, indicating that unfrozen cells were not sensitive to NaCl.

In both cultures, a liberation of nucleic acids and proteins were observed both in M25 and in M37 after freeze-thawing. However, the leakage was less noticeable in M25 cells than in M37 cells after freeze–thawing (data not shown).

Effect of Growth Temperature on Lipid and Fatty Acid Composition

The lipid composition was determined after growing at 25 and 37°C respectively, before freeze–thawing. The fraction of cardiolipin (CL) in the total lipid extract was higher in cells grown at 37°C with respect to phosphatidylglycerol (PG) in comparison to those grown at 25°C (Fig. 2, upper panels). In parallel, an increase in the fraction of triglycosyldiglyceride (TGDG) in M37 cell was observed with respect to the diglycosyldiglyceride (DGDG) fraction in M25 cells.

FAMEs patterns from *L. acidophilus* CRL 640 show that the hexadecanoic (C16:0) and the octadecadienoic (C18:2) acid methyl esters increased two and fivefold, in cultures at 25°C, respectively, with respect to 37°C. In addition, a twofold decrease in both 10-hydroxyoctadecanoic acid (C18:0, 10-OH) and C19-cyc acid was also observed. An increase in C16:0, as well as a decrease in C19 cyc and C18:0 10OH were observed in neutral lipids, PG and CL of M25 with respect to M37 (Figs. 3A–3C). The neutral lipid fraction showed a fivefold increase in C18:2 fatty acids (Fig. 3A). The fragmentation by electronic impact in mass spectrometry indicated that it was a 18:2 derivate. Although this fatty acid could not be identified, it can be said that it is not linoleic acid, since the samples were coinjected with this acid as a pattern and did not run with the same elution time. The cardiolipin fraction (CL) also showed a 2.9- to 7-fold increase in C18:0, being this fatty acid also increased in the PG fraction (Figs. 3B and 3C).

Comparison of FAMEs profiles for the DGDG fraction (Fig. 3D) revealed the increase in long chain fatty acids, such as, octadecanoic (C18:0) acid and C16:0, as well as an important decrease (from 1.5 to 9.5 times) in C18:1 and C19 cyc in M25. The FAME profile in the TGDG (Fig. 3E) fraction was quite similar at both temperatures. The fatty acid C18:0 10-OH was absent or in traces (less than 1%) within glycolipids.

DISCUSSION

The results presented in this paper demonstrate that different structural damages are suffered by lactobacilli after freeze–thawing depending on they were grown at 25 or at 37°C. According to the results shown in Fig. 1, freeze–thawing of *L. acidophilus* CRL 640 grown at 25°C results in a high number of damaged cells, recoverable in rich media, and undamaged cells. Cultures of cells grown at the optimal temperature (37°C) showed a low percentage of damaged cells and a high ratio of dead cells. Thus, in average M25 cells are more resistant to freeze–thaw than M37 cells. That is, the growing of cells at sub-

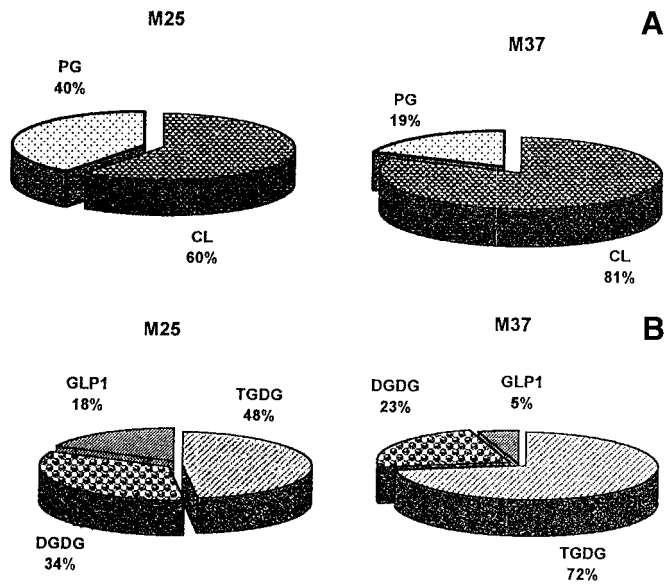

FIG. 2. Polar lipid composition of *Lact. acidophilus* CRL 640 grown at 25 and 37°C. (A) phospholipids fraction and (B) glycolipids fractions. PG, phosphatidylglycerol; CL, cardiolipin; DGDG, diglycosyldiglyceride; TGDG, triglycosyldiglyceride; GLPI, nonidentified glycolipid.

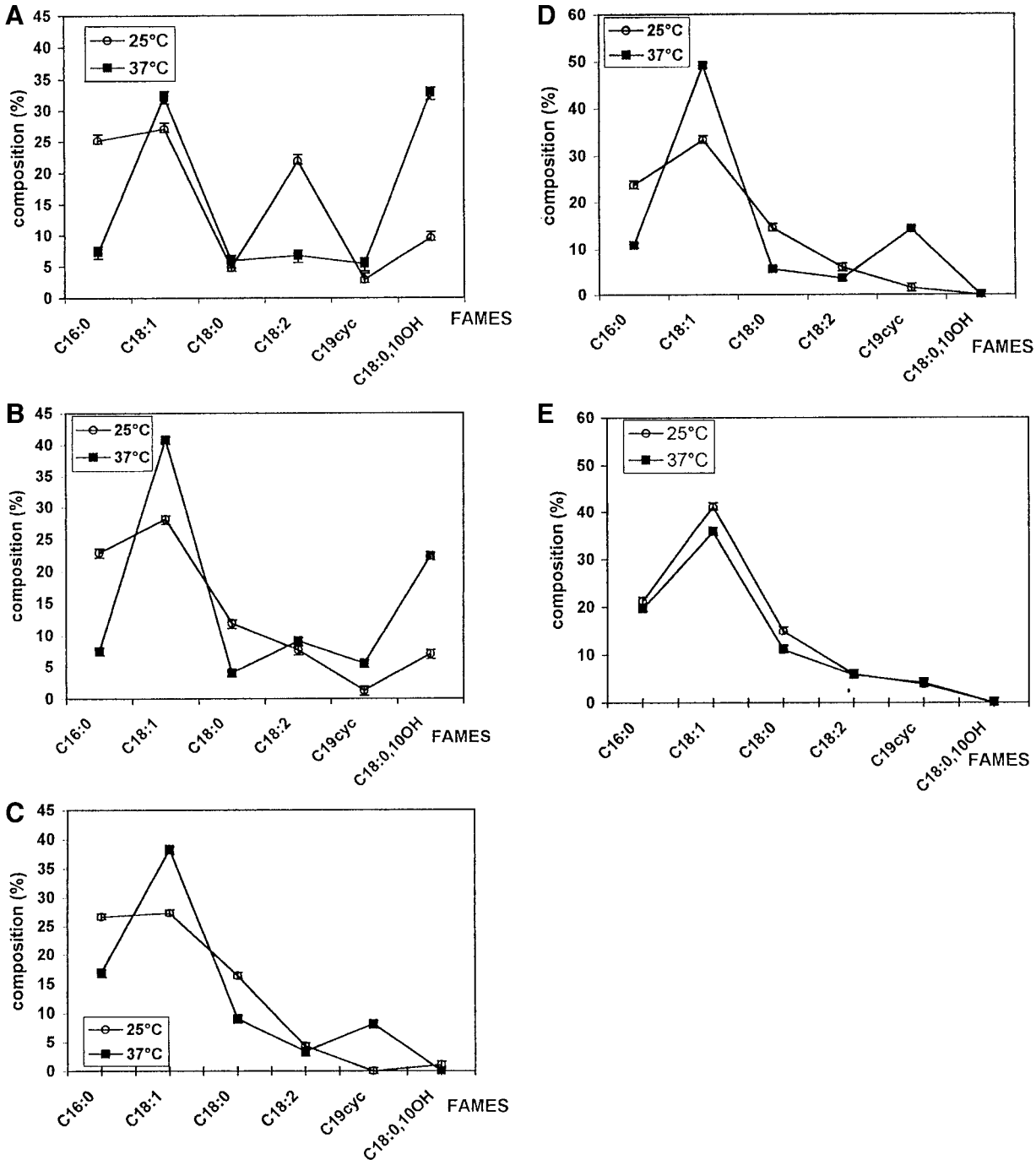

FIG. 3. Percentage of FAMES composition (%) of *Lact. acidophilus* CRL 640 grown at 25 and 37°C. (A) Neutral lipids, (B) Cardiolipin, (C) Phosphatidylglycerol, (D) Diglycosyldiglyceride, and (E) Triglycosyldiglyceride.

optimal temperatures increases the resistance to freeze–thawing. A similar response to cold shock has been reported for *Escherichia coli*, *Bacillus subtilis*, and *Enterococcus faecalis* after several hours of adaptation at 10°C (15–17).

The increased sensitivity of sublethally injured bacteria to NaCl has been related to damage in the cytoplasmic membrane (18). This may be the reason of the high percentage of damaged cells in M25 cultures after freeze–thawing. This damage is correlated with the decrease in CL, TGDG with a corresponding increase of C16:0, C18:0 in the CL, PG and DGDG fractions, and the decrease of C19 cyc and C18:0, 10-OH in the neutral lipids and CL fractions. Thus, it appears that dam-

age is correlated with an increase in the saturated ratio of the fatty acids.

The leakage of intracellular material measured by the increase of the absorbance at 260–280 nm in the supernatant is greater in cells grown at 37°C than in M25 (19). This might be related to changes in the barrier properties of the bilayer due to conformational changes as reported in other systems (1, 8). The membrane stability has been explained by the stabilization of the lipids in a bilayer conformation (1). This conformation is favored by the cylindrical shape of the constituent lipids, given by the similarity of the areas in the head groups and the hydrocarbon tail regions. Thus, a change from the bilayer to a nonbilayer conformation might be involved when the CL or the TGDG increases, because CL has a large tail area in comparison to the head group and the TGDG viceversa. DGDG and PG having similar areas in the head group and the hydrocarbon chains would stabilize in bilayers, TGDG and CL would trend to form nonbilayer structures by separate. Thus, the excess of one of these species with respect to DGDG and PG would determine the destabilization of the bilayer. However, due to their complementary shapes, they would stabilize in a bilayer when TGDG and CL are in similar proportion. From the fractions in Fig. 2, it can be derived that the TGDG/CL ratio is nearly the same (c.a. 0.8) in M25 and in M37 and, although the ratio DGDG/PG is slightly higher in M37 it would not affect drastically the bilayer conformation considering their geometrical shapes as described above. Thus, it seems that the leakage produced is not a consequence of the bilayer conformational change.

Cryotolerance studies of psycrophylic and mesophylic bacteria (6) have shown that the increase of resistance is related to an increase of the unsaturation degree in the fatty acid. Unsaturation increases the permeability to water and the viscoelasticity of the bilayer (23, 24). In lactobacilli an additional decrease in C19 cyc has been reported (20).

In terms of the homoviscous adaptation, which postulates the increase of the unsaturation level for the lower temperatures of growth, the only species appearing to increase the unsaturation ratio in M25 is 18:2 fatty acids in the neutral lipid fraction. This finding is congruent with the data reported in several lactobacillus. As described elsewhere the detection of this specie is strongly dependent on the lipid extraction and chromatography conditions (14, 21). Lactobacillus is gram-positive, therefore, all the lipids seem to be in the membrane. Thus, this fatty acid can contribute to the stabilization of the membrane.

The increase in the percentage in M25 cells sensible to NaCl after freeze/thawing are parallel to a decrease in the CL/PG and TGDG/DGDG ratios from 4.3 and 3.13 at 37°C to 1.5 and 1.4 at 25°C, respectively. This indicates a great decrease in negative charges and in hydrogen bonding groups in M25.

Direct evidence that the charge/hydrogen bond ratio is related to the different response to NaCl of *L. acidophilus* CRL 640 has been demonstrated in a previous study (21). Unilamellar vesicles prepared with lipids extracted from lactobacilli were able to trap fluorophores, denoting the formation of closed vesicles. The fluorescence anisotropy in the polar head groups and the hydrocarbon region were significantly lower in vesicles prepared with lipids extracted from lactobacilli grown at 25°C (containing the highest glycolipid ratio and the 18:2 species) in comparison to those of bacteria grown at 37°C. The vesicles prepared with lipids of bacteria grown at 37°C were more unstable than those from bacteria grown at 25°C when subjected to NaCl.

This might determine a higher permeability to Na^+ ions of the membrane and therefore some instability against osmosis of M37 in comparison to M25. However, the higher sensitivity to NaCl of M25 would denote that destabilization by Na is not only due to a higher permeability to these ions but to the resistance of the cell membrane to osmotic stress. Probably the saturation level of the fatty acids could make a more rigid membrane that is more fragile when cells swell by the Na influx.

In M37 cells 18:2 is reduced and there is a great increase in OH in the lipid matrix due to the presence of C18:0 10-OH. This could be the reason of a higher number of dead cells in the M37 cultures after freeze–thawing, since membrane would be mechanically more fragile in the presence of these species.

In conclusion, under the working conditions employed the acclimation of cells at 25°C can hinder permeability changes of the cytoplasmic membrane during freeze–thawing, which results in a loss of viable cells. The difference in cryotolerance between the frozen cultures emphasizes the importance of selecting appropriate conditions of growth of microorganisms for use as dietary adjuncts. Any increase in the cellular resistance to freeze injury induced by culture conditions before freezing would be of practical value in developing methods for lactic acid bacteria cryopreservation.

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