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Osmotic response in *Lactobacillus casei* ATCC 393: biochemical and biophysical characteristics of membrane

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Abstract

The biochemical and biophysical properties of the membrane and some general characteristics of the response of *Lactobacillus casei* ATCC 393 (reclassified *Lactobacillus zeae*) to hyperosmotic conditions were studied. Under hypertonic conditions, the hydrophobicity and the bile salt sensitivity of the cultures were increased. The glycolipid AcylH3DG is only present in membranes of NaCl containing medium, whereas, H4DG undergoes a significant increment and H2DG a significant decrease. The fluidity of both the purified membranes and the total lipid vesicles, as determined with the fluorescent probe DPH, did not change in conditions of high salinity. This was coincident with changes in the fatty acid (FA) composition where an increase in the saturated/unsaturated FA ratio was compensated by a rise in the fluidifying 11,12-methyleneoctadecanoic FA (cyc 19:0). Under osmotic stress conditions, Laurdan and acridine orange in total lipid vesicles showed increased lateral lipid packing and proton permeability, respectively.

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Lactobacillus are Gram-positive bacteria with important applications in the food industry. Their normal habitat is the oral cavity and the digestive tract. In their habitat and also during the industrial process, these microorganisms are exposed to variations in the external concentration of solutes, while the internal concentration of nutrients must remain relatively constant. A sudden increase in the external osmolarity results in the water removal from the cell to the exterior, producing loss of turgor as well as changes in the intracellular solute concentration and in the cellular volume [1]. It has been described that osmotic, thermal, and oxidative stresses and the variations of environmental pH produce several changes on the cellular envelopes of *Lactobacillus*. In particular, changes in the lipid and fatty acid composition of their membranes have been observed [2–4].

The stability and permeability of the cellular membranes play a fundamental role on the adaptation to different kinds of stresses and they might be closely related to the lipid and fatty acid composition. Nevertheless, little is known about the permeability and stability properties of the lactic bacterial envelopes [5].

Lactobacillus species are used as a probiotic in humans and must survive and settle in the intestine. The capacity of this strain to withstand the action of the bile salts is an important criterion of selection since lactic bacteria are responsible for a larger part of the hydrolysis of conjugated bile salts observed in the intestinal tract. It has been shown [6] that the synthesis of bile salt hydrolases is not essential for the resistance and intestinal colonization of the lactic acid bacteria. Apparently, the resistance of these bacteria to the action of the bile salts appears to be dependent on the physico-chemical properties of the cellular envelopes and these characteristics

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could be modified by the different growth conditions of the cultures.

The aim of this work was to compare some of the biochemical and biophysical properties of the envelopes of *Lactobacillus casei* ATCC 393(formerly reclassified as *Lactobacillus zeae*), grown in normal and hyperosmotic conditions. In particular, we focus our study on the variations in polar lipid and fatty acid composition produced by the hypertonic conditions of growth and the physico-chemical characteristics of vesicles formed by the total lipid extracted from cultures grown in either MRS[2] or a hyperosmotic NaCl–MRS medium.

Materials and methods

Materials

Man, Rogosa, and Sharpe broth (MRS) was purchased from Biokar Diagnostics (Bauvais, France). Sucrose, ethylenediaminetetraacetic acid (EDTA), 1,6-diphenyl-1,3,5-hexatriene (DPH), bile bovine (Oxgall Powder) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Bacterial Acid Methyl Esters (Mix 4780-U) were from Supelco (Bellefonte, PA), 2-dimethylamino-6-lauroyl-naphthalene (Laurdan) was purchased from Molecular Probes (Junction City, OR). Stock solutions of 125 μM DPH in ethanol and 200 μM Laurdan in methanol were prepared. Acridine orange was obtained from Calbiochem (La Jolla, CA). Thin-layer chromatography plates (TLC) (Silicagel 60 F_{254}, 0.25 mm thick Art. 5735) were from Merck. All the reagents and solvents employed were of analytical grade.

Bacterial growth

Lactobacillus casei strain ATCC 393 (reclassified as *L. zeae*) was grown at stationary phase without shaking at 37 °C, in MRS broth (control culture, C) or in hyperosmotic medium by adding NaCl 1.0 M final concentration (culture N) to the basal MRS. The cells were harvested by centrifugation after 24 or 48 h, respectively and the pellets were used for membrane preparations and lipid extraction. The cellular pellets obtained from the control cultures were washed twice in buffer C (48 g K_2HPO_4, 98 g KH_2PO_4 per liter distilled water, pH 6.4) and once in buffer PUM (22.2 g K_2HPO_4, 7.26 g KH_2PO_4, 1.8 g urea, and 0.2 g $MgSO_4 \cdot 7\,H_2O$ per liter distilled water, pH 7.1). The cellular pellets from the cultures grown in hyperosmotic conditions were washed twice in buffer N (48 g K_2HPO_4, 98 g KH_2PO_4, and 58.4 g NaCl per liter distilled water, pH 6.4) and once in buffer PUM.

Bacterial membranes

Bacterial membranes were prepared as described by Lysenko et al. [7]. Protein concentrations of cellular pellets and purified membranes were determined according to the method of Lowry et al. [8] using BSA as standard. Total glycolipids in membrane preparations were determined by the phenol–sulfuric method [9] and the lipid phosphorous content was measured according to Bartlett [10].

Cell surface hydrophobicity

Cellular hydrophobicity was determined using a small modification of the rapid and simple assay methods described by Op den Camp [11]. Briefly, culture pellets were washed and re-suspended in PUM buffer and absorbance was measured at 600 nm. To 2 ml of a turbid bacterial suspension (absorbance at 600 nm 1.0–1.5) hexane was added by aliquots until a final volume of 2 ml. Samples were incubated for 30 min at 37 °C, mixed uniformly for 120 s, and allowed to stand until phase separation. The aqueous phase was carefully removed and its absorbance at 600 nm was measured. The decrease of absorbance of the aqueous phase was used as a measure of cell surface hydrophobicity as described by different authors [11–13].

Lipid extraction and purification

Total lipids were extracted from cellular pellets, according to the original method of Folch et al. [14] with some modifications. Briefly, culture pellets were washed, suspended in distilled water, and extracted with 19 volumes of chloroform/methanol (2:1 v/v) containing HCl 6N (0.84 ml/L) to improve the lysilPG extraction [15]. After 2 h at room temperature with agitation in the solvent system, 0.2 volumes of distilled water were added and mixed powerfully. The phases were separated by centrifugation and the aqueous phase was discarded. The lower chloroformic phase was filtered, dried under vacuum, and conserved at −20 °C under a nitrogen atmosphere.

Polar lipid analysis

The total lipid extract was analyzed by TLC, with the following solvent systems: (a) chloroform/methanol/acetic acid (65:25:6, v/v/v); (b) chloroform/acetone/methanol/acetic acid/water (50:20:10:10:4.7, v/v/v/v/v), and (c) chloroform/methanol/ammonium/water (65:35:3.5:2.5, v/v/v/v). Phospholipids and glycolipids were detected with iodine vapors and identified by comparison with authentic purified standards and by treatment with specific reagents (ninhydrin, α-naphthol, and/or ammonium molybdate). Zones of the plates corresponding to each phospholipid were scraped off and quantified. Lipid phosphorous was determined according to Bartlett [10]. The glycolipids were quantified by treatment with α-naphthol/H_2SO_4 [16]; the plates were scanned and the intensity of the spots was measured by densitometric analysis in a LAS 1000-Fuji Analyzer.

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[2] *Abbreviations used:* MRS, Man, Rogosa, and Sharpe medium; MRS–NaCl, MRS medium supplemented with 1.0 M NaCl; DPH, 1,6-diphenyl-1,3,5-hexatriene; Laurdan, 2-dimethylamino-6-lauroyl-naphthalene; GP, generalized polarization; GP_{em}, emission generalized polarization; GP_{ex}, excitation generalized polarization; r_s, steady-state anisotropy; AO, acridine orange.

Fatty acid analysis

Total lipid extract was suspended in 2 ml of a fresh solution of KOH 10% in ethanol and digested during 45 min at 80 °C. One milliliter of distilled water was added and extracted three times by shaking with 2 ml of hexane. The upper organic phase was discarded. The aqueous layer was acidified with 0.5 ml of concentrated HCl and extracted twice with 2 ml of hexane. Free fatty acids were dried under a nitrogen stream and subjected to methyl esterification with 2 ml of 2% H_2SO_4 in methanol at 60 °C for 120 min under a nitrogen atmosphere. Then, 2 ml of chloroform and 0.67 ml of distilled water were added. The aqueous phase was discarded and the organic phase was washed twice with distilled water. After washing, the organic phase was evaporated under a nitrogen stream and re-suspended in hexane and fatty acid methyl esters were analyzed by Gas Chromatography using a GLC-FID HP6890 capillary GC (Hewlett–Packard, Palo Alto, CA) with an Omegawax Capillar (30 m × 250 μM, 25 μM nominal) (Supelco 11090-02A, USA) [17]. A mixture of bacterial fatty acid methyl esters was used as standard.

Oxgall resistance

Several Oxgall concentrations between 0 and 0.35% (w/v) at a final volume of 2 ml were added into tubes containing either fresh MRS or MRS 1 M NaCl medium for the bile salt challenge. Ten microliters of cellular cultures grown for 24 h in MRS medium (control C) and 48 h in MRS 1 M NaCl (hyperosmotic conditions N) was used to inoculate these tubes (dilution 1/200). The samples were incubated again at 37 °C for 24 h (control cultures) and 48 h (cultures grown in NaCl containing media). The toxic effect of the bile salts was analyzed by monitoring at 600 nm the cellular optical density reached by the cultures. Data were expressed by normalizing the absorption values obtained by the cultures grown in the absence of Oxgall.

Vesicle preparation and labeling procedures

For proton permeability assays, multilamellar lipid vesicles were prepared by mixing appropriate amounts of total lipids (54 μM final concentration) with buffer 120 mM $(NH_4)_2SO_4$, pH 7, in glass tubes and by vortexing vigorously for 5 min. These vesicles were extruded through polycarbonate filter (100 nm pore diameter) mounted in a miniextruder (Avestin, Ottawa, Canada), fitted with two 0.5 ml Hamilton syringes (Hamilton, Reno, NV). To obtain the large unilamellar vesicles, the samples were subjected to 13 passes through two filters in tandem [18]. In all experiments, the absorbance of the samples never exceeded an OD 0.1 at 360 nm. Preparation of vesicles with DPH or Laurdan was performed by dissolving the appropriate amount of total lipids (50 μM final concentration) in 2.5 ml of buffer sucrose 0.25 M, EDTA 1 mM, pH 7.0. The final probe/phosphorous ratios were: DPH 1/200 and Laurdan 1/400.

Steady-state fluorescence with DPH

Steady-state anisotropy of DPH was measured at a temperature range between 10 and 55 °C using a Kontron-SMF 25 spectrofluorometer, Kontron instruments SpA (Milan, Italy), equipped with standard polarization accessories and a circulating water bath. The excitation and emission wavelengths were 355 and 425 nm, respectively. The steady-state fluorescence anisotropy was calculated as

$$r_s = (I_{//} - GI_{\perp})/(I_{//} + 2GI_{\perp}), \tag{1}$$

where $I_{//} = I_{vv}$ and $I_{\perp} = I_{vh}$.

$G = I_{hv}/I_{hh}$ is a correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. The value is given by the ratio of the fluorescence intensities of the horizontal to vertical components when the exciting light is polarized in the horizontal direction [19].

Laurdan generalized polarization (GP)

The generalized polarization (GP) of Laurdan was measured with the same equipment mentioned above. Laurdan fluorescence intensity and GP spectra were obtained with monochromator bandpasses of 10 nm in excitation and emission wavelengths as described by Nichols et al. [20]. An excitation wavelength of 350 nm and an emission wavelength of 435 nm were used to obtain the emission and excitation spectra, respectively. The emission GP parameter was calculated according to

$$GP_{em} = (I_{390} - I_{350})/(I_{390} + I_{350}), \tag{2}$$

where I_{390} and I_{350} are the intensities at each emission wavelength from 430 to 510 nm, obtained using fixed excitation wavelength of 390 and 350 nm, respectively.

The excitation GP parameter was calculated according to

$$GP_{ex} = (I_{435} - I_{490})/(I_{435} + I_{490}), \quad (3)$$

where I_{435} and I_{490} are the intensities at each excitation wavelength from 330 to 410 nm obtained using fixed emission wavelength of 435 and 490 nm, respectively. The hydration level was calculated as the GP_{ex} value obtained at 350 nm [21].

Proton permeability of total lipid vesicles

Acridine orange dye (AO) was employed to monitor the proton permeability of the total lipid vesicles. Measurements were performed in the same equipment mentioned above. The fluorescence emission intensity of AO at 525 nm was measured for the excitation wavelength of 490 nm in a 2.3 ml magnetic-stirred quartz barrel. The fluorescence intensity of an AO 3.5 μM solution was recorded for 25 s; these determinations were used as the 100% of initial fluorescence. The measurement of proton permeability started with the addition of the lipid vesicles (50 μl) in four different buffer-dye solutions (see below). Samples were in permanent agitation during the data collection. The fluorescence intensity was monitored at periods of 5 s each and fluorescence percentage was calculated as

$$\% F = I_0/I_t \times 100, \quad (4)$$

where % F represents the percentage of fluorescence, I_0 the initial fluorescence intensity measured in the absence of vesicles, and I_t the fluorescence intensity measured in the presence of vesicle suspension at time t.

The assays were made at 20 °C using the following buffers:

(a) 120 mM $(NH_4)_2SO_4$, pH 7; (b) 0.4 mM $(NH_4)_2SO_4$ + 119.6 mM Na_2SO_4, pH 7; (c) 0.4 mM $(NH_4)_2SO_4$ + 119.6 mM Li_2SO_4, pH 7; and (d) 0.4 mM $(NH_4)_2SO_4$ + 119.6 mM K_2SO_4, pH 7.

The AO is accumulated into the lipid vesicles in response to ammonium gradient and the formation of dimers induces a decrease of the fluorescence intensity [22].

Results and discussion

Cell surface hydrophobicity

The affinity of *L. casei* towards hydrocarbons was used to measure the cell surface hydrophobicity of the bacteria [12]. Fig. 1 shows the values of the hydrophobicity obtained for the cultures grown under normal (C) and hyperosmotic conditions (N). The decrease of absorbance of the aqueous phase was used as a measure of cell surface hydrophobicity as was described [11–13]. On one hand, the control cultures showed a very low hydrophobicity, indicating that *L. casei* ATCC 393 has a low adherence to hydrocarbons and is relatively hydrophilic. On the other hand, the cultures grown under hyperosmotic conditions showed a significantly higher hydrophobicity than control cultures.

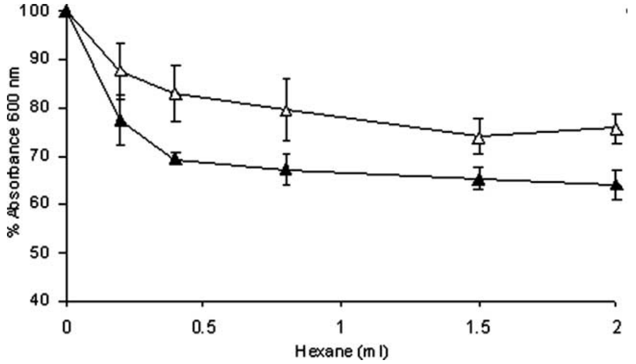

Fig. 1. Cell surface hydrophobicity of *L. casei* ATCC 393 cultures grown under normal C (△) or hyperosmotic N (▲) conditions. The data represent the average of three different cultures ± SD.

These results indicate that the adaptation of *L. casei* ATCC 393 to grow in hyperosmotic conditions leads to important variations of its envelope. Our results confirm the hydrophilic character of this bacterium [13]. Furthermore, cells grown in hyperosmotic medium evoked an increased hydrophobicity of their surface indicated by a higher adherence to the organic solvent (Fig. 1). This increased hydrophobicity should enable these bacteria to endure the stress imposed by the hypertonicity of the medium. Similar results were obtained in *Bacillus subtilis* grown in hyperosmotic medium [23]. The nature of the molecules conferring hydrophobicity to the bacterial surface has not yet been identified. Nevertheless, it has been suggested [24,25] that in Gram-positive bacteria the lipoteichoic acid, together with proteins, constitutes the most important wall component responsible for surface hydrophobicity.

Phospholipid and glycolipid composition

The composition of the crude lipid extract from *L. casei* ATCC 393 was analyzed by mono- and bidimensional thin-layer chromatography. We found that more than 87% of the polar lipids present in the *L. casei* ATCC 393 membrane are glycolipids and in a lesser amount, (≈ 12%) phospholipids. Monohexosyldiacylglycerol (HDG), dihexosyldiacylglycerol (H2DG), trihexosyldiacylglycerol (H3DG), and tetrahexosyldiacylglycerol (H4DG) are the principal glycolipids, in addition to the acylated acyldihexosyldiacylglycerol (AcylH2DG) and acyltrihexosyldiacylglycerol (AcylH3DG) (Fig. 2).

Phosphatidylglycerol (PG) and its lysil derivative lysilphosphatidylglycerol (lysilPG) are the major phospholipids present in this strain. In addition, a small

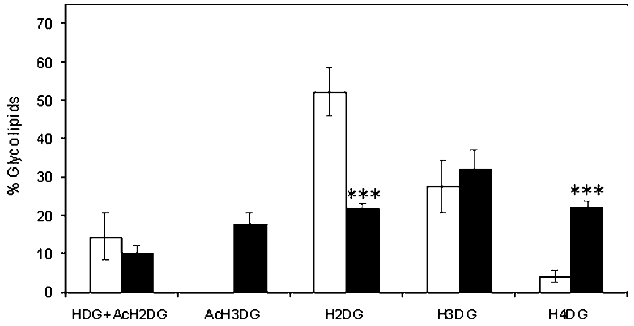

Fig. 2. Glycolipid composition of *L. casei* ATCC 393 membranes from cultures grown under normal (□) and hyperosmotic (■) conditions. Values are expressed as % and represent the mean of five determinations ± SD. Unpaired Student's test was applied to statistic analysis: $*** \equiv p < 0.0001$. HDG, monoglucosyldiacylglycerol; AcH2DG, acyldihexosyldiacylglycerol; AcH3DG, acyltrihexosyldiacylglycerol; H2DG: dihexosyldiacylglycerol; H3DG: trihexosyldiacylglycerol; and H4DG: tetrahexosyldiacylglycerol.

amount of cardiolipin (CL) and an unidentified phosphoglycolipid (PGL) were also detected (Fig. 3).

The glycolipid/phospholipid ratio of the total lipid extracted from purified membrane preparations, shows that glycolipid concentration is approximately seven times greater than the phospholipid one. There are no significant differences in the glycolipid/phospholipid ratio between C and N cultures (Table 1). Nevertheless, the individual glycolipid and phospholipid composition shows some significant variations between both cultures. In the NaCl containing medium, H4DG undergoes a significant increment (more than five times) and the AcylH3DG is present only in the membranes of *L. casei* ATCC 393 grown in hyperosmotic medium (Fig. 2). Also, there appears to be a small increase of H3DG and in parallel, a significant decrease (58.5%) of H2DG.

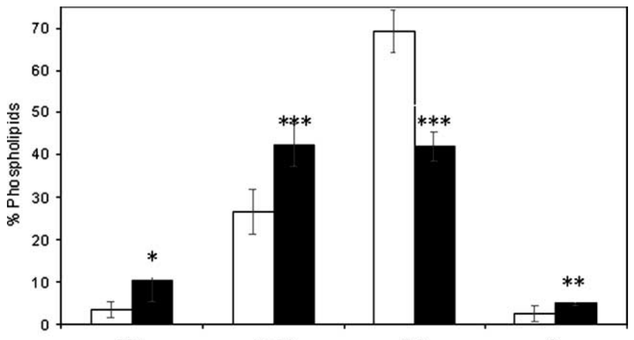

Fig. 3. Phospholipid composition of *L. casei* ATCC 393 membranes from cultures grown under normal (□) and hyperosmotic (■) conditions. The data represent the average percentage of nine different batches of lipids from nine different cultures of bacteria ± SD. Unpaired Student's test was applied to stadistic analysis: $* \equiv p < 0.05$; $** \equiv p < 0.0002$; and $*** \equiv p < 0.0001$. PGL, phosphoglycolipid; LysPG, lysilphosphatidylglycerol; PG, phosphatidylglycerol; and CL, cardiolipin.

Table 1
Relationship between phospholipid–glycolipid and phospholipid–protein in *L. casei* ATCC 393 purified membranes

	Phospholipid–glycolipid	Phospholipid–protein
Control	0.14 ± 0.02	0.067 ± 0.01
NaCl 1.0 M	0.17 ± 0.04	0.056 ± 0.02

Results are the average of four replicate assays ± SD.

It was described that the lipid moiety of the lipoteichoic acid, which is a molecular component of the membrane and cell wall in *L. casei*, is formed by both the H3DG and the AcylH3DG glycolipids present in the membrane [26]. Our results demonstrated a small though not significant increase in H3DG and that the AcylH3DG is synthesized only in the cultures grown in hyperosmotic conditions (Fig. 2). These glycolipids would be related to the increased cell surface hydrophobicity observed during osmotic stress. Moreover, this high content of glycolipids was reported previously for other *L. casei* strain [14] as well as for *Lactobacillus acidophilus* [5].

Besides, phospholipids showed a significant increase in the unidentified PGL (environ three times) and smaller but significant increases in both LysilPG and CL, whereas PG showed a significant decrease (Fig. 3). Similar variations have been described in *B. subtilis* [23] and in *Staphylococcus aureus* [27] grown in hypersaline media.

Regarding the phospholipid and glycolipid composition, we observed a very similar lipid pattern as those described in other *L. casei* strains [14]. Moreover, cells grown in hyperosmotic medium present quantitative differences in comparison with the cultures grown under normal conditions.

Membrane lipids are some of the most adaptable molecules in response to perturbations. Furthermore, membrane stability has been explained by the stabilization of the lipids in a bilayer conformation depending on the geometrical shape of the lipids. The changes observed in the bilayer and non-bilayer forming lipids in *L. casei* ATCC 393 in an hyperosmotic medium might tend to compensate the original membrane stability to maintain the bilayer structure. In fact, the ratio between phospholipids and glycolipids remains unchanged in both growing conditions, in spite of the differences observed in the percentage of each lipid. In addition, no significant differences were obtained for the quantitative ratio μg membrane proteins to μg lipid phosphorous between both culture conditions (Table 1), although the membrane protein profile observed by gel electrophoresis was qualitatively different (unpublished results).

On the other hand, it has been suggested [28] that the hydrophilic moiety of the glycolipids forms regions (pores) in the membrane through which small molecules, e.g., water, may pass. The presence of variable amounts of trisaccharide chains in these regions may represent a mechanism by which the size of these hypothetical pores may be regulated. The changes in the polar head group composition observed during osmotic stress might modify the lipid charge distribution affecting lipid–protein and lipid–lipid interactions involved in the stress response. Compensation changes on the structure, composition, and dynamic properties of lipids and biological membranes affected by various stressors have been previously described in Gram-negative [29,30] and in Gram-positive bacteria [20,31,32]. Likewise, alterations of the lipid–protein interactions can affect membrane processes such as protein assembly and folding, as well as transport activities. Recent reports on the ABC transport system of *Lactococcus lactis* showed that this system senses osmotic stress through alterations in membrane physico-chemical properties, with the electrostatic interactions between ionic lipids and the transporter being essential to the osmosensing mechanism [33].

Fatty acid composition

Hexadecanoic (16:0), 11,12-methyleneoctadecanoic (cyc 19:0), octadecenoic (18:1 n-7 and n-9), hexadecenoic (16:1 n-7 and n-9), and tetradecanoic (14:0) acids, are the principal fatty acids present in the total lipid extracted from *L. casei* ATCC 393. The composition analysis showed that 16:0, 18:1 n-7 and n-9, and 11,12-methyleneoctadecanoic (cyc 19:0) accounted for more than 76% of the total fatty acids. No polyunsaturated fatty acids were found, in accordance with a previous publication [34] (Table 2).

Osmotic strength induced an increase in the 16:1 n-9 content and a decrease of 16:1 n-7 in comparison to the control conditions. In agreement with previous observations [3,4], we found that the presence of NaCl in the medium results in an increase of the saturated/unsaturated ratio and promotes the formation of lactobacilic acid (cyc C19:0) from the corresponding monounsaturated precursor 18:1 n7 (Table 2). The formation of cyclopropane-containing fatty acids is induced when certain microorganisms enter the stationary phase of their growth cycle or environmental conditions such as osmotic pressure or temperature are modified [35].

The role of cyclic fatty acids in the membranes of lactic acid bacteria is poorly understood. However, fatty acid cyclization is generally regarded as a mechanism to reduce membrane fluidity and prevent the penetration of undesirable molecules [3]. In *Escherichia coli*, cyclic fatty acids are one of the factors that protect cells from acid shock [30]. On the other hand, it has been reported that cyclic fatty acids increase the fluidity of the cytoplasmic membrane as they retain the ability to slide past each other as they cannot form crystalline structures [36]. Furthermore, cyclopropane fatty acyl chains seem to confer a kind of tolerance towards disturbances in many microorganisms submitted to different stresses [3,30,32,37,38] and this could be their role in *L. casei*. The analysis of saturated/unsaturated fatty acids ratio suggests that under osmotic stress conditions, there may be a slight decrease in the fluidity of lipid paraffinic chains of the membranes. However, this would be compensated by the increase in cyc 19:0 fatty acid. Perly et al. [35] have shown that a decrease in the temperature of the gel to liquid-crystalline phase transition is observed upon introduction of a cyclopropane ring in model systems. In addition, a very significant broadening of the transition profile is observed. These observations are consistent with the poor packing ability of mixed saturated and cyclopropane-containing chains due to the bulky substituent affects. Hence, the membranes of *L. casei* ATCC 393 subjected to osmotic stress would not change their fluidity.

Table 2
Major fatty acids of *L. casei* ATCC 393 grown at control and hyperosmotic conditions

Fatty acid	Control	NaCl 1 M
13:0	Tr	Tr
14:0	8.05 ± 0.24	6.94 ± 0.29
14:1	1.09 ± 0.33	1.74 ± 0.35
15:0 ai	Tr	Tr
15:0	Tr	Tr
16:0	21.24 ± 0.42	19.30 ± 0.65
16:1n-9	Tr	2.97 ± 1.77
16:1n-7	9.78 ± 0.45	4.41 ± 0.48
17:0 ai	Tr	Tr
17:0	Tr	Tr
18:0	1.34 ± 0.03	2.46 ± 0.41
18:1n-9	17.34 ± 0.95	18.59 ± 1.04
18:1n-7	15.17 ± 0.17	5.53 ± 0.68
Cyc-19	22.26 ± 0.14	30.02 ± 3.16
Total saturated	30.63 ± 0.69	28.70 ± 1.27
Total unsaturated (without cyc-19)	43.37 ± 1.93	33.24 ± 4.49
Saturated/unsaturated	0.71	0.86
Saturated/unsaturated (incl. cyc-19)	0.47	0.45
Unsaturation index *	0.64	0.61

Values are the mean of triplicate analyses as mol % ± SD.

Tr: fatty acids with less than 1%. * Unsaturation index: (∑ number unsaturation mol × number double bonds)/∑ number total mol.

Oxgall resistance of L. casei cultures

Bile salts are amphiphilic molecules possessing different relative hydrophobicities. These compounds may solubilize phospholipids to form thermodynamically stable micelles.

To determine whether the variations in polar lipids and fatty acids observed at the membrane level under hypersaline conditions (see below) could affect the membrane sensitivity to bile salts, we studied the Oxgall resistance of both cultures. For this purpose, cultures of

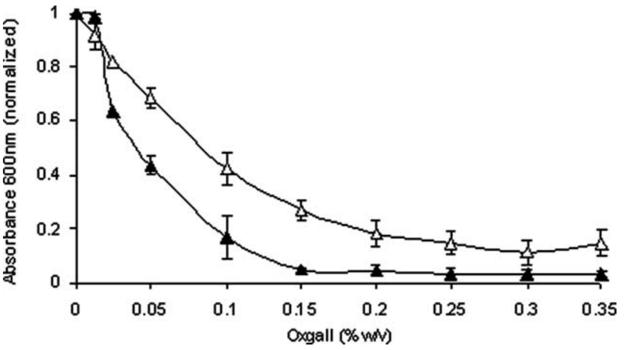

Fig. 4. Oxgall resistance of *L. casei* ATCC 393 cultures grown under normal (△) and hyperosmotic (▲) conditions. The data represent the average of three different determinations ± SD.

L. casei ATCC 393 grown either 24 h in MRS (C) or 48 h in MRS supplemented with 1 M NaCl (N) were diluted into fresh MRS and MRS 1 M NaCl respectively, containing different concentrations of the crude bile salt extract. The resulting curves observed in Fig. 4 indicate a decrease in the growth of both bacterial cultures, showing a typical dose–response curve. However, the cells grown under hypersaline condition were more sensitive to the action of the bile salts.

Lactobacilli have long been used as probiotics in human and must survive the low pH of the stomach and the conjugated bile acids in the duodenum. With regard to the bile salt resistance, our results indicate an increased sensitivity of the *L. casei* ATCC 393 cells grown in hyperosmotic medium (Fig. 4). Albalak et al. [39] have shown that bile salts increased membrane permeability to small ions, such as calcium, protons, sodium, and ferrous ions, but the mechanisms of action by which these solutes pass through the membranes is still unclear. Furthermore, it was suggested that the diffusion rate of bile acids through model-membranes is not only dependent on the hydrophobicity of these molecules, but also on both bile acid oligomeric associations and membrane lipid composition [40]. Disalvo et al. [41] have demonstrated that in pure phospholipid membranes in the gel state, a high lateral packing of lipids produces an increase in defects that enhances the penetration of molecular harpoons, causing the disruption of the membrane. Our results indicate that the *L.casei* ATCC 393 cultures grown in hyperosmotic conditions were more sensitive to the addition of bile salts to the medium (Fig. 4). These results, together with the fact that vesicles constructed with total lipids of these cultures showed a higher lateral packing (see below), suggest that their higher sensitivity against bile salts could not only be due to the differences in lipid and fatty acid compositions but also to the altered packing of the membrane into which oxgall may insert. However, these results could also be related to changes in the partition coefficients of the bile salts between the aqueous medium and the cell surface affecting the bile salt accessibility inside the cell. On the other hand, it has been described that in *Lactobacillus reuteri*, the presence of bile salts in the culture medium induces changes in the barrier properties of the membrane [42].

Steady-state anisotropy of DPH

Steady-state fluorescence spectroscopy was employed to study the thermotropic behavior of intact bacterial membranes and vesicles prepared from total lipids isolated from cultures of *L. casei* ATCC 393 grown at 37 °C in normal and hyperosmotic conditions. We selected DPH, a neutral fluorescent probe that is located deeply in the hydrocarbon core of the bilayer for this study. Fig. 5 shows the temperature-dependent profiles of DPH anisotropy (r_s), in total lipid vesicles and in intact membranes within a temperature range from 10 to 55 °C. In all these samples, the increasing temperature produced a gradual decrease in the r_s values indicating a thermotropic increase of the lipid acyl chain motion within the bilayer [43]. The absence of an abrupt change in the slopes of these profiles indicates that in *L. casei* ATCC 393 there was no phase transition of bulk lipids within the tested temperature range. Similar observations have been made in *L. acidophilus* [5]. The r_s values for DPH in membranes and in total lipid vesicles were similar when the isolated membranes and the vesicles in each culture condition were compared. No significant differences were observed in the r_s values in either membranes or vesicles, due to the presence of high ionic strength (Fig. 5) and in spite of the different fatty acid and polar lipid compositions presented by these preparations. Nevertheless, the isolated membranes obtained from both cultures showed higher r_s values for DPH than for lipid vesicles, indicating that the membrane proteins decrease the fluidity of the bilayer regardless of

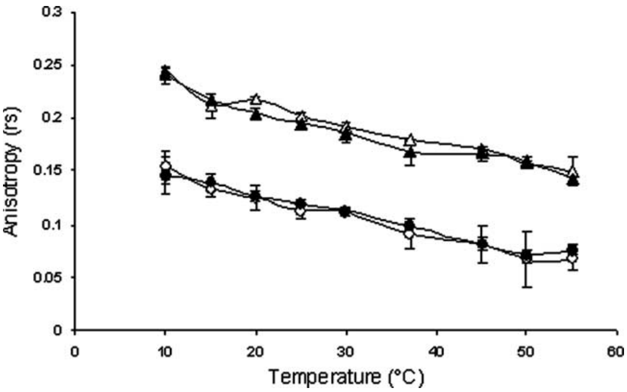

Fig. 5. DPH anisotropy (r_s) of membranes and total lipid vesicles of *L. casei* ATCC 393 grown under normal and hyperosmotic conditions. (△) Membranes from cultures grown under normal conditions; (▲) membranes from cultures grown under hyperosmotic conditions; (○) vesicles from cultures grown under normal conditions; and (●) vesicles from cultures grown under hyperosmotic conditions. Values represent the mean of three determinations ± SD.

the growing conditions. Similar results have been previously reported by our laboratory for *B. subtilis* grown under hyperosmotic stress [44]. The conservation of membrane fluidity under stress conditions was observed in a wide variety of organisms [45]. Since 1974, Sinensky [46] has proposed that modifications in the fatty acid composition enable the cells to maintain the membrane fluidity invariable, a process originally termed "homeoviscous adaptation," fundamental for the maintenance of cellular functions. Nevertheless, this concept is not applicable to all microorganisms or to all types of stresses.

Laurdan fluorescence measurements

Different biophysical characteristics such as lipid lateral packing and proton permeability of lipid vesicles from *L. casei* were studied. Lateral packing, stability, and permeability properties of cell membranes are mostly related to their fatty acid composition and its molar ratio. However, little is known about the permeability and stability of membranes with such complex compositions as those of lactic acid bacteria. The structural properties of the total lipid vesicles were studied by Laurdan's fluorescence spectroscopy. Laurdan's dipolar relaxation properties were used to study the influence of osmotic stress on the phase behavior and interface properties of total lipid vesicles. Laurdan spectral shifts are usually quantified in the form of GP. The GP_{ex} parameter reports on the emission spectral shift and it is thus indicative of the dipolar relaxation and lipid phase packing.

The excitation and emission spectra and the wavelength-dependence for Laurdan excitation (GP_{ex}) and emission (GP_{em}) parameters were measured at two different temperatures (20 and 37 °C). The excitation spectrum obtained from the vesicles of total lipids of *L. casei* grown under normal and hyperosmotic conditions is characterized by two maxima, at 356 nm (blue band) and 380 nm (red band). However, the intensity of the red band is lower than that found in the phospholipid gel state [47]. Furthermore, the emission spectrum predominantly exhibited a blue band at ~435 nm, this band being characteristic not only of a lipid bilayer in the gel state but also of a lipid bilayer in the liquid-ordered state usually found in membranes containing cholesterol. Similar results were also described in *Shewanella gelidimarina* grown under temperature and salinity stress [20] and in *E. coli* membranes [48]. Upon decreasing the temperature from 37 to 20 °C, the red excitation band becomes more important in both samples, whereas there is a maximum slight shift to the blue in the vesicles extracted from the cells cultured under hyperosmolarity. The wavelength dependence of the GP pattern in these samples changed gradually as a function of temperature showing with increasing wavelength a negative and positive slope for the GP_{ex} and GP_{em}, respectively (Fig. 6). Nevertheless, the GP_{ex} values are higher than those characteristic of the liquid-crystalline phase. This behavior indicated that dipolar relaxation occurred and that there was no selective excitation of subpopulations of Laurdan molecules in coexisting gel and liquid-crystalline domains [49–51]. In addition, the GP_{ex} pattern showed significant differences between C and N vesicles both at 37 and 20 °C, the GP_{ex} values for N being higher than for C samples at the tested temperatures. Since the ability of water molecules to penetrate into the lipid bilayer is affected by the charge and size of the lipid molecule, the results obtained could be attributed to a change in the water hydration of the membranes due to the remarkable change at the polar lipid composition of the cultures.

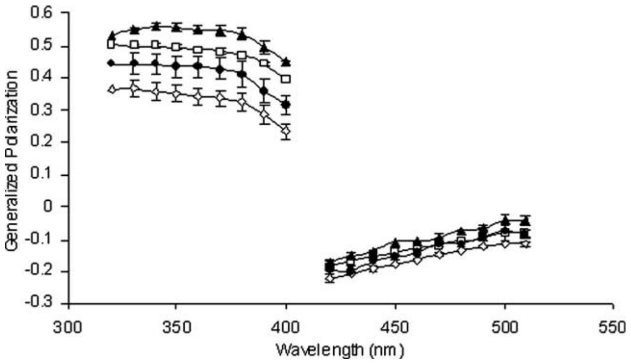

Fig. 6. Excitation and emission generalized polarization of Laurdan in total lipid vesicles of *L. casei* ATCC 393 grown under normal and hyperosmotic conditions. Measurements were performed at 20 and 37 °C. (◇) normal conditions, 37 °C; (□) normal conditions, 20 °C; (●) hyperosmotic conditions, 37 °C; and (▲) hyperosmotic conditions, 20 °C. The data represent the average of lipid batches extracted from three independent cultures of bacteria ± SD.

Proton permeability

The fluorescent probe AO has been used extensively as a cytochemical dye. The assays were made in vesicles constructed with total lipids prepared from cultures grown in control and hyperosmotic media. The changes in the fluorescence emission observed in the experiments were in accordance with the incorporation and liberation of AO by the vesicles. The proton permeability in the vesicles was analyzed in media containing different cationic compositions (Na^+, Li^+, and K^+).

Figs. 7A and B shows that vesicles formed with lipids extracted from cells grown in the hyperosmotic medium display a higher proton permeability than the control ones. Moreover, the pH equilibrium gradient in those vesicles is reached faster than in the control vesicles. Nevertheless, both kinds of vesicles are unstable once the pH equilibrium gradient has been reached. A gradual increase of fluorescence percentage is shown due to the liberation of the probe from the interior of the

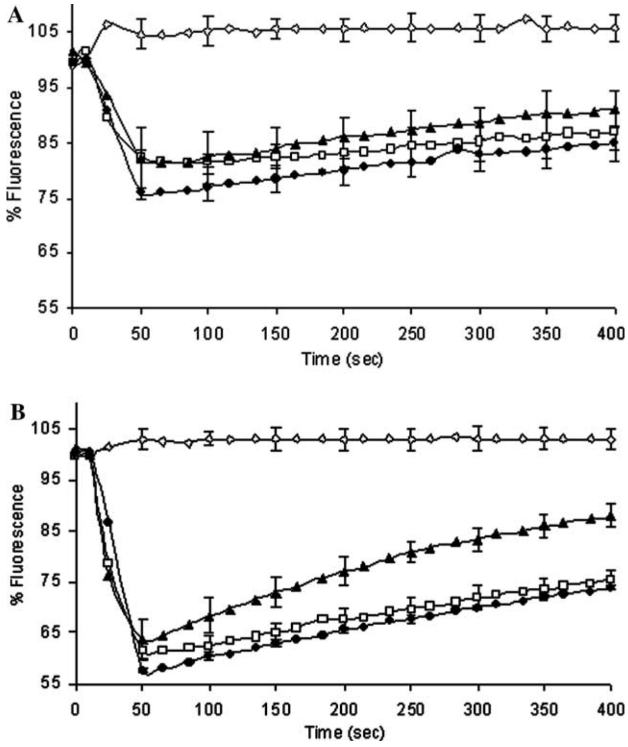

Fig. 7. Proton permeability of vesicles constructed from total lipids extracted from *L. casei* ATCC 393 cultures grown at normal (A) and hyperosmotic conditions (B). The composition of the buffers tested was: (◇) 120 mM $(NH_4)_2SO_4$; (□) 0.4 mM $(NH_4)_2SO_4$ + 119.6 mM Na_2SO_4; (▲) 0.4 mM $(NH_4)_2SO_4$ + 119.6 mM Li_2SO_4; (●) 0.4 mM $(NH_4)_2SO_4$ + 119.6 mM K_2SO_4, pH 7. The data represent the average of lipid batches extracted from three independent cultures of bacteria ± 1 SD.

vesicles. In the control vesicles, the percent of leakage is independent of the ionic composition of the medium, but in the vesicles constructed with the lipids purified from the cells grown under hyperosmolarity the leakage is higher in the presence of Li^+ than in the presence of Na^+ or K^+.

The proton permeability study showed that vesicles constructed with lipids from cells grown under hyperosmotic conditions reached the equilibrium gradient faster and present a higher permeability than the control ones (Figs. 7A and B). Moreover, both types of vesicles present dissimilar bilayer instability after the equilibrium gradient. The increased permeability and instability of the lipid vesicles from the cells grown under osmotic stress could be explained by the high lateral lipid packing observed in these samples, since this physical characteristic induces structural disorders as mentioned previously. The polar lipid modifications observed in osmotic stress conditions could be related to the changes in permeability shown by the lipid vesicles prepared from cultures of *L. casei* grown in hypertonic conditions. Similarly, it has also been demonstrated that the lipid vesicles of *L. acidophilus* cultures grown at different temperatures present a differential permeability according to the ionic composition of the medium [5]. Our results also demonstrate that the lipid vesicles of *L. casei* ATCC 393 grown under hyperosmotic conditions appear to have selective properties, which enable them to discriminate between Li^+, Na^+, and K^+. This phenomenon could be attributed not only to a different binding of the ions to the surface of the vesicle, but also to the permeability across the lipid vesicles.

In summary, *L. casei* ATCC 393 changes its membrane biochemical composition as a function of the external osmolarity. Most of these variations compensate each other to maintain membrane fluidity. However, the proton permeability and packing of lipid vesicles were altered in response to the external ionic strength of the medium. These changes are likely to be critical to cope with the stress conditions produced by the osmotic imbalance between the cells and their environment.

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